STRATEGIES TO IMPROVE NEWCASTLE DISEASE VACCINES: DEVELOPMENT OF GENOTYPE-SPECIFIC AND CYTOKINE-EXPRESSING VACCINE VIRUSES

by

NANY STIVALIS CARDENAS GARCIA

(Under the Direction of Corrie C. Brown and Claudio L. Afonso)

ABSTRACT

Despite decades of research, Newcastle disease (ND) persists as a continual threat to the poultry industry. Current vaccines have not been able to markedly reduce the global prevalence of disease and cannot prevent replication and shedding of the challenge virus; therefore, improved vaccines are required. To evaluate the effect of genotype-specific ND vaccines, an attenuated, chimeric Newcastle disease virus (NDV) expressing the F and HN genes from a genotype XIII virulent NDV (rLS-PK33) was developed. Specific-pathogen-free chickens were vaccinated with LaSota or rLS-PK33 live vaccines at different doses, and challenged with virulent PK33 to evaluate and compare their performance on preventing mortality and challenge virus shedding. In addition, a plasmid and an attenuated ND virus expressing chicken IFN-γ (rZJ1*L/IFNγ), and an attenuated NDV expressing chicken IL-10 (rZJ1*L/IL-10) were developed to investigate their effect on modulating the immune response in chickens and their effect on protection and reduction of virus shedding after challenge with vZJ1. Cloning and reverse genetic techniques were utilized for the development of all recombinant viruses. These were

characterized by sequencing of the fusion protein cleavage site and inserted genes, intracerebral pathogenicity index assay, mean death time assay in eggs, ELISA and Western blotting for cytokine determination, virus isolation and titration in eggs, hemagglutination inhibition assay for antibody quantification, and lymphocyte proliferation assays and flow cytometric analysis for evaluation of cellular immune response. Our results reveled that rLS-PK33 decreased viral shedding more efficiently than LaSota; additionally, it was able to increase survival rates better than LaSota when administered at suboptimal doses. Evaluation of the effects of chIFN-γ delivered by plasmid DNA, live or inactivated rZJ1*L/IFNγ, demonstrated that, regardless of the delivery system, chIFN-γ did not enhance the immune response. On the contrary, evaluation of the effect of chIL-10 delivered by inactivated rZJ1*L/IL-10, resulted in an increased antibody response and lower antigen-specific T cell response, without increasing morbidity and mortality after challenge. In conclusion, these results provide new strategies to improve ND vaccines, and provide new insights to better understand the chicken immune response, as well as the effects of two key avian cytokines on immune response modulation.

INDEX WORDS: Newcastle disease, Newcastle disease virus, APVM-1, genotype-specific vaccines, recombinant vaccines, chicken interferon-gamma, chicken IL-10, live vaccines, inactivated vaccines, decreased challenge virus shedding, decreased mortality

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# DEDICATION

I would like to dedicate all of my research work and the present dissertation to my Lord Jesus Christ! He has opened many scientific and non-scientific doors for me, gave me the strength, courage, patience, and knowledge I needed, helped me push through all of those times of stress and uncertainty, gave me my wonderful family, and directed my path where I could meet all of the wonderful people that have helped me and supported me along my journey. With all my love, I also dedicate this work to my beloved parents Rosa and Antonio, and to my beloved sister Nadia, for their sacrifice, relentless support, understanding and endless love. The thought of you also gave me the strength to continue until the end. In addition, I want to include my entire family in this dedication, especially my grandparents Elena and Antonio for their unconditional love and support.

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# CHAPTER 1

# INTRODUCTION

Vaccination is currently one of the most important methods used in the attempts to control Newcastle disease (ND). Regrettably, vaccination alone has neither completely controlled, nor eradicated virulent Newcastle disease virus (vNDV) from countries with endemic disease (Israel, Egypt, China, Pakistan, Korea, South Africa, etc.) that utilize extensive vaccination programs [1-12]. This underscores the need to revise the efficacy of NDV vaccines and current vaccination approaches in order to identify possible weaknesses and apply epidemiologic knowledge about each region to develop a better approach. Several studies have concluded that existing vaccines are capable of protecting against clinical disease caused by infection with vNDV from most of the current circulating genotypes [5, 12-17], but it has also been concluded that factors such as immune suppression caused by other biologic agents and/or the use of non-effective vaccination schemes compromise the efficacy of field vaccination [12, 14, 18].

Even though all NDV strains belong to a single serotype, there are significant phylogenetic differences between the current vaccine strains that belong to genotypes I (Ulster) or genotype II (LaSota and B1), and the currently circulating vNDVs from genotypes V, VI, VII, and XIII, among others [19-21]. This may explain why live, inactivated, and subunit vaccines have not been able to completely prevent infection, virus replication, and shedding [16, 20, 22]. Previous studies performed in our laboratory and others have demonstrated decreased oropharyngeal virus shedding after challenge

with vNDV when the genotype of the vaccine virus was homologous to the genotype of the challenge virus, when compared with the LaSota vaccine strain [20, 21, 23, 24].

Live attenuated vaccines have been used against respiratory viruses, including NDV, since the 1950s due to their capability to replicate in mucosal membranes, and therefore, induce effective cell-mediated and humoral immune responses [25-28]. Cell mediated immunity (CMI) is important for viral clearance, while humoral immunity is crucial for viral neutralization, and both contribute to decreasing viral replication [29-31]. Unfortunately, immune responses induced by the administration of live vaccines are often not optimal to satisfy industry needs, since there is an inverse correlation between live virus vaccine efficacy and adverse vaccine reactions impacting production. Typically, the most attenuated (safer) vaccines are the least effective, whereas the most effective vaccines often lead to clinical respiratory diseases (side effects) [32-35]. Because ND continues to affect poultry causing devastating economic losses, there is an urgent need to improve ND vaccines. **An optimal vaccine would increase virus clearance and virus neutralization, and also as a result, decrease replication and shedding of the challenge virus, thereby reducing the environmental viral load and transmission**.

Cytokines are crucial regulators of the immune response. Interferon-gamma (IFN-γ) is a key cytokine secreted as a result of the activation of a Th1 response, known to counteract intracellular pathogens such as viruses [36, 37]. Previous studies in chickens have reported increased antigen-specific antibody production and enhanced cellular immune responses to different pathogens when chicken IFN-γ (chIFN-γ) was administered during or after vaccination [38-47]. Interleukin-10 (IL-10) is a cytokine secreted mostly during Th2 responses, and it is also known as a regulatory cytokine for

its anti- inflammatory effects [37, 48]. Information on the effects of chicken IL-10 (chIL-10) is more limited. This cytokine was cloned and studied for the first time by Rothwell and collaborators in 2004 [49]. They demonstrated its ability to block both chIFN-γ production and activation of macrophages. Studies performed in mammals have shown that IL-10 is able to increase antigen-specific antibody responses [50-53]. Its ability to modulate the cellular response was also reported [54-56]. Based on these observations, the use of avian cytokines as vaccine adjuvants may improve ND vaccine efficacy.

In the present research work, we evaluated two strategies to improve ND vaccines; therefore, two different methodologies were implemented:

1. Development of a genotype-specific, live attenuated, recombinant NDV vaccine bearing the fusion (F) and hemagglutinin-neuramidase (HN) genes from a genotype XIII virulent virus (PK33) circulating in Pakistan.
2. Use of recombinant NDV vaccines expressing chIFN-γ and chIL-10 to improve cellular and humoral immune responses.

Here, we hypothesized that: 1) the F and HN genes matching the virulent challenge virus into a vaccine backbone will be enough to reduce challenge virus shedding more efficiently than the standard LaSota vaccine; and 2) delivery of chIFN-γ and chIL-10 using inactivated vaccine viruses will enhance CMI and/or antibody-mediated (AMI) immune responses, respectively, leading to better protection after challenge and to more efficient reduction in challenge virus shedding that vaccine viruses not expressing cytokines.

In order to test these hypotheses, three recombinant viruses were developed and used in a series of vaccination and challenge experiments. All three recombinant viruses

were generated employing reverse genetic techniques. The virus bearing the F and HN gene from PK33 (rLS-PK) was created by swapping the F and HN genes from the LaSota backbone with the F and HN genes from PK33. Before gene swapping, the F gene from PK33 was mutated, so its fusion protein cleavage site was identical to the cleavage site from the LaSota vaccine virus. This virus and the LaSota vaccine virus were then used in a first experiment as a live vaccine to immunize 1-day-old, specific-pathogen-free chickens (SPF). Birds were then challenged with PK33 to determine which vaccine would protect better against the challenge virus, and to evaluate and compare reduction in challenge virus shedding. A second experiment was performed in 4-week-old SPF chickens, where birds were immunized with different doses of rLS-PK or LaSota to compare protection against morbidity and mortality.

Viruses expressing chIFN-γ (rZJ1*L/IFNγ) and chIL-10 (rZJ1*L/ IL-10) were generated by inserting the chIFN-γ and chIL-10 genes into an attenuated virus from genotype VIId (ZJ1*L). A series of inactivated vaccine experiments involving the two cytokine-expressing viruses, ZJ1*L (the backbone virus of low virulence without the cytokines inserted), and LaSota, were performed in order to study the ability of rZJ1*L/IFNγ to modulate CMI and AMI responses, and to evaluate the ability of rZJ1*L/ IL-10 to enhance the AMI response and downregulate the CMI response. The effect of both viruses on protection against morbidity and mortality after challenge with vZJ1 (parental virulent virus) was also evaluated.

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# CHAPTER 2

# LITERATURE REVIEW

## Newcastle disease definition and significance

Newcastle disease (ND) is caused by virulent strains of Newcastle disease virus (NDV) that affects over 241 species of birds [1]. In terms of international trading of poultry and poultry products, the World Organization of Animal Health (OIE) has defined ND 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 [2]. 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' refers to at least three arginine or lysine residues between residues 113 and 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterization of the isolated virus by an ICPI test [2].

Newcastle disease (ND) is one of the most important diseases that affect the poultry industry around the world [3]. It can engender devastating economic losses for producers due to its ability for rapid spread, high rates of mortality in birds, decreased growth rates, drop in egg production, and continuous losses represented by vaccination

programs and other strategies that have to be implemented to control the disease [3]. Also, ND contributes to malnutrition by impacting the availability and quality of food, especially in developing countries with endemic disease, where many people depend on village or backyard chickens as a source of dietary protein [3, 4]. According to the World livestock atlas, NDV was ranked in second place, just after rabies, having 56 countries with reported outbreaks between 2006 and 2009, and seventh for the livestock losses with a total loss of 60,370 livestock units [5]. In developing countries, it is difficult to control the disease due to social and financial restrictions. Therefore, the development of countries' commercial poultry production, as well as the establishment of trading links, become compromised [4].

## History

The first outbreaks of Newcastle disease (ND) were reported by Kraneveld and Doyle in 1926, when the disease caused two separate outbreaks in poultry farms of different regions of the world: the island of Java in Indonesia [6] and Newcastle-upon-Tyne in England [7]. There has been a lot of speculation about the link between these two outbreaks, but in general, it has been considered that the presence of the virus in England resulted from transportation to the port of Newcastle from Africa [6]. Later on, some evidence suggested that ND could have been circulating before 1926 in Korea. [6]. In July, 1927, an outbreak occurred in Ranikhet, India, and by 1928, the disease had spread through all provinces in British India [8]. It is probable that ND was present before 1926 but was unnoticed due to lack of expertise in recognizing the disease. It is important to note that a highly virulent virus of poultry spread in a very short time through England, Java, Philippines, India, Ceylon, Korea, and Japan [9].

In the 1930s, a relatively mild respiratory disease, often accompanied with neurological signs was first described in California, USA, and named pneumoencephalitis [10, 11]. This disease had very low rates of mortality compared to the ND described by Doyle, but later on, it was discovered that the etiological agent was serologically compatible with NDV [11]. Researchers were not certain how long the virus had been circulating in the USA, but some evidence suggested the presence of the virus also on the east coast at the same time. This was further investigated by retroactive virus identification, and NDV was confirmed in isolates as early as 1938 [12].

In the next few years, several NDV isolates obtained from chickens around the world were found to produce mild or no disease at all, determining that there were different subtypes according to the grade of pathogenicity, as seen by clinical disease and mortality rates in chickens [13].

Since the first recognized appearance of NDV, four ND panzootic episodes have been recognized. The first panzootic episode started in 1926 and spread slowly through the world, and it took about 16 years to become a true panzootic [4, 6]. The second panzootic event was associated with a viscerotropic velogenic NDV, originated in the Middle East in the late 1960s; it took only 4 years for it to spread around the world, including the United States of America and Mexico. The much more rapid spread of the disease was attributed to the increased transportation and commercialization of poultry, and in particular, exotic wild birds, such as psittacines, which raised the question about natural reservoirs of ND [14]. Based on antigenic and genetic analyses, a third panzootic took place in the late 1970s, but its origins and spread were inconclusive [4]. The most recent panzootic originated in the 1980s and mostly affected racing and show pigeons

with some spread into poultry through feed contaminated with pigeon feces. This virus was antigenically different from previous NDV and was named pigeon paramyxovirus type 1 (PPMV-1). The clinical manifestation was characterized by neurological signs on affected pigeons [4, 6]. It spread rapidly into Europe (by 1981) due to bird-to-bird contact during races and shows, with some spread into wild Columbiformes [4].

The efforts to control and understand ND gave origin to multiple studies and the development of new techniques to identify and classify NDV. These studies led to the discovery of determinants of virulence (role of the fusion protein and its cleavage site) in the late 1970s [15-17], utilization of monoclonal antibodies to differentiate isolates according to their antigenicity in the early 1980s [18], and the development of nucleotide sequencing, which enabled estimation of genome size and gene order [19]. Most importantly, it revealed the difference at the fusion protein cleavage site between virulent NDV (vNDV) and low virulent NDV (loNDV) (late 1980s) [17]. Nucleotide sequencing continues to evolve and has been used to classify NDV isolates and conduct phylogenetic studies to understand virus evolution. Within the 2000s, real-time polymerase chain reaction techniques (RRT-PCR) to detect NDV from clinical samples, and differentiate between virulent and low virulent ND viruses are some of the most recent developments [20, 21], in addition to the use of reverse genetic techniques to study gene interactions and develop recombinant vaccines [22-32].

## Newcastle disease virus (NDV)

NDV is an avian paramyxovirus type 1 (APMV-1) classified in the genus *Avulavirus*, sub-family Paramyxovirinae, family Paramyxoviridae, order Mononegavirales [3, 33-35]. It has a negative-sense, single-stranded, non-segmented,

enveloped RNA genome of about 15.2 kb in length, with six genes (3'-NP-P-M-F-HN-L-5') that encode for six structural proteins from 3' to 5': nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), and RNA-dependent RNA polymerase (L) [3, 33, 35]. Transcriptional editing of the phosphoprotein mRNA at nucleotide position 402 gives origin to two non-structural proteins, V and W [36, 37].

The NP is 489 amino acids long and has a molecular weight of 55 kDa [33]. It is an RNA binding protein that serves several functions such as transcription, replication and encapsidation of the viral genome to protect it against RNAse activity. Under electron microscopy, the NP bound to the viral genomic RNA has the appearance of a "herring bone", measuring 18 nm across [3, 33]. Each monomer of NP binds six nucleotides, which explains why the length of the NDV genome is always a multiple of six (the "rule of six") [33, 38]. The NP associates with P and L proteins during transcription and replication, and with M protein during viral assembling [39]. The intracellular concentration of unassembled NP is thought to be a determinant in controlling the rates of transcription and replication, but this still remains uncertain [39]. The phosphoprotein (P) is 395 amino acids long, and it is heavily phosphorylated at specific serine and threonine residues, with a molecular weight of approximately 42 kDa [33]. It works as a non-catalytic subunit of the viral RNA polymerase (also called the large (L) protein) and also functions as a chaperone to avoid uncontrolled encapsidation. It associates with NP and L and is essential for transcription and virus replication [13, 33, 39]. L is the largest protein in the NDV genome with 2204 amino acids and a molecular weight of approximately 250 kDa. It is also the least concentrated protein in infected

cells because the L gene is the most distal gene from the promoter, and therefore, the last to be transcribed [33, 40]. The L protein is the RNA-directed RNA polymerase and is responsible for the enzymatic processes, such as nucleotide polymerization, mRNA capping, methylation, and polyadenylation of mRNAs, among others, which are all involved in synthesis of viral mRNAs and viral replication [33]. Together, NP, P, and L, in association with the viral RNA, form the "Ribonucleoprotein complex" (RNP), which serves two major functions: 1) RNA synthesis or replication, and 2) RNA transcription into mRNA [33, 40]. The M protein is involved in virus assembly, envelope formation, and budding of the new viral particles [33]. It is 364 amino acids long and has a molecular weight of 40 kDa [33]. The F protein is responsible for inter-membrane fusion to allow viral entry into the host cell [33]. It is a type I transmembrane protein located in the lipid viral envelope, with 553 amino acids in length and it is synthesized as an inactive precursor called F0, which has to be cleaved into F1 (fusion peptide) and F2 in the trans-Golgi to become active and able to initiate inter-membrane fusion [33, 40, 41]. The HN protein mediates attachment of viral particles to the host cell by binding specific receptors on the cell surface that contain sialic acid, and thus facilitates membrane fusion. It also prevents virion self-association through its neuraminidase activity by cleaving sialic acid from the surface of the virions [33, 39, 40]. It is required to interact with the F protein to allow inter-membrane fusion [42] and also provides the ability to agglutinate red blood cells (RBCs) from different species, such as: chickens (and other avian species), amphibian, reptilian, human, horse, mouse, and guinea pigs [13, 43, 44]. Its size is variable depending on the NDV strain and can range from 571 to 616 amino acids in length, with an approximate molecular weight of 74kDa [33]. The V protein is 239

amino acids long with a molecular weight of 36 kDa [33]. It plays an important role on viral pathogenicity by blocking the antiviral effect of interferon alpha (IFN-α) [45-47]. The role of NDV W protein remains uncertain [36].

Newcastle disease virions are pleomorphic and quite variable in size, usually ranging between 100 nm to 500 nm. It possesses a lipid envelope derived from the host's plasma membrane, and its surface is covered with projections of about 8 nm and 12 nm in length that correspond to the F and HN glycoproteins. Associated with the inner side of the viral envelope is the M protein and inside the viral particle is the nucleocapsid [3, 33].

**Virus replication, assembly, and release**

The replication strategy of NDV follows the same order as all the other non-segmented, negative-sense, RNA viruses from the genus *Avulavirus* [13]. The replication process has two primary roles: 1) to produce messenger RNAs that can be translated into structural proteins, and 2) to produce multiple copies of the genomic RNA that will constitute the future viral particles.

First, NDV attaches to the epithelial cells through the binding of the HN viral glycoprotein with cell surface receptors containing sialic acid, such as gangliosides and N-glycoproteins [33]. After the attachment, the F protein undergoes conformational modifications, and the fusion peptide is inserted into the host cell membrane to initiate fusion of the viral and host membranes [33]. After fusion, the RNP complex is released into the cytoplasm of the cell and starts to synthesize leader RNAs and mRNAs encoding viral proteins during a process called "primary transcription". After translation of the first transcripts, when the viral protein concentrations in the cytoplasm have reached high levels, positive full-length copies of the viral genome (antigenomes) are synthesized from

the input RNP complex and coated with NP protein to be used as templates to replicate the viral genome by producing new, negative-sense, genomic RNA molecules. When abundant progeny genomes have been produced, they serve as templates to produce more mRNAs encoding viral proteins during the growth phase called "secondary transcription". Replication cannot be performed if the genomes and antigenomes are not encapsidated; therefore, the continuing transcription and translation during viral replication reflects the need of high levels of unassembled NP protein to encapsidate the oncoming progeny genomic RNA, along with lower levels of P and L proteins [40]. Therefore, it is believed that the levels of unassembled NP protein in the cytoplasm determine when genome replication starts, but it has not yet been proven [40, 48].

The F and HN mRNAs are transported to and translated in the endoplasmic reticulum and undergo post-translation modifications, such as glycosylation and fatty acid acylation during intracellular trafficking [33, 40, 42]. As mentioned before, the inactivated F0 is cleaved in the trans-Golgi into F1 and F2, but both subunits remain linked through disulfide bonds [33, 40, 42]. Both glycoproteins are then transported to the plasma membrane through the exocytic pathway [40], especially to lipid raft regions [49]. The M protein, translated in the cytoplasm, moves to the cell membrane and interacts with the inner leaflet, probably through amphipathic α-helixes and with the F and HN cytoplasmic tails. It also interacts with the RNP complex through acid and basic amino acid interactions and uses the host machinery to bud and pinch off from the cell [40, 50]. The neuraminidase activity of the HN prevents reattachment of the viral particle to the infected cell from which it originated, and also prevents the viruses from self-binding [40].

## NDV Molecular basis for pathogenicity

The virulence of NDV strains varies widely in chickens and is determined by different factors of which the fusion protein cleavage site has been considered the primary determinant. The F protein is synthesized as an inactive precursor F0 that must undergo post-translational cleavage into two disulfide-bounded subunits (F1 and F2) by host cell proteases [3, 33, 40, 51]. Virulent NDV strains have multiple basic amino acids (at least 3) between positions 113 and 116, and a phenylalanine at position 117 ($_{113}$R-Q-R/K-R ↓ $F_{117}$), which is cleaved by furin, a subtilisin-like endoprotease that is a ubiquitous protease present in almost every tissue, and this allows the infection with vNDV to occur in most body tissues. In contrast, loNDV strains have a monobasic cleavage motif and a leucine at position 117 ($_{113}$K/R-Q-G/E-R ↓ $L_{117}$) that can be cleaved only by trypsin, which naturally exists only in respiratory and gastrointestinal tissues, thus limiting the infection to these mucosal surfaces [33, 51-54]. Several studies mutating the cleavage site of the LaSota vaccine strains (low virulence NDV) into a virulent cleavage site have shown increased virulence of the virus, as demonstrated by pathogenicity assays, such as ICPI and MDT, and from the severity of the lesions and tissue distribution of the virus [30, 55-57]. On the other hand, when the F gene from virulent strains, such as Texas GB (Turkey/US(ND) 43084/92) and California (Game fowl/US(CA)/212510/02) were inserted into a mesogenic backbone (Anhinga), the expected increase in virulence was not observed by ICPI and MDT [23], suggesting the presence of other factors involved in virulence determination for NDV.

The role of the HN protein in virulence has also been studied, and it was shown to determine tropism and also contribute to virulence [23, 29, 58]. The HN can be

translated in three different sizes (571, 577, or 616 amino acids) depending on the position of stop codons in the gene, but no effect of its length in virulence has been demonstrated [56]. Experiments conducted by Wakamatsu showed that insertion of the HN proteins from the virulent strain Beaudette C into a LaSota Backbone did not increase the virulence of the virus [59] and neither did the insertion of the HN gene from virulent strains into a mesogenic backbone [23].

The role of internal proteins, such as NP, P, and L, has also been studied through reverse genetics and some evidence of their effect on virulence has been demonstrated [60, 61].

## NDV classification and characterization

NDV is classified based on phylogenetic analysis and also based on pathogenicity and tissue tropism. According to phylogenetic analysis, NDV is divided into two major groups designated as class I and class II [62-64]. Class I contains mainly avirulent viruses commonly isolated from wild birds and is sub-divided into one genotype [62]. Class II comprises most of the virulent viruses circulating around the world divided into eighteen genotypes (I-XVIII) [62, 65, 66].

The length of the NDV genome varies depending on the class, time of emergence and genotype, and three different lengths have been described [67]. Class I viruses have the longest of the APMV-1 genomes, approximately 15,198 nucleotides [67]. Within class II viruses, the genotypes that are considered as "early" (1930-1960) contain 15,186 nucleotides, and the "late" genotypes (after 1960) contain 15,192 nucleotides [63, 67].

The severity of the disease caused by NDV varies widely depending on virus-related factors (virus strain, dose, and route of inoculation) and host-related factors (species, age,

immunological status, and individual susceptibilities). Originally, NDV was classified into four pathotypes, based on the severity of the disease and the tropism of the virus in susceptible chickens: Doyle, Beach, Beaudette, and Hitchner [13, 35]. Currently, there are five recognized pathotypes:

1. **Enteric asymptomatic NDV**, including avirulent viruses that replicate in the intestinal epithelium without causing any clinical signs related with NDV [3, 68].
2. **Lentogenic NDV**, characterized by mild respiratory disease (Hitchner's form) [3, 69].
3. **Mesogenic NDV**, characterized by low rates of mortality in young birds and acute respiratory infection with occasional neurological signs (Beaudette's form) [3, 69].
4. **Viscerotropic velogenic NDV (VVNDV)**, comprises viruses that are able to produce acute disease with high rates of mortality and hemorrhages in multiple organs, especially in the gastrointestinal tract, and may produce neurological signs such as torticollis and tremors (Doyle's) [3, 69].
5. **Neurotropic velogenic NDV (NVNDV)**, mainly characterized for the presence of neurologic signs and high mortality without hemorrhagic lesions in the gastrointestinal tract (Beach's form) [3, 69].

Classification and characterization of NDV strains is based on capability to cause disease and severity, demonstrated by different laboratory tests. Currently, ICPI and the sequence of the fusion protein cleavage site are the international standard tests to determine NDV virulence [2]. The ICPI is required by the OIE for *in vivo* determination of NDV virulence. It consists of inoculating one-day-old chickens intracerebrally and

scoring birds as: 0 if normal, 1 if sick, or 2 if dead over a period of 8 days. The resulting score ranges from 0.0 to 2.0. Viruses scoring between 0.0 and less than 0.7 are considered lentogenic, between 0.7 and less than 1.5 are mesogenic, and equal or greater than 1.5 are velogenic. All of those viruses with an ICPI equal to 0.7 or greater are considered virulent and are notifiable to the OIE due to the threat those pose to international trading [2, 35].

The amino acid sequence of the fusion cleavage site helps to determine the virulence of NDV strains. Virulent strains usually carry three basic amino acids between positions 113 and 116, along with a phenylalanine at position 117 [2, 3, 35].

Some other tests, such as mean death time (MDT) and intravenous pathogenicity index (IVPI), are commonly used but are not part of the international standards recognized by the OIE [35]. MDT is still widely used and entails inoculating 9 to11-day-old, specific-pathogen-free (SPF), embryonated chicken eggs (ECEs) with 10-fold dilutions of the virus and recording the time to death of each embryo. Velogenic viruses are those that present a MDT of up to 60 hours, those that kill the embryos between 60 and 90 hours are classified as mesogenic NDV, and the viruses that take more than 90 hours for a lethal effect are classified as lentogenic [2, 35].

The IVPI also helps to determine the virulence of NDV isolates, and it is performed by inoculating 6-week-old SPF chickens intravenously with 100 µl of a 1:10 dilution of infectious allantoic fluid; the birds are monitored every 24 hours for 10 days and scored as: 0 if normal, 1 if sick, 2 if paralyzed, or 3 if dead. The score ranges from 0.0 to 3.0 [70].

## Clinical signs and pathologic findings

NDV infections have been reported in at least 241 species of birds, representing 27 of the 50 phylogenetic orders of birds [1]. Chickens are probably the most susceptible host. The clinical signs seen with NDV infection vary widely depending on several factors such as: virulence of the strain, tropism of the virus, host species, host age, route of exposure, dose, immune status, and environmental factors among others. General signs of NDV include: depression, loss of appetite, severe dehydration, emaciation, and fever. Birds infected with velogenic strains may result in sudden and high rates of mortality, with very few apparent clinical signs prior to death. VVNDV can cause severe respiratory signs, edema, congestion of the face, conjunctivitis, greenish diarrhea, muscular tremors, torticollis, paralysis of limbs, and opisthotonos. With NVNDV, the neurological signs are often more evident [2, 3, 6, 35]. Mesogenic NDV strains cause respiratory disease in adult chickens, with rare neurological signs, and sometimes cause death in young chickens [2, 3, 35]. Usually lentogenic strains of NDV do not cause disease in young or adult chickens, but on rare occasions may cause respiratory disease in young birds [3, 33].

Pathologic findings are variable depending on the virulence and tropism of the strain. Macroscopic findings from chickens infected with VVNDV are usually characterized by multifocal necrosis and hemorrhage corresponding to lymphoid-associated tissues in theproventriculus, proventricular-ventricular junction, small intestine, cecal tonsils, as well as in the ocular conjunctiva, spleen, and thymus. Atrophy of thymus and bursa are reported, increasing in degree as the disease progresses [69, 71-75]. Macroscopic lesions associated to VNNDV and mesogenic NDV are rare, but

splenic and proventricular congestion [69, 71], and mild splenomegaly and conjunctivitis [69] may be reported, respectively. Lentogenic strains may cause variable degrees of respiratory disease in young commercial chickens, a lesion associated with these strains may be airsacculitis and reddening of the trachea, but these findings may be also associated with secondary infections [69, 76].

Microscopic findings are mostly associated with lymphoid tissue. Tissues from birds infected with VVNDV will show severe necrosis of the spleen and lymphoid-associated tissues (particularly in the gut), and lymphoid depletion in the thymus and bursa. Multifocal necrosis in the pancreas and liver can also be observed, with occasional neuronal necrosis and perivascular cuffing in the brain. Infection with VNNDV will cause multifocal perivascular cuffing, moderate gliosis, and multifocal necrosis of cerebellar Purkinje cells. [69, 71-75].

## Diagnosis

Over the years, different diagnostic strategies for NDV have been developed, but virus isolation in embryonated chicken eggs (ECEs) continues to be the preferred diagnostic tool. Virus can be isolated from oropharyngeal, tracheal, and cloacal swab samples (or feces), as well as from supernatant from homogenized tissue samples (gastrointestinal tract, spleen, liver, or any tissue with visible lesions) [2, 3]. Cleared supernatants from swabs or tissue homogenates are inoculated into the choriallantoic cavity of 9 to 11-day-old ECEs (0.1 – 0.2 mL), using either SPF or NDV-free embryos. Thereafter, ECEs are incubated at 37°C for 4-7 days and candled daily for mortality. Allantoic fluids (AFs) are then collected from dead embryos and from all those that survived until the end of the 7-day incubation period, after a short period of refrigeration

[2, 3, 69]. Alternatively, virus isolation in cell culture can be performed as some viruses may only grow in certain cell types, such as the Ulster strain that grows in chicken liver or chicken kidney cells but not on ECEs [2]. Allantoic fluids (or cell culture supernatants) are then tested for viral presence by hemagglutination (HA) assay, but should also be tested by hemagglutination inhibition (HI) assay using specific antisera or monoclonal antibodies to differentiate from the presence of avian influenza virus and other APMV types, such as APMV-2, APVM-3, APMV-4 and APMV-7 [2, 3, 69]. If samples test negative after the first passage in eggs, subsequent passages are required [2, 69]. If mortality is observed in inoculated embryos, but the HA test results are negative, the AFs should be tested for bacterial contamination. It is important to keep in mind that Cormorant viruses, isolated after 2002 in the United States, do not hemagglutinate but do cause mortality; therefore, those AF samples will require further tests for identification and characterization [3].

Molecular techniques offer a very rapid detection of NDV. In the instance of an outbreak, when a prompt diagnosis is desired, virus isolation may be less desirable due to the period of time required for the identification of the etiological agent. In these instances, the use of real-time RT-PCR (RRT-PCR) has been proven to be a useful tool. This consists of the development of gene-specific primers and probes that bind to a conserved region of the genome, so several strains can be detected with a single set. Primers and probes targeting the M gene are generally used to detect most of the class II viruses, but have failed to detect class I viruses [77] and do not differentiate between vNDV and loNDV. Some modifications to the technique have been made in order to enable this technique to detect class I viruses, including the design of primers and probe

that target the L gene and a few modifications to cycle conditions compatible with the M gene test [21, 78]. For differentiation between vNDV and loNDV, a RRT-PCR test using primers and probes targeting the F gene was developed and validated [20]. Unfortunately, RRT-PCR techniques are affected by genetic variability of NDV isolates as was observed with a NDV isolated from Columbiformes. These viruses escaped detection due to specific mismatches on the probe [79]; therefore a pigeon-specific probe was designed [80]. Usually, the M gene and F gene tests are used together in order to detect NDV and rapidly identify its virulence, which allows timely notifications when appropriate.

Even though molecular techniques offer a faster identification and classification of the virus, it is advisable to also perform parallel virus isolation in ECEs. Detection of infectious virus is still required, and the materials can be used for further characterization using sequencing analysis and ICPI to determine its virulence classification as defined by the OIE [2]. Further details of these and other diagnostic methods have been fully described and discussed in the past and can be reviewed in the following references [2, 3, 35, 63, 69, 81].

## Newcastle disease vaccines

Since ND's first appearance, several studies have been carried out to try to develop vaccines that are able to control the disease. Some of the first attempts consisted of the use of inactivated viruses as vaccines, but were proven to provide insufficient protection [6]. The need for better vaccines to control the ongoing outbreaks incited researchers to try the attenuation of field isolates by multiple passages in embryos to be used as live vaccines. Some examples include strain H (from attenuated Hertz 1933) [82]

and the mesogenic vaccine Mukteswar, which caused disease and mortality [83] (still used today in some regions of Asia) [2]. Continuing the search for better vaccines, Beaudette and collaborators screened several field isolates and selected the Roakin strain, which unfortunately proved virulent when used as a vaccine [84]. Some years later, strains B1 and LaSota were selected [85, 86]. The latter two have become the most widely-used vaccines in the poultry industry over the years and have replaced inactivated vaccines. More than 60 years after their introduction, B1 and LaSota are still the most used as live or inactivated vaccines in poultry.

Live NDV vaccines are inexpensive to produce, tend to induce a strong cell-mediated immune response, some levels of neutralizing antibodies, and they are generally known to induce mucosal immunity. These vaccines are usually administered in drinking water, aerosol, or direct eye drop, and so they enter the avian organism by the oral and ocular mucosae, inducing mucosal immunity. These vaccines are able to induce an immune response within two weeks after administration. However, one of the downsides with live vaccines is that some (LaSota strain) may cause mild to moderate respiratory disease and/or decrease productivity in commercial poultry [87]. Also their effectiveness relies on maintaining the cold-chain, which can be difficult in some regions. But this might be less critical when using more thermostable strains, such as I-2 [88], which has a better thermostability than LaSota and B1. Another issue with live vaccines is their lethality to embryos, and unfortunately, no one has been able to provide a reliable live NDV vaccine that can be administered *in ovo* without causing unacceptable levels of mortality before and after hatch.

Inactivated vaccines do not replicate in the host, and therefore, do not induce clinical disease and do not elicit a strong cell-mediated immune response [89]; however, they usually induce a long-lasting antibody response [90]. These vaccines are more expensive to produce and require more labor during vaccination, since they require subcutaneous or intramuscular administration. Inactivated vaccines do not rely on col-chain maintenance and therefore, are more suitable for areas where it is difficult to maintain.

Current vaccines such as LaSota can protect against clinical disease and mortality but do not completely prevent challenge virus replication and shedding upon infection. It has been suggested by Miller and collaborators that the amount of virus shed into the environment correlates with rates of transmission through the flock [91]. In addition, there is limited use of live vaccines during the chicken's early life due to interference by maternally-derived antibodies. Several attempts have been made to develop improved vaccines, including development of vaccines homologous to the vNDV strains. This strategy has been proven to be effective by inducing higher reduction in challenge virus shedding than the common LaSota vaccine strain, but only when the replication levels are comparable to those for the LaSota vaccine [28, 32, 92, 93]. Unfortunately, not many of these vaccines are available on the market.

Vectored vaccines are another alternative to prevent disease related to vaccination in chickens. Commercial vectored vaccines are now available and consist of a recombinant fowl pox virus (rFPV) or a recombinant herpes virus of turkey (rHVT) expressing NDV's surface glycoproteins [3]. The rHVT expressing NDV-F confers long-lasting protection against challenge with vNDV after a single application without causing clinical signs [94, 95]. Unfortunately, with this vaccine, it takes at least 4 weeks to

mount a protective immune response [94, 96], which might compromise the vaccinated flocks during an outbreak. Also, there is no reliable test to determine and monitor the antibody response of the vaccinated.

*In ovo* vaccination has been explored as an option to overcome maternal-antibody interference and confer early protection. As of now, there are two vaccines that can be administered *in ovo*. The first option is the rHVT expressing the F protein from NDV, but as mentioned above, it requires a protracted period of time to induce a protective response. The second option is a live NDV conjugated with an antibody. The antibody is slowly released from the virus over time, which prevents the usual mortality observed when using a live NDV vaccine administered *in ovo* [97], but no commercial product has reached the market.

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# CHAPTER 3

# DEVELOPMENT OF AN IMPROVED VACCINE EVALUATION PROTOCOL TO COMPARE THE EFFICACY OF NEWCASTLE DISEASE VACCINES[1]

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## Abstract

While there is typically 100% survivability in birds challenged with vNDV under experimental conditions, either with vaccines formulated with a strain homologous or heterologous (different genotype) to the challenge virus, vaccine deficiencies are often noted in the field. We have developed an improved and more stringent protocol to experimentally evaluate live NDV vaccines, and showed for the first time under experimental conditions that a statistically significant reduction in mortality can be detected with genotype matched vaccines. Using both vaccine evaluation protocols (traditional and improved), birds were challenged with a vNDV of genotype XIII and the efficacy of live heterologous (genotype II) and homologous (genotype XIII) NDV vaccines was compared. Under traditional vaccination conditions there were no differences in survival upon challenge, but the homologous vaccine induced significantly higher levels of antibodies specific to the challenge virus. With the more stringent challenge system (multiple vaccine doses and early challenge with high titers of vNDV), the birds administered the homologous vaccine had superior humoral responses, reduced clinical signs, and reduced mortality levels than those vaccinated with the heterologous vaccine. These results provide basis for the implementation of more sensitive methods to evaluate vaccine efficacy.



***Keywords***

NDV; Vaccine efficacy; Newcastle disease; Homologous vaccination; Mortality


## Introduction

Newcastle disease (ND) is one of the most important diseases affecting poultry world-wide. It is caused by virulent strains of Newcastle disease virus (vNDV), also

known as avian paramyxovirus serotype 1 (APMV-1) [1, 2]. NDV belongs to the genus Avulavirus of the family Paramyxoviridae [1, 2]. The virus genome consists of a single-stranded, negative sense, non-segmented, RNA molecule with approximately 15.2 kb which encodes six structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion (F), hemagglutinin-neuraminidase (HN), and RNA polymerase (L) [1, 2]. Genome sequence analysis of multiple NDV isolates allowed their classification into two major classes (class I and II). Class II is subdivided into at least eighteen genotypes (I to XVIII), and contains most of the vNDV strains circulating in poultry around the world [3, 4, 5]. According to the OIE, virulent strains are defined as those NDV containing an F protein cleavage site with at least three basic amino acids between position 113 and 116, and a phenylalanine at position 117, or an intracerebral pathogenicity index ≥0.7 [6]. Infection with vNDV in countries with endemic disease results in significant economic losses to the poultry industry due to decreased growth rates and to drop in egg production in vaccinated birds, or due to high levels of mortality in naïve or poorly vaccinated birds. Control of ND requires implementation of expensive culling measures, preventive vaccination and biosecurity measures to prevent the disease from spreading [1, 7].

Several studies have concluded that classical live or inactivated vaccines made of viruses of genotype I or II (heterologous), when administered to healthy birds in adequate doses, are capable of preventing clinical disease and death caused by infection with vNDV strains from most of the current circulating genotypes, but do not completely prevent viral replication and shedding [8, 9, 10, 11, 12, 13, 14]. Previously, advantages of the use of genotype matched (homologous) vaccines have been demonstrated only at the

level of control of viral shedding [15]. Studies performed in our laboratory, and by others, demonstrated decreased oropharyngeal virus shedding after challenge when the genotype of the vaccine virus was homologous to the genotype of the challenge virus compared to vaccines that did not match the genotype of challenge virus [11, 16, 17, 18]. In addition, it was demonstrated that decreasing shedding of the challenge virus can potentially reduce horizontal transmission of vNDV [18]. Unfortunately, Title 9 of the Code of Federal (9 CFR) regulations does not take into account the determination of viral shedding after challenge as part of the NDV vaccine evaluation process as it does with other vaccines such as avian infectious bronchitis vaccine [19].

Under optimal experimental conditions, ensuring the administration of appropriate doses of vaccine and the sufficient time to induce an immune response, no statistical differences in morbidity and mortality rates between homologous and heterologous NDV vaccines have been observed. It has been argued that new NDV vaccines are not necessary because all NDV strains belong to a single serotype. Furthermore, since the current commercial NDV vaccines protect equally well against morbidity and mortality caused by any virulent NDV strain, again, there is no reason to discontinue the use of the NDV vaccine strains formulated with strains that were isolated in the late 1940s [20]. This justification, based on the use of vaccine evaluation protocols that only measured survival under optimal conditions (using a high vaccine dose and challenging after three weeks post vaccination), along with the fact that the currently circulating vNDV stains belong to genotypes for which no natural lentogenic variants exist, have hampered the development of new NDV vaccines.

Failure to control vNDV with current vaccines and vaccination programs in countries where the virus is endemic (Israel, Egypt, China, Pakistan, Korea, South Africa) [14], [21, 22, 23, 24, 25, 26, 27, 28, 29, 30], challenges the previous justification and underscores the need to improve NDV vaccines, and to re-evaluate the current system for evaluating NDV vaccine efficacy. While results from experimental conditions document the ability of NDV vaccines formulated with NDV strains heterologous to challenge virus to prevent morbidity and mortality, the results in the field are not as convincing. This disconnect between the experimental and the field efficacy of vaccines has encouraged us to develop a more stringent vaccine evaluation protocol, and to demonstrate that it is possible to measure survival differences after challenge in birds vaccinated with homologous vs. heterologous NDV vaccines.

In the present study, we have developed a live attenuated chimeric ND virus that expresses the surface glycoproteins (F and HN) from a recent genotype XIII NDV isolate to document that the NDV vaccine protocol can be improved. Virulent ND viruses from genotypes XIII have been circulating and causing important outbreaks in Pakistan and are very closely related to viruses circulating in Iran and India (unpublished observations, Afonso C. L.). The resulting chimeric vaccine (homologous) was compared to the LaSota vaccine (heterologous) for its effect on preventing clinical signs and virus shedding after challenge following standard and suboptimal dose vaccination schemes on specific-pathogen-free (SPF) birds.

Because of the rapid mortality caused by vNDV (4–6 days post infection) [12, 31, 32], it is also important to develop vaccines that induce rapid immune responses. Here we demonstrate that when an experimental evaluation scheme mimics field conditions (early

and strong challenges); it is possible to measure significant differences in morbidity and mortality between homologous and heterologous NDV vaccines, being the homologous vaccine significantly more effective.

## Materials and methods

### *Viruses*

Virulent NDV isolate Chicken/SPVC/Karachi/NDV/33/2007 (GenBank: GU182331) (PK33) was used in the present study as source of the F and HN genes to generate the chimeric vaccine candidate and as challenge virus. PK33 was isolated in 2007 from commercial poultry in Karachi, Pakistan during a ND outbreak [33, 34, 35], and has been classified into genotype XIII [4]. NDV strain LaSota (LS-wt) is used worldwide as a live or inactivated vaccine and was used here as a control vaccine in the immunization-challenge experiments, comparing its performance to that of the new chimeric vaccine candidate developed and tested in the present study. These two viruses, along with a recombinant LaSota (rLS) virus used as a backbone for the vaccine, were obtained from the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA) repository and propagated in 9–11 day-old specific-pathogen-free (SPF) embryonating chicken eggs (ECEs). The recombinant modified vaccinia virus Ankara expressing the T7 RNA polymerase (MVA/T7) (a gift from Bernard Moss, National Institute of Health) was propagated in primary chicken embryo fibroblast cells (CEF) and used to rescue the chimeric viruses.

***Construction of the full length LaSota clone containing the F and HN genes from PK33***

Total RNA was extracted from allantoic fluids of ECEs inoculated with vNDV isolate PK33 using Trizol-LS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. The F and HN gene coding sequence of PK33 was amplified in a single fragment by reverse transcription polymerase chain reaction (RT-PCR) using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen). PCR amplicons were analyzed by electrophoresis in 0.7% agarose gels and DNA bands of about 4 kb were excised from the gels and purified using a DNA gel extraction kit (GenScript, Piscataway, NJ). The F-HN fragment was cloned into TOPO pCR2.1 (Invitrogen) and grown into Top 10 chemical competent cells using the TA cloning system (Invitrogen). The resulting intermediate plasmid was subjected to site-directed mutagenesis to attenuate the fusion protein cleavage site using the Phusion Site-Directed Mutagenesis kit (New England Biolabs Inc., Ipswich, MA) according to the manufacturer's instructions. The recombinant plasmid containing the NDV LaSota complete genome (pFLC-LaSota) [36] was used as the backbone to construct the recombinant cDNA clone containing the attenuated F-HN fragment from PK33. The vector plasmid and the attenuated F-HN insert were amplified by polymerase chain reaction (PCR) using the pfuULTRATM II Fusion HS DNA polymerase (Stratagene, La Jolla, CA). The vector plasmid was amplified in a single piece excluding only the F and HN genes. The full-length clones were constructed by PCR cloning with the In-Fusion® Advantage PCR Cloning system (Clontech, Mountain View, CA). The reactions were performed as directed by the manufacturer and resulted in the full-length clone pPKLSL.

Every step was confirmed by sequencing analysis with ABI BigDye Terminator 1.1 Reaction Mix & ABI 3730XL DNA Analyzer.

***Virus rescue***

The chimeric virus was rescued by reverse genetic techniques using Hep-2 cells grown and maintained in Dulbeco's Modified Eagle Medium (DMEM) (Corning cellgro, Invitrogen) supplemented with 5% Fetal Bovine Serum (FBS) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) at 37 °C with a 5% CO2 atmosphere. Briefly, the cells were seeded in 6-well plates (1 × 106 cells per well) and infected with MVA/T7 at a multiplicity of infection (MOI) of 3. One hour following infection, the inoculum was removed and the cells were gently washed twice. Five hundred μl of Opti-MEM containing 1 μg of cDNA (pPKLSL), 0.5 μg of pTM-NP (expression vector containing the NP gene from NDV), 0.25 μg of pTM-P (expression vector containing NDV P gene) and 0.1 μg of pTM-L (plasmid expressing the L gene from NDV) were mixed with 500 μl of Opti-MEM containing Lipofectamin 2000 (Invitrogen) and the total 1 mL mixture was added to the MVA/T7 infected cells. Six hours post-transfection, the supernatants were replaced by 2 mL of fresh DMEM containing no FBS, 1% antibiotics and supplemented with porcine pancreatic trypsin (1 μg/mL) (Sigma–Aldrich, St. Louis, MO). At 72 h post-transfection, the cells were harvested after 3 rapid freeze-and-thaw cycles and the cell lysates were cleared by centrifugation at 1200 × g for 10 min. Thereafter, 300 μl of cleared cell lysates were inoculated into 9–10 day old ECE's. The allantoic fluids were collected 6 days post-inoculation and tested by hemagglutination assay (HA) with chicken red blood cells [1], [2] and [6]. All HA positive samples were subjected to RNA extraction, RT-PCR and sequencing to confirm the identity of the rescued virus. The

rescued virus was designated as NDV/rPK-FHN*L-LaSota/102611/SCG. From now on this chimeric vaccine candidate will be referred to as rLS-PK33 [36].

***Chickens and eggs***

All four-week-old, or one-day-old White Leghorn chickens and 9-11 day-old SPF ECEs were obtained from the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA) SPF flocks. Birds were housed in negative pressure isolators in Biosecurity Level 3 Enhanced (BSL-3E) facilities and received food and water ad libitum.

***In vivo characterization experiments***

**Intracerebral pathogenicity index (ICPI) assay**. One day-old SPF chicks were inoculated intracerebrally with 50 µl of a 1:10 dilution of allantoic fluid harvested from ECEs infected with rLS, LS-wt, rLS-PK33 and PK33. Birds were monitored every 24 h during 8 days and scored as follows: 0 = normal, 1 = sick or 2 = dead [2, 6, 37].

**Mean death time (MDT).** Nine to eleven day-old SPF ECEs were inoculated as preciously described [2, 6] with rLS-PK33. Allantoic fluids were harvested after death or at the end of the experimental period (6 days post-inoculation) from chilled eggs and used to determine virus titers by HA test and using the Spearman-Kärber method to calculate the $EID_{50}$/mL [38].

***Vaccination and challenge experiment I***

Thirty six one-day-old White Leghorn SPF chickens were randomly allocated into 3 experimental groups (n = 12) identified as follows: 1) Sham control, 2) rLS-PK33 and 3) LS. Birds in sham group 1 received 100 µl of BHI, group 2 was vaccinated with rLS-PK33 and group 3 was given of LS-wt, both with a target dose of $10^{6.5}$ $EID_{50}$/bird

(vaccine back titers: $10^{6.5}$ $EID_{50}$/bird and $10^{6.1}$ EID50/bird, respectively). All vaccines were administered as live viruses, inoculating 50 µl via eye drop into the right eye and 50 µl into choanal cleft. Birds were monitored daily for characteristic ND clinical signs. Oropharyngeal and cloacal swab samples were collected on days 2 and 4 post-vaccination (pv) for VI of the vaccine virus. Blood samples were collected on day 14 pv from each bird via wing-vein for serology and all groups were challenged with 100 µl of PK33 at $10^{7.3}$ $EID_{50}$/bird following the same protocol as in vaccination. Each group was observed until day 14 post-challenge (dpc); blood samples were collected at this time point for serology. At 2 and 4 dpc all remaining birds were swabbed by oropharynx and cloaca for VI and titration.

***Vaccination and challenge experiment II***

One hundred and sixty 4-week-old White Leghorn SPF chickens were randomly allocated into 16 groups (n = 10). All groups were vaccinated following the same protocol mentioned above. Group 1 received BHI (Sham control). Groups 2, 3 and 4 were vaccinated with live LS-wt at $10^4$, $10^5$ and $10^6$ $EID_{50}$/bird, respectively. Groups 9, 10, 11 and 12 were vaccinated with LS-wt at $10^3$, $10^4$, $10^5$ and $10^6$ $EID_{50}$/bird, respectively. Groups 5 and 13, 6 and 14, 7 and 15, and 8 and 16 received rLS-PK33 at $10^3$, $10^4$, $10^5$ and $10^6$ $EID_{50}$/bird, respectively. Seven days after vaccination, sera were harvested on birds from groups 1 through 8, and thereafter were challenged with vNDV isolate PK33 at $10^{8.5}$ $EID_{50}$/bird, half dose by eye drop and half dose by choanal cleft. Those birds were observed daily for up to 14 days after challenged to record mortality and ND clinical signs. Fourteen days after vaccination sera from birds in groups 9 through 16 were collected and the birds were challenged with PK33 at $10^{8.5}$ $EID_{50}$/bird, the same as the

previous groups, and were monitored daily until day 14 after challenge (Table 3.1). Mortality was recorded and the sera were tested by HI test to determine titers of antibodies specific to vNDV PK33.

***Virus isolation, titration and serological assays***

Virus isolation was performed in 9–11day old SPF ECE and the allantoic fluids were tested by HA as per standard protocol [1, 2, 6]. All positive swab samples were titrated using 9–11 day-old ECEs as previously described [1, 2, 6]. Virus titers were calculated using the Spearman-Kärber method [38]. Antibody levels were determined by hemagglutination inhibition (HI) assay from pre- and post-challenge serum samples using round-bottomed 96-well micro titer plates as previously described [1, 2, 6].

***Evolutionary divergence analysis***

The evolutionary distance between the LaSota vaccine and vNDV strain PK33 genotypes II and XIII, respectively, was estimated using the full amino acid sequence of the F and HN genes. Analyses were conducted using the JTT matrix-based model [39]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analyses were performed on July 2013 using MEGA software version 5.21.

***Statistical analysis***

HI and virus titers are expressed as arithmetic means plus or minus the standard error of the mean for each vaccine group. Animals negative for VI were also included in the group mean. Group means were analyzed by ANOVA and either Tukey's or Sidak's tests for multiple comparisons, and using Student's t-test when comparing only two groups at a time. Also, correlation and simple linear regression were performed. The survival curves were analyzed using the Log-rank test. The level of significance used to

determine statistical differences among groups was 5% ($\alpha = 0.05$). The data was analyzed using Prism software version 6.0.

***Animal care statement***

All experiments were conducted complying with protocols reviewed and approved by the SEPRL institutional biosafety committee and were conducted with appropriate measures to maintain biosecurity and biosafety. General care of chickens was provided in accordance with the procedures reviewed and approved by the SEPRL Institutional Animal Care and Use Committee, as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

## Results

***Evolutionary divergence analysis***

The amino acid divergence of the predicted F and HN proteins from NDV LaSota and vNDV strain PK33 was quantified. The LaSota vaccine strain differs from PK33 by 11.3% in amino acid sequence at the F protein. For the HN protein, the difference is of about 13.5% between LaSota and PK33.

***Development and in vivo characterization of candidate vaccine viruses***

A chimeric vaccine candidate was developed by replacing the F and HN genes from NDV LaSota vaccine strain with the F and HN genes from vNDV PK33. This chimeric vaccine was successfully rescued by reverse genetics and the attenuation of the cleavage site was confirmed by nucleotide sequencing and determination of the ICPI in one day-old chickens and the MDT in ECEs. The ICPI results, MDT values and the cleavage site amino acid sequences from the chimeric vaccine and the parental virulent virus are shown in Table 3.2. The vaccine candidate and LS-wt had ICPI values

compatible with NDV strains of low virulence (from 0.06 to 0.30) while the parental virulent virus exhibited high ICPI (1.85). The cleavage site of the vaccine rLS-PK33 was confirmed to be identical to the low virulence cleavage site from the LaSota vaccine (112G R Q G R L117). Furthermore, the MDT value for rLS-PK33 (144.4 h) classified this virus as low virulent NDV. The chimeric vaccine replicated at titers greater than $10^9$ in eggs, comparable to the titers of the LaSota vaccine (Table 3.2).

***Vaccination and challenge experiment I***

To confirm the protection conferred by the chimeric live vaccine upon challenge with homologous vNDV, one-day-old SPF chickens were vaccinated with rLS-PK33 and were challenged with the vNDV strain PK33. In addition, one more group was vaccinated with LS-wt and challenged with PK33 to compare the previous group to the LaSota vaccinated group. Our observations indicated that all the SPF birds were protected from disease and mortality in all the vaccinated groups after challenge, but 100% of birds from the sham control succumbed by day 4 post-challenged (dpc) with PK33 (Fig. 3.1). The bird MDT of the non-vaccinated sham group after challenge, suggested that the challenge vNDV strain was highly virulent to naïve birds; however no clinical signs were observed in any of the vaccinated animals during two weeks of observation after the challenge.

Pre- and post-challenge serum samples were tested for HI to determine differences in levels of specific antibodies between LS-wt and rLS-PK33 vaccinated birds. Results are presented in Table 3.3. When comparing pre-challenge HI titers for the same sera tested against both LS-wt and PK33 antigens, sera from LS-wt vaccinated birds had significantly higher HI titers specific to LS-wt than those specific for PK33 ($P = 0.0014$); vice versa, sera from rLS-PK33 vaccinated birds had higher HI titers against

PK33, however the difference was not statistically significant (P = 0.1180). When comparing between groups, the HI titer of antibodies specific to PK33 virus present in serum taken before challenge was significantly higher (P < 0.049) in SPF birds vaccinated with rLS-PK33 than in LS-wt vaccinated birds, indicating that rLS-PK33 produced better antibody response against the challenge virus. In the post-challenge serum, the antibody titers against PK33 were higher in LS-wt group challenged with PK33 than that for the rLS-PK33 group, thus suggesting that this homologous vaccine decreased better the viral shedding of PK33 following challenge ( Table 3.3).

Oropharyngeal and cloacal swab samples were collected in order to determine and compare the amount of vaccine virus shed from vaccinated and non-vaccinated birds, and to compare the number of birds shedding and the viral titers between groups challenged with the same virulent virus. When viral shedding from LS-wt and rLS-PK33 groups were compared at 2 and 4 dpv, no statistical differences were found in the number of birds shedding vaccine virus from oropharynx and the amount of virus being shed between those two groups was not significantly different, neither at 2 dpv (P = 0.5799) nor at 4 dpv (P = 0.8646), suggesting that our rLS-PK33 vaccine virus replicates at the same level as the LS-wt vaccine strain (Table 3.4 and Fig. 3.2). However, as it is shown in Table 3.4, at both 2 and 4 dpc the rLS-PK33 group had significantly fewer birds (2 at both time points) shedding challenge virus compared to the LS-wt group (8 and 7 birds respectively) (2 dpc P = 0.002; 4 dpc P = 0.045). There were no significant differences in number of birds shedding challenge virus from cloaca between vaccine groups (P = 0.478); however, both vaccine groups had significantly fewer birds shedding challenge virus from cloaca when compared to the sham control (P < 0.05). Although both vaccine

groups shed significantly lower amount of virus than the sham control ($P < 0.001$) after challenge, the amount of challenge virus being shed from oropharynx was significantly lower in the group vaccinated with rLS-PK33 than in the group vaccinated with LS-wt at 2 dpc ($P = 0.0046$) and 4 dpc ($P = 0.0084$) ( Fig. 3.3). When comparing the amount of virus shed from cloaca, both vaccinated groups shed significantly less virus than the sham control ($P < 0.0001$); however, no significant differences between vaccine groups were found neither at 2 dpc ($P = 0.9999$) nor at 4 dpc ($P = 0.3282$).

***Vaccination and challenge experiment II***

The objective of this experiment was to generate vaccination conditions that might reflect what happens in the field, where the birds may receive very high challenges and lower doses of vaccine than intended. Along with multiple vaccine doses ($10^3$–$10^6$) we challenged the birds with a very high dose of vNDV PK33 ($10^{8.5}$) at two early time points (day 7 and 14 post vaccination). Here we wanted to determine differences in protection against mortality between LS-wt (heterologous) and rLS-PK33 (homologous) vaccines in addition to comparing the specific humoral immune response induced by vaccination. When the birds were challenged at 7 days after vaccination, it was observed that just 10% of the birds in the group vaccinated with rLS-PK33 at $10^3$ $EID_{50}$ survived after the challenge. There was no LS-wt group vaccinated at the same dose to compare. At an $EID_{50}$ of $10^4$ there was no statistical significant difference as 40% of the birds survived in the LS-wt vaccinated group and just 30% survival was observed in the rLS-PK33 vaccinated group. Most important, when groups vaccinated at an $EID_{50}$ of $10^5$ were compared, a higher percent survival was observed in the homologous vaccinated group (100%) than in the heterologous vaccinated group (70%), but due to the low number of

animals there was not enough power to detect significant difference between these two curves (P = 0.0679). At a dose of $10^6$, there was no statistical difference between groups as 100% survival for both LS-wt and rLS-PK33 vaccinated groups was observed (Fig. 3.4-A). In general, all birds in the non-vaccinated group, 3 birds from group LaSota $10^4$ and 4 birds from group rLS-PK33 $10^4$ presented moderate to severe conjunctivitis and severe depression before death, while all survivors had no visible clinical sign.

When the challenge was given at 14 days after the vaccination, we observed a significant difference in the percentage of survival between LS-wt and rLS-PK33 vaccinated groups at a dose of $10^3$. While the LS-wt vaccinated group had 0% survival, the rLS-PK33 vaccinated group had 40% survival ($P < 0.001$). As the vaccine dose increased also did the percent survival, from 70% to 100% and 100% for vaccine doses $10^4$ and $10^5$ and $10^6$, respectively for both LS-wt and rLS-PK33 vaccinated groups, showing no statistical difference between vaccine strains ($P > 0.05$) at these doses (Fig. 3.4-B). The effect of timing on the protective efficacy of the vaccines was assessed by comparing groups challenged at 7 dpv with the groups challenged at 14 dpv. As expected, results showed better protection against clinical signs and mortality in the 14 dpv challenged groups (Fig. 3.4-A and 3.4B). In these groups, all birds vaccinated with LS-wt at a dose of $10^3$, 6 birds from those vaccinated with rLS-PK33 at $10^3$, and 3 birds from each group either vaccinated with LS-wt or rLS-PK33 at a dose of $10^4$ also presented moderate to severe conjunctivitis and depression before death. Two of the survivors from the rLS-PK33 group vaccinated at $10^3$ had mild depression and one of them presented moderate unilateral conjunctivitis by 3 dpc, but gradually recovered within the next 5 days.

In order to explain the differences in survival for groups vaccinated with LS-wt $10^5$ versus rLS-PK33 $10^5$ challenged at 7 dpv (Fig. 3.5-A), and in groups vaccinated with LS-wt $10^3$ versus rLS-PK33 $10^3$ challenged at 14 dpv (Fig. 3.5-B), the HI titers of antibodies specific to the challenge virus (PK33) and LS-wt induced by vaccination were determined from the serum samples collected before the challenge. The pre-challenge antibody titers against PK33 and LS-wt and the corresponding times of death per bird after challenge are shown in Table 3.5. Birds that survived until the end of the experiment (14 days after the challenge) were recorded with a death time of 14 dpc for analysis purposes. As it is shown (Table 3.5), those birds that presented HI titers against PK33 equal or greater than 2 survived until the end of the experiment for both challenge time points. When birds were challenged at 7 dpv, birds 225, 226 and 230 that were vaccinated with LS-wt $10^5$ presented HI titers (PK33) lower than 2 and died; for most of the birds vaccinated with rLS-PK33 $10^5$ the HI titers were greater or equal than 8 showing a better antibody response against PK33 than the LS-wt vaccinated group and with a 100% of survival compared with 70% survival for the LS-wt vaccinated group. For groups challenged at 14 dpv, the LS-wt 103 group had no survivors and the HI titers (PK33) for all those birds were lower than 2. On the other hand, the rLS-PK33 103 group had 40% survival; with survivor HI titers greater or equal than 2 and non-survivor HI titers lower than 2. All birds (dead ones and survivors) from these 4 groups were proven to have antibodies specific to LS-wt, showing that the birds were vaccinated and that the mortality may have depended on whether or not they developed specific antibody response against the challenge virus (PK33) (Table 3.5). In addition, a significant positive correlation was found between the HI titers of antibodies specific to the challenge virus

and the time to death, indicating that the higher the specific antibody titers against the challenge virus, the later the time of death ($P = 0.0037$; $r = 0.4488$).

When analyzing the pre-challenge HI titers specific to PK33 from all the groups, it was confirmed that the homologous-vaccinated groups, no matter the dose, had higher titers of antibodies specific to PK33 than the LaSota vaccinated groups. As it is shown in Fig. 3.5-A for the groups sampled 7 dpv, groups vaccinated with rLS-PK33 at a dose of $10^5$ and $10^6$ presented significantly higher HI titers against PK33 than the LaSota vaccinated groups at the same vaccine doses ($P = 0.033$ and $P < 0.001$, respectively). For the groups sampled 14 dpv, we did observe an increment on HI titers with respect to the groups sampled at 7 dpv, but must importantly we did see again that the groups vaccinated with rLS-PK33 at $10^6$ had significantly higher HI titers specific to PK33 than the LS-wt vaccinated group at $10^6$ ($P = 0.014$) ( Fig. 3.5-B).

## Discussion

We present here the first demonstration of increased survival conferred by a homologous NDV vaccine in comparison to a classical heterologous vaccine. The advantages of using homologous vaccines to decrease shedding of vNDV after challenge have previously been shown [15, 16, 17, 18, 40]; however, none of these studies have shown superior protection against mortality. As an example, a recent publication from Kim et al. using similar chimeric vaccines demonstrated that the F and HN genes were sufficient to provide 100% survival under optimal vaccination conditions. However, this study did not address the problems that occur when vaccinated flocks present with morbidity and mortality events, nor did it demonstrate any difference in survival in comparison to heterologous vaccines. Unfortunately, additional evidence supporting the

existence of statistical differences in antibodies levels was inconclusive, and differences in viral shedding was not statistically supported as only 3 animals per group were analyzed [40].

As Newcastle disease continues to spread around the world despite massive vaccination efforts, demonstration of increased survival is critically important because the lack of differences in protection against clinical disease and death falsely validates the continuous use of heterologous vaccines worldwide [9, 10, 12, 13, 16]. One of the problems in testing vaccines is that 100% protection under laboratory conditions do not always translate in an effective field vaccine. Until now it has not been possible to replicate in an ABSL-3E setting what field conditions may contribute to vaccine failure or to document how to abrogate those conditions. Furthermore, some authors have found no difference between the effect of homologous and heterologous vaccines on viral shedding following challenge, and have attributed field vaccine failures mainly to inadequate vaccination or to secondary immunosuppressive infectious agents [10], arguing that simply improving vaccine application protocols rather than improving the antigenic similarities between vaccine viruses and virulent field viruses will improve ND control [10]. Even though mass application of vaccine in water or by spray is not an ideal system to achieve the 85% immunity level necessary for herd immunity necessary to control ND [41], as only 53–60% of birds will develop a proper immune response [42], it is unlikely that producers will use more labor intensive methods.

In experiment II birds vaccinated with the homologous vaccine at doses similar to what occasionally may be found in the field (suboptimal) and challenge with vNDV 7 and 14 days later, before the immune response has peaked, had a significant increase in

survival rates and in antibody levels compared to the birds vaccinated with the heterologous vaccine. The novelty of this work stems from the development of a protocol to evaluate NDV vaccines based on four different elements: vaccination using multiple suboptimal doses, a high dose challenge, an early challenge, and live vaccines that replicate at comparable rates as the control LaSota vaccine. The combination of this very sensitive vaccination-challenge protocol with the development of a vaccine that replicates at the same rate as the control vaccine has allowed the demonstration of statistical significant differences in survival between the two types of vaccines.

Our data suggest that the differences in the F and HN glycoproteins between challenge and vaccine viruses may be sufficient to affect the performance of the vaccine during early challenges or when vaccine doses are low, as often occur in the field. Two of the most widely used NDV vaccine strains, LaSota and B1 (genotype II), have been in use for more than 60 years [43]. These vaccine viruses are genetically distant from the genotypes of vNDV currently causing outbreaks in several regions of the world (V, VI, VII, XIII, etc.) [4] and [15]. In experiment I using standard vaccine doses, we demonstrated the typical laboratory outcome of vaccine evaluation. The homologous vaccine expressing the F and HN genes identical to the challenge virus, like the heterologous vaccine, produced 100% survival. However, it was significantly more efficient than the LaSota vaccine at reducing the amount of virus shed from oropharynx after challenge. In addition, experiment II provides for the first time statistically significant differences in survival and demonstrates significantly higher levels of humoral antibodies specific to the challenge virus. The next step would be to repeat this experiment in commercial birds with known titers of maternal antibodies and determine

whether this experimental vaccine evaluation protocol can identify differences between vaccines.

It is also important to emphasize that the live chimeric NDV vaccine evaluated here performed as our hypothesis suggested; however, not all live chimeric vaccines may perform as well. Therefore, all newly developed vaccines should be critically evaluated since the efficacy of live vaccines may depend on the ability of the virus to replicate in birds.

In conclusion, our results confirm once more that there can be significant advantages conferred by the use of vaccines homologous to circulating vNDV such as improved potential to reduce environmental viral load [18], increased level of specific humoral antibodies, and increased survival rates after challenge. As a result, vaccine companies may consider the implementation of more sensitive methods to evaluate vaccine efficacy, such as the use of different vaccine doses, high titers of challenge virus, different challenge times, and measurement of virus shedding following challenge to better predict the outcome under field conditions. The implementation of an improved evaluation system for NDV vaccines together with the use of NDV vaccines that decrease the amount of vNDV shed from vaccinated birds may offer an opportunity to stunt the number of ND outbreaks. The current problem with the evaluation of NDV vaccines is likely to apply to the evaluation of other respiratory viral diseases vaccines; therefore, we are suggesting that the capacity to reduce viral shedding should be consider as part of the vaccine design and evaluation criteria.

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## Author contributions

C.L.A., P.J.M., S.C.G, E.L.D. and C.C.B were involved in the conception and design of the study. S.C.G., C.L.A., P.J.M., D.G.D., and L.S. participated on sample collection. S.C.G. was responsible for sample processing. S.C.G., C.L.A. and P.J.M. analyzed and interpreted the data. Q.Y. developed and donated the recombinant plasmid containing the NDV LaSota full genome (pFLC-LaSota). All authors contributed to the development of this manuscript.

## Conflict of interest

Dr. Eduardo Lucio Decanini is the General Director of Investigacion Aplicada, S.A. de C.V. (IASA), the company that partially funded this research as part of a Cooperative Research and Development Agreement between the USDA and IASA. This CRADA enables IASA to have the right to market this product.

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**Table 3.1 Summary of vaccination and challenge experiment II**. Four-week-old SPF birds allocated into 16 groups ($n$ = 10) and all groups were vaccinated at the same time either with LS-wt or rLS-PK33 at 4 different doses ($10^3$–$10^6$); group 1 was designated as the sham control and received BHI instead of vaccine. All groups were challenged with vNDV PK33 at either 7 dpv (groups 1–8) or 14 dpv (9–16).

| Group | Vaccine | Dose ($EID_{50}$[a]/bird) | Challenge (PK33 $10^{8.5}$ $EID_{50}$/bird) |
|---|---|---|---|
| **1** | BHI[b] | 0.0 | 7 dpv |
| **2** | LS-wt | $10^4$ | 7 dpv |
| **3** | LS-wt | $10^5$ | 7 dpv |
| **4** | LS-wt | $10^6$ | 7 dpv |
| **5** | rLS-PK33 | $10^3$ | 7 dpv |
| **6** | rLS-PK33 | $10^4$ | 7 dpv |
| **7** | rLS-PK33 | $10^5$ | 7 dpv |
| **8** | rLS-PK33 | $10^6$ | 7 dpv |
| **9** | LS-wt | $10^3$ | 14 dpv |
| **10** | LS-wt | $10^4$ | 14 dpv |
| **11** | LS-wt | $10^5$ | 14 dpv |
| **12** | LS-wt | $10^6$ | 14 dpv |
| **13** | rLS-PK33 | $10^3$ | 14 dpv |
| **14** | rLS-PK33 | $10^4$ | 14 dpv |
| **15** | rLS-PK33 | $10^5$ | 14 dpv |
| **16** | rLS-PK33 | $10^6$ | 14 dpv |

[a]Median embryo infective dose.

[b]Brain heart infusion.

**Table 3.2 ICPI values, MDTs, amino acid sequence of the Fusion protein cleavage site and viral titer reached in embryonating chicken eggs ($Log_{10}$ $EID_{50}$/mL).**

| Virus strain | ICPI[a] value | MDT[b] (hrs.) | Fusion protein cleavage site | $EID_{50}$[c]/mL |
|---|---|---|---|---|
| **PBS** | 0.00 | – | – | – |
| **LS-wt** | 0.30 | 153.25 | $_{112}$G R Q G R $L_{117}$ | $10^{9.3}$ |
| **rLS** | 0.43 | – | $_{112}$G R Q G R $L_{117}$ | $10^{9.5}$ |
| **rLS-PK33** | 0.06 | 144.4 | $_{112}$G R Q G R $L_{117}$ | $10^{10.1}$ |
| **PK33** | 1.85 | 54.5 | $_{112}$R R Q K R $F_{117}$ | $10^{10.1}$ |

[a]Intracerebral pathogenicity index; <0.7, low virulence, ≥0.7, virulent.

[b]Mean death time in eggs; >90 h low virulence, <60 h highly virulent.

[c]Median embryo infective dose.

**Table 3.3 Pre- and post-challenge cross mean HI antibody titers per vaccine group.** Sera form vaccinated and/or challenged birds were tested against both LS-wt and PK33 viral antigens. The HI titers are presented as the group mean plus or minus the standard error of the mean. Mean HI titers were statistically analyzed between vaccine groups and by viral antigen within groups using two-way ANOVA and Sidak's multiple comparison test. The mean titers of antibodies specific to the vaccine or the challenge viral antigen are indicated in **bold** face. Values sharing symbol (*, £, †, ¥ or ‡) were non-significantly different from one another.

| **Serum/vaccine group** | **Pre-challenge HI titer** | | **Post-challenge HI titer** | |
|---|---|---|---|---|
| | LS-wt[a] | PK33[b] | LS-wt | PK33 |
| **LS-wt** | **24.0 ± 5.7***  | 6.2 ± 1.2† | **320.0 ± 76¥** | 257.3 ± 84.1¥ |
| **rLS-PK33** | 9.0 ± 1.4£,† | **17.0 ± 2.9***,£ | 114.7 ± 26.3‡ | **149.3 ± 36.4‡** |

[a]LaSota viral antigen.

[b]Challenge virus antigen.

**Table 3.4 Number of one-day-old SPF birds shedding virus after vaccination and after challenge.**

| Group | 2Dpv[a] | 4Dpv | 2 Dpc[b] | | 4 Dpc | |
|---|---|---|---|---|---|---|
| | OP[c] | OP | OP | CL[d] | OP | CL |
| **Sham x PK33** | 0/12 | 0/12 | 12/12 | 12/12 | NS[e] | NS |
| **LS-wt x PK33** | 12/12[f] | 12/12[f] | 8/12[f] | 2/12[f] | 7/12 | 1/12 |
| **rLS-PK33 x PK33** | 12/12[f] | 12/12[f] | 2/12[f,g] | 0/12[f] | 2/12[g] | 0/12 |

[a]Days post-vaccination.

[b]Days post-challenge.

[c]Oropharyngeal.

[d]Cloacal.

[e]No survivors.

[f]Denotes significant difference from the sham control.

[g]Denotes significant difference from LS-wt group.

**Table 3.5 Time of death and vaccine-induced pre-challenge antibody titers per bird on four-week-old SPF chickens.[k]**

| Challenged 7 dpv[a] | | | | | | | | Challenged 14 dpv | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **LaSota $10^{5}$[e]** | | | | rLS-PK33 $10^{5}$[f] | | | | LaSota $10^{3}$[g] | | | | rLS-PK33 $10^{3}$[h] | | | |
| | | HI[c] titer | | | | HI titer | | | | HI titer | | | | HI titer | |
| **Bird ID** | Death (dpc)[b] | LS-wt[i] | PK33[j] | Bird ID | Death (dpc) | LS-wt | PK33 | Bird ID | Death (dpc) | LS-wt | PK33 | Bird ID | Death (dpc) | LS-wt | PK33 |
| **221** | 14 | 32 | 4 | 261 | 14 | 8 | 16 | **281** | **4** | **16** | **<2** | **321** | **5** | **16** | **<2** |
| **222** | 14 | 32 | <2[d] | 262 | 14 | 8 | 8 | **282** | **4** | **16** | **<2** | **322** | **4** | **16** | **<2** |
| **223** | 14 | 32 | 4 | 263 | 14 | 8 | 8 | **283** | **4** | **16** | **<2** | **323** | **4** | **16** | **<2** |
| **224** | 14 | 32 | 8 | 264 | 14 | 8 | 8 | **284** | **4** | **16** | **<2** | 324 | 14 | 16 | 32 |
| **225** | **7** | **16** | **<2** | 265 | 14 | 8 | 32 | **285** | **4** | **16** | **<2** | **325** | **4** | **16** | **<2** |
| **226** | **4** | **16** | **<2** | 266 | 14 | 4 | <2 | **286** | **5** | **16** | **<2** | 326 | 14 | 16 | 64 |
| **227** | 14 | 64 | 8 | 267 | 14 | 16 | 8 | **287** | **4** | **16** | **<2** | **327** | **4** | **16** | **2** |
| **228** | 14 | 16 | <2 | 268 | 14 | 16 | 16 | **288** | **5** | **16** | **<2** | **328** | **4** | **16** | **<2** |
| **229** | 14 | 32 | 8 | 269 | 14 | 16 | <2 | **289** | **2** | **32** | **<2** | 329 | 14 | 32 | 2 |
| **230** | **11** | **8** | **<2** | 270 | 14 | 16 | 8 | **290** | **4** | **16** | **<2** | 330 | 14 | 16 | 8 |

[a]Days post-vaccination.

[b]Days post-challenge.

[c]Hemagglutination inhibition.

[d]<2= titer below limit of detection.

[e]I would rather: Birds vaccinated with LaSota at a dose of $10^5$ EID50/bird.

[f]Birds vaccinated with rLS-PK33 at a dose of $10^5$ EID50/bird.

[g]Birds vaccinated with LaSota at a dose of $10^3$ EID50/bird.

[h]Birds vaccinated with rLS-PK33 at a dose of $10^3$ EID50/bird.

[i]LaSota HI antigen.

[j]PK33 HI antigen.

[k]Birds with a death time of 14 dpc are those which survived until the end of the experiment. Fatalities are indicated in **bold** face.

**Fig. 3.1 Percent survival of one-day-old SPF vaccinated chickens after challenge**. Percent survival of one-day-old SPF vaccinated chickens was recorded daily after they were challenged with vNDV strains.

**Fig. 3.2 Oropharyngeal virus shedding at 2 and 4 days post-vaccination in one-day-old SPF chickens.** Oropharyngeal swab samples were collected after vaccination to measure the amount of vaccine virus shed in all vaccinated groups. The mean virus titers expressed as log10 EID50/mL were compared between groups using the multiple comparisons Tukey's test. Bars with equal letter mean non-statistically different from one another ($P > 0.05$).

**Fig. 3.3 Oropharyngeal virus shedding at 2 and 4 days post-challenge in one-day-old SPF chickens.** Oropharyngeal swab samples were collected after the birds were challenged with vNDV to determine the amount of challenge virus shed per group. The mean virus titers were compared between groups by the multiple comparisons Tukey's test. Bars with different letter are statistically different from one another ($P < 0.05$). NS = no survivors.

**Fig. 3.4 Percent survival in 4-week-old SPF vaccinated chickens after challenge.** SPF birds were vaccinated with LaSota or rLS-PK33 at an EID50/bird of 103, 104, 105 or 106 and challenged with PK33 at 7 (A) or 14 (B) after vaccination. Percent survival was recorded for up to 14 days after challenge. Survival curves were compared between groups. The star (*) denotes groups statistically different form one another ($P = 0.028$).

**Fig. 3.5 Pre-challenge HI titers specific to the challenge virus (PK33) per vaccine group.** Serum samples were taken at 7 dpv (A) or 14 dpv (B) and were tested against vNDV-PK33 to measure levels of specific antibodies. Columns sharing letters were not significantly different from one another after analyzed with the Tukey's test for multiple comparisons with a level of significance of 5%.

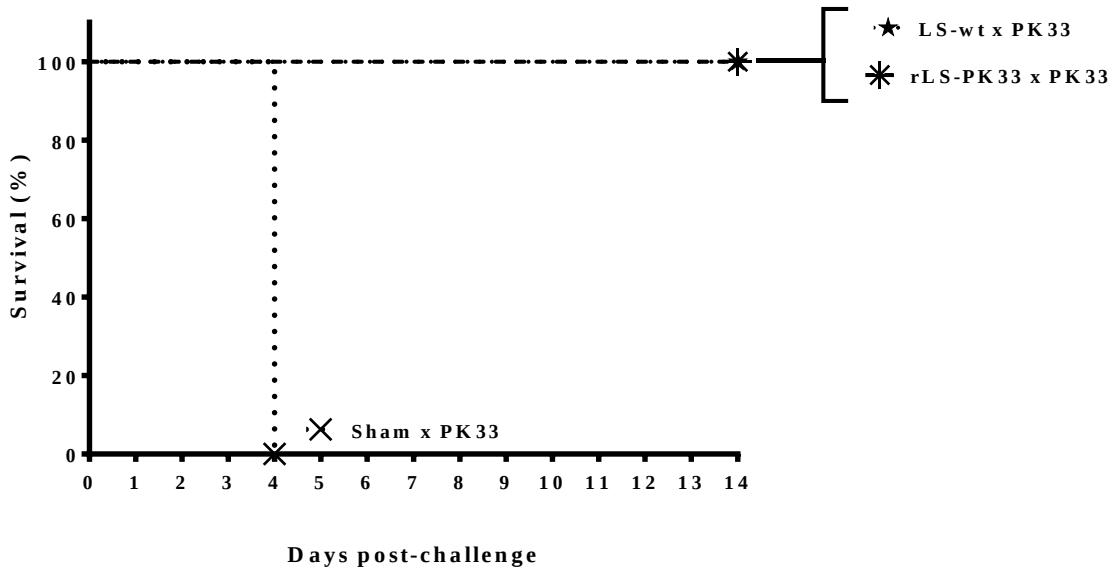

LS-wt x PK33
rLS-PK33 x PK33
Survival (%)
100
80
60
40
20
0
Sham x PK33
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days post-challenge

**Fig. 3.1**

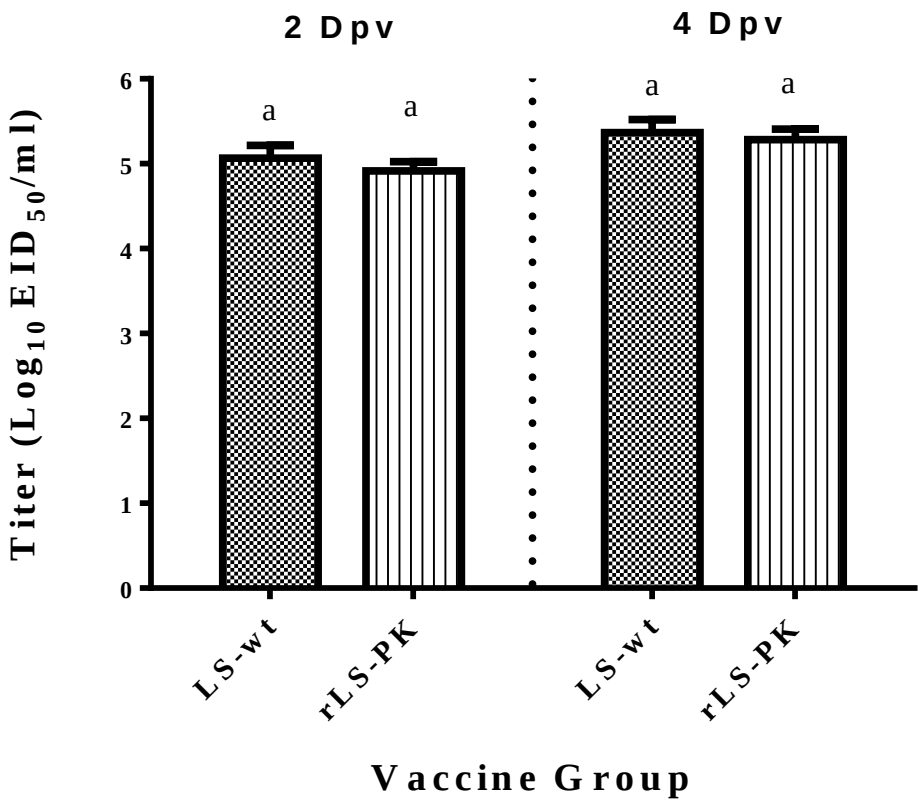

2 Dpv
4 Dpv
Titer (Log10 EID50/ml)
6
5
4
3
2
1
0
a
a
a
a
LS-wt
rLS-PK
LS-wt
rLS-PK
Vaccine Group

**Fig. 3.2**

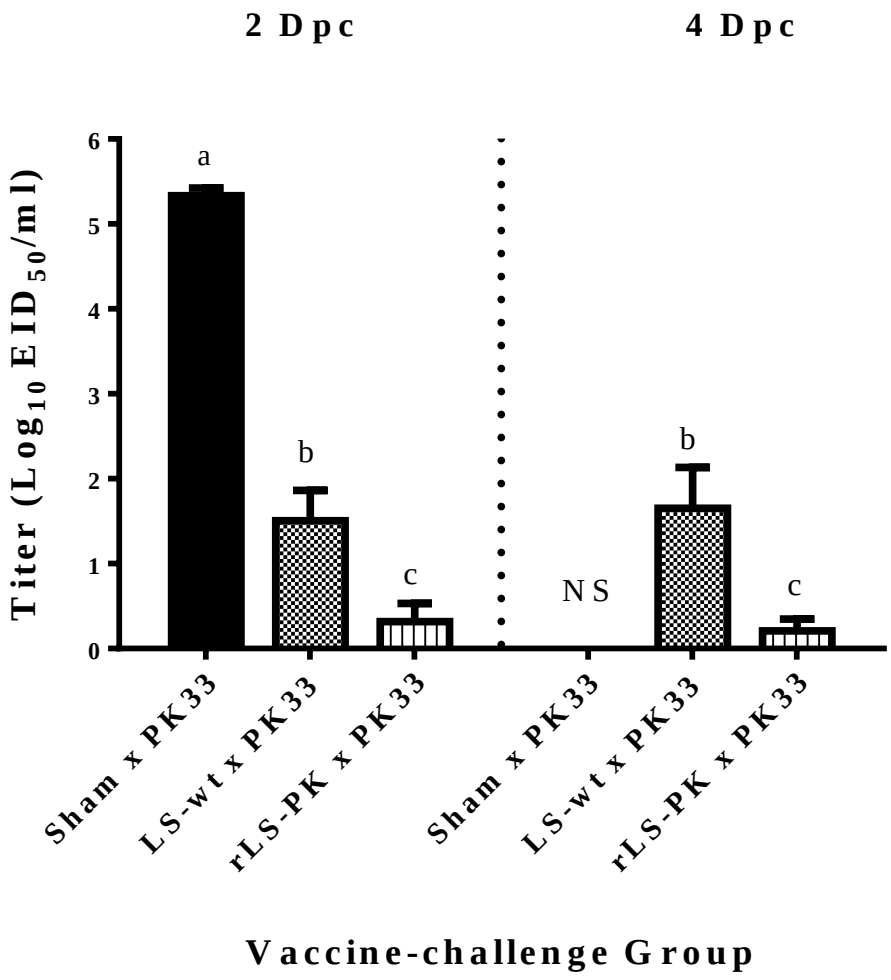
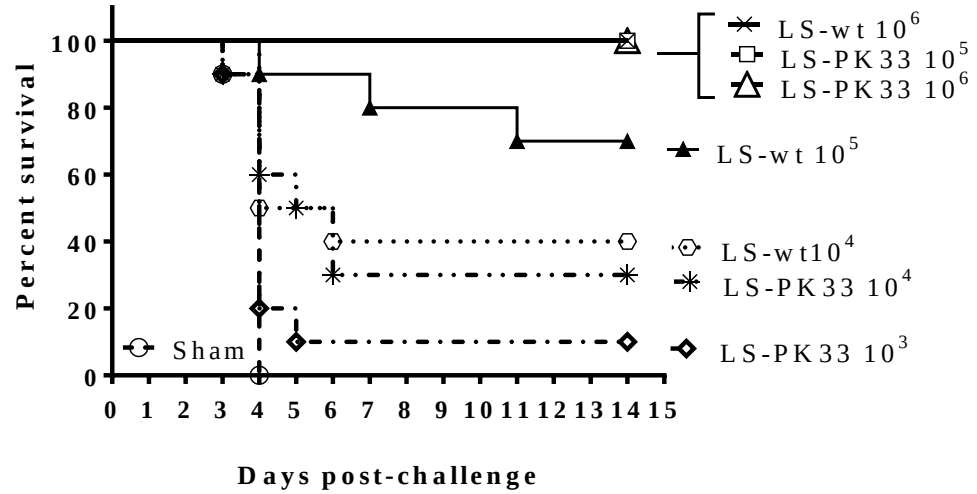

2 Dpc
4 Dpc
Titer (Log10 EID50/ml)
6
5
4
3
2
1
0
a
b
c
NS
b
c
Sham x PK33
LS-wt x PK33
rLS-PK x PK33
Sham x PK33
LS-wt x PK33
rLS-PK x PK33
Vaccine-challenge Group

**Fig. 3.3**

A.


Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days post-challenge
LS-wt 10^6
LS-PK33 10^5
LS-PK33 10^6
LS-wt 10^5
LS-wt10^4
LS-PK33 10^4
LS-PK33 10^3
Sham

B.

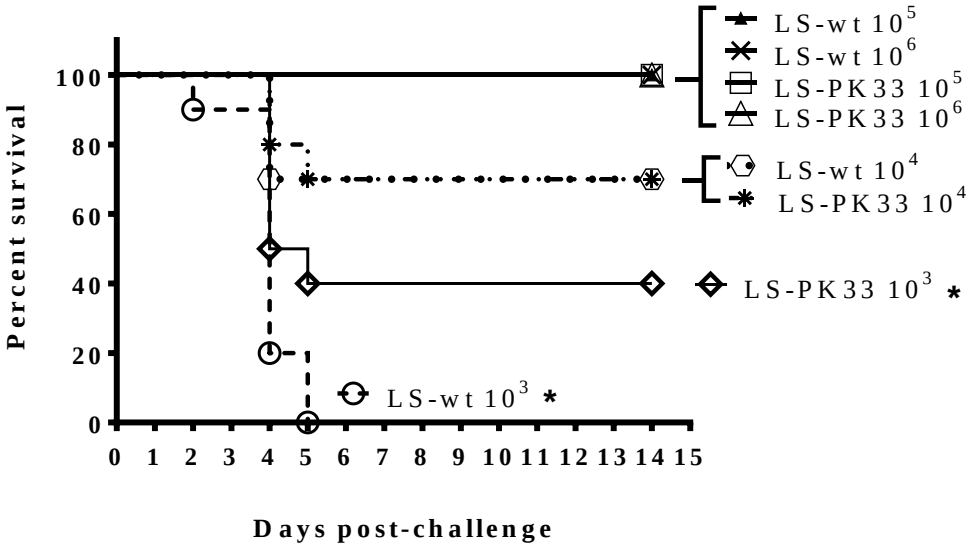
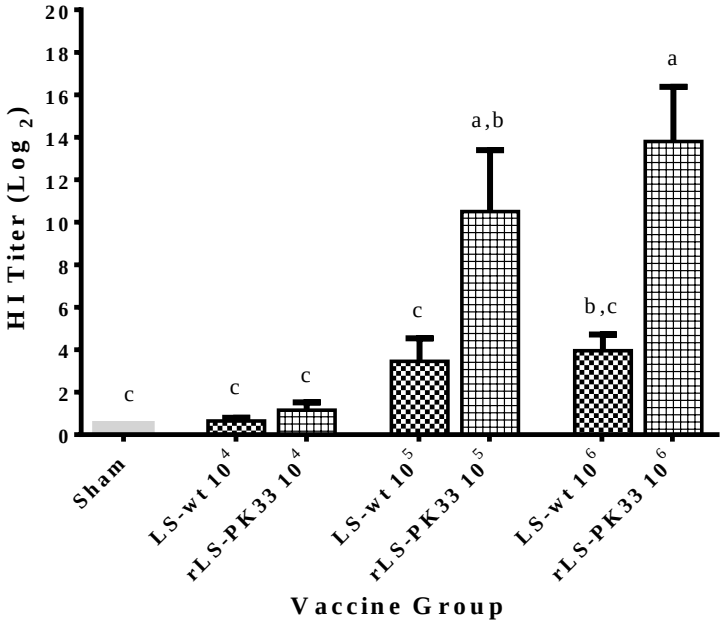

Percent survival
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Days post-challenge
LS-wt 10^5
LS-wt 10^6
LS-PK33 10^5
LS-PK33 10^6
LS-wt 10^4
LS-PK33 10^4
LS-PK33 10^3 *
LS-wt 10^3 *

**Fig. 3.4**

**A**


HI Titer (Log 2)
20
18
16
14
12
10
8
6
4
2
0
c
c
c
c
a,b
b,c
a
Sham
LS-wt 10 4
rLS-PK33 10 4
LS-wt 10 5
rLS-PK33 10 5
LS-wt 10 6
rLS-PK33 10 6
Vaccine Group

**B**

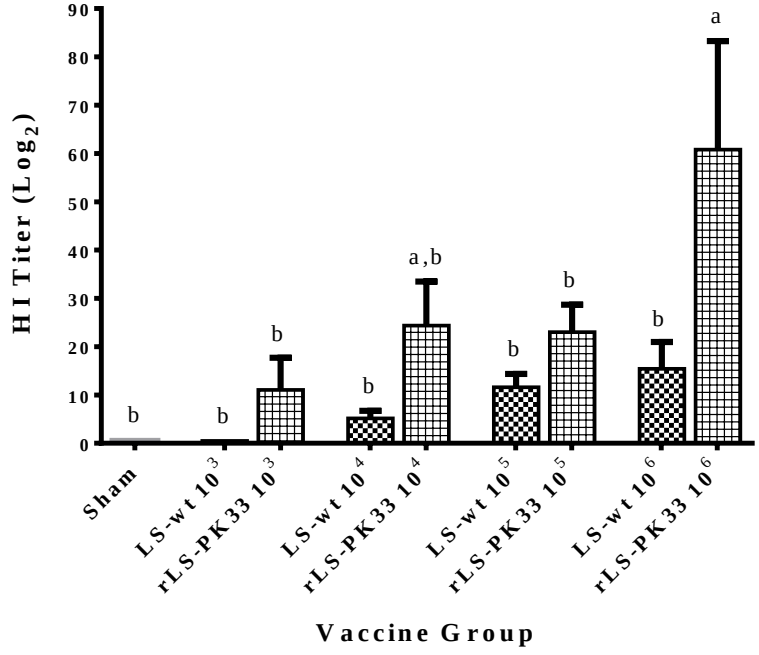

HI Titer (Log2)
90
80
70
60
50
40
30
20
10
0
b
b
b
b
a,b
b
b
b
a
Sham
LS-wt 10 3
rLS-PK33 10 3
LS-wt 10 4
rLS-PK33 10 4
LS-wt 10 5
rLS-PK33 10 5
LS-wt 10 6
rLS-PK33 10 6
Vaccine Group

**Fig.3.5**

# CHAPTER 4

# CO-ADMINISTRATION OF CHICKEN INTERFERON-GAMMA WITH THE VACCINE ANTIGEN DOES NOT SIGNIFICANTLY ENHANCE PROTECTION AND IMMUNITY AGAINST NEWCASTLE DISEASE VIRUS

[1] Cardenas-Garcia, S., Marcano, V., Dunwoody, R.P., Diel, G.D., Williams, R.J., Gogal Jr., R.M., Brown, C.C., Miller, P.J. and Afonso, C.L. 2015. To be submitted to *Plos One*.

**Abstract**

Newcastle disease is one of the most important diseases that affect the poultry industry. Vaccination is one of the main methods used to control clinical disease; however, vaccines do not control virus replication and shedding. The most effective live vaccines in the market also induce mild to moderate respiratory side effects that decrease productivity, while other more attenuated vaccines with fewer side effects are often slow to mount strong immune responses. The current situation underscores the need to develop more effective vaccines that are able to boost the immune response without compromising the health and productivity of the vaccinated chickens. In the present study, three different systems to co-deliver chicken IFN-γ (chIFN-γ) along with vaccine antigen were evaluated for their effectiveness in enhancing the avian immune response and their protective capacity upon challenge with virulent Newcastle disease virus. These systems consisted of: 1) a DNA vaccine carrying NDV-F gene co-administered with an expression vector carrying the chIFN-γ gene used as *in ovo* vaccine and booster vaccine for 2-week-old chickens, 2) a recombinant Newcastle disease virus expressing the chIFN-γ gene (rZJ1*L/IFNγ) used as a live, and 3) the rZJ1*L/IFNγ virus used as an inactivated vaccine. Co-administration of chIFN-γ and DNA vaccine expressing the F gene resulted in higher virus shedding after challenge and higher morbidity and mortality when compared to the group that did not receive chIFN-γ. In addition, the live vaccine system co-delivering chIFN-γ did not enhance the post-vaccination antibody response, neither improved survival after hatch when administered *in ovo*, and neither had a significant effect on survival after challenge when administered in juvenile chickens. Finally, the inactivated vaccine system co-delivering fixed amounts of chIFN-γ did not

exert any significant effect on challenge virus shedding, antigen-specific memory response or survival after challenge. Our results showed that, regardless of the delivery system, chIFN-γ did not enhance the immune response.

**Key words:** chicken interferon-gamma, Newcastle disease virus, vaccine adjuvants, live recombinant vaccine, DNA vaccination, inactivated vaccine, cell-mediated immune response, antibody-mediated immune response.

## Introduction

Despite several years of research, Newcastle disease (ND) remains a significant problem worldwide. Vaccination alone has not been able to eliminate the occurrence of outbreaks with virulent Newcastle disease virus (vNDV) in countries where the disease is endemic [1-8]. Live-attenuated and inactivated vaccines are the primary vaccines used for flock immunization around the world. These vaccines are usually administered by ocular-nasal instillation and replicate in the mucosa stimulating effective cell-mediated (CMI) and antibody-mediated (AMI) immune responses. However, some of these vaccines (i.e. LaSota) may cause a mild respiratory disease decimating flock productivity [9]. Inactivated vaccines eliminate the risk of vaccine reaction and induce a long-lasting antibody response [10]; however, due to the lack of replication in the tissue, they do not induce a strong CMI response [11]. In general, these vaccines are able to protect against clinical disease, but two main problems remain: 1) their inability to prevent replication of the challenge virus and shedding, and 2) live vaccines confer suboptimal protection during early life due to maternal-antibody interference.

The poultry industry needs more reliable vaccines to overcome maternal immunity and prevent infection, replication, and shedding of vNDV. Several attempts

have been made to develop improved vaccines, including the development of vaccines homologous to vNDV strains, which reduce vNDV shedding more efficiently than the standard LaSota vaccine [12-14]. *In ovo* vaccination has also been explored to achieve early protection and overcome maternal antibody interference. Two *in ovo* vaccines have been used: a recombinant herpes virus from turkey expressing the F protein from NDV (rHVT/F), which confers long-lasting protection but takes at least takes at least 4 weeks to mount a protective immune response [15-17]; and a live NDV conjugated with an antibody, which is slowly released from the virus over time preventing embryo mortality [18], but its effectiveness is inconsistent.

Chicken IFN-γ is a cytokine with pleotropic functions and with multiple similarities to its orthologue in mammals. It contains 169 amino acids (aa) including a 19-aa-signaling segment; the secreted protein contains 145 aa with a molecular weight of approximately 16.8 kDa [19]. It is primarily secreted by T lymphocytes [20] and NK cells. It is the major modulator of macrophage activation in birds [19, 21, 22], it is capable of inhibiting viral replication [23, 24], promotes development of the Th1 response by inhibiting Th2 cytokine production (IL-4 and IL-10) [25, 26], promotes expression of MHC I [27] and MHC II [21, 27], and it enhances antigen presentation and processing, and destruction of intracellular pathogens [26]. Interferon-gamma has been identified in other bird species, such as: duck [24], goose [28], turkey [29], pigeon [30], pheasant, quail, and Guinea fowl [29]. As with the other chicken cytokines, chicken IFN-γ (ch IFN-γ) signaling pathways are not well investigated, but it is presumed to follow the classical JAK-STAT signaling pathway as the mammalian cytokines [26, 31, 32].

The use of IFN-γ as vaccine adjuvant may improve vaccine efficacy. Studies performed in mammals have revealed an advantageous effect of the use of IFN-γ as a vaccine adjuvant. For instance, increased survival of immunocompromised vaccinated mice upon challenge with malaria was reported in 1989 [33]. Interferon-gamma was also able to decrease allergic responses and lung inflammatory responses upon challenge in a mice model for asthma [34], and it has been reported that it was able to increase the antigen-specific antibody response to hepatitis B virus and HIV gp120 protein, increasing antigen-specific T cell proliferative response as well [35, 36]. Improved protection and enhanced immune responses in avian species have also been reported. Lowenthal and collaborators proved that chIFN-γ was able to enhance antigen-specific humoral immune response in chickens when co-administered with sheep red blood cells. The maximum effect of chIFN-γ was observed 4 to 6 weeks after vaccination and required a high dose of purified protein (10 μg) inoculated intraperitoneally [37]. In addition, increased antibody and cellular responses, and improved overall protection against vNDV challenge, have been previously reported after co-administration of chIFN-γ with DNA and recombinant fowl pox virus vaccines in chickens and turkeys [38-40]. Chicken IFN-γ also improved protection against other avian pathogens, such as *Eimmeria tenella*, *Eimmeria acervulina*, chicken anemia virus and Marek's disease virus [41-46]. However, no commercial products are yet available in the market.

There is need for the development of a practical method to deliver cytokines to be used as vaccine adjuvants for poultry. The use of chIFN-γ as a vaccine adjuvant implicates the use of an adequate system to produce and deliver the cytokine in a reliable and economic manner. Cytokine use as vaccine adjuvant in poultry would require

massive production in order to supply the millions of doses needed every year. Production of cytokines in large scale can be expensive and laborious, which can potentially increase poultry production costs. That is one of the main reasons why the poultry industry has not implemented the use of cytokine adjuvants during vaccination. Chicken IFN-γ production and delivery using recombinant viruses and expression vectors can reduce the costs and facilitate administration. Recombinant NDV expressing chIFN-γ can be grown in eggs and produce the cytokine in every replication cycle. These offer the advantage of delivering both vaccine antigen and the adjuvant at the same time. Plasmid DNA expressing the cytokine will be naturally transfected into the host cells, and the cytokine gene will be transcribed and translated by the host machinery. Both systems have the advantage of not requiring extra steps for cytokine purification, which make them suitable delivery systems to fulfill the demands of the poultry industry and reduce the costs of vaccine/cytokine production.

In the present study, we focused on the development and characterization of three different low cost alternative systems to deliver chIFN-γ during vaccination, in order to study its effects on CMI and AMI enhancement and protection upon challenge with vNDV. These systems consist of the development of a DNA vaccine system expressing a NDV F gene with chIFN-γ co-administration, and the development of a recombinant NDV expressing chIFN-γ, which was used as a live and inactivated vaccine. In addition, the effects of chIFN-γ on viral shedding, morbidity, and mortality were also evaluated. Based on previous reports, we initially hypothesized that these three vaccination systems delivering chIFN-γ would improve CMI and AMI responses, as well as the overall

protection after challenge with vNDV, but our results showed that delivering chIFN-γ in combination with these three vaccination systems did not exert any significant effect.

## Materials and Methods

### *Viruses*

Virulent NDV ZJ1 (Goose/China/ZJ1/2000; GB AF431744.3) was used as a challenge virus in the vaccination experiments. NDV strain LaSota (LS) is used worldwide as a live or inactivated vaccine and was used here as a control vaccine in the immunization-challenge experiments. Recombinant ZJ1*L (rZJ1*L) is an attenuated version of ZJ1 that was previously generated in our laboratory through reverse genetics; this virus was also included as a control vaccine virus for all of the characterization and immunization experiments reported here. All three viruses were obtained from the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA) viral stocks or repository and were propagated in 9 to 11-day-old, specific-pathogen-free (SPF) embryonated chicken eggs (ECEs). The recombinant modified vaccinia virus Ankara, expressing the T7 RNA polymerase (MVA/T7) (a gift from Bernard Moss, National Institute of Health), was propagated in primary chicken embryo fibroblast cells (CEF) and was used to rescue the recombinant viruses.

### *Chickens, eggs, and cells*

Southeast Poultry Research Laboratory White Leghorn SPF flocks were the source of all 9 to 11-day-old ECEs and 2-week-old chickens in every characterization and immunization-challenge experiment. Birds were housed in brooder cages or negative pressure isolators in a biosecurity level 2 enhanced animal (ABSL-2E) facility at

vaccination and transferred into negative pressure isolators in a ABSL-3E facility to be challenged with vNDV ZJ1. Birds were provided with food and water *ad libitum*.

Hep-2 and DF-1 cells were grown and maintained in high glucose Dulbecco's modified Eagle's media (DMEM) supplemented with 5% fetal bovine serum (FBS), 100 U/mL of Penicillin, and 100 µg/mL of Streptomycin, and incubated at 37°C under 5% $CO_2$ atmosphere. These cell lines were used for virus rescue procedures and protein expression assays, respectively. HD11 cells, a chicken macrophage-like cell line, were grown and maintained in RPMI 1640 media supplemented with 10% FBS, 2 mM L-Glutamine, 100 U/mL of Penicillin, and 100 µg/mL of Streptomycin, and incubated at 37°C under 5% $CO_2$ atmosphere. This cell line was used for the IFN-γ bio-activity assay.

***Development and characterization of plasmids expressing NDV F and chIFN-γ genes***

The F gene was amplified by PCR from NFV ZJ1 cDNA using Phusion polymerase (New England Biolabs). The amplicons were then digested with Nco I and Not I restriction enzymes and further ligated into the cloning site of the Novagen pTriEx-3 expression vector (cat# 70823; Millipore, Billerica, MA) matching the same restriction sites. The expression plasmid contaning the F gene was named pTriEX-ZJ1F. The recombinant plasmid expressing chINFγ was generated from the plasmid pCRINFγ (mentioned above) by transferring the chINFγ gene. The resulting plasmid was named pTriEX-INFγ. The recombinant expression plasmids were transformed into Nova Blue E. coli (Millipore) following manufacturer's instructions. Thereafter, single colonies were grown overnight in LB broth supplemented with 100 µg/ mL of ampicillin and purified using the Endotoxin Free plasmid Giga Prep Kit (Cat. # 12391; Qiagen)

following manufacturer's protocol for use in protein expression and vaccination experiments.

**chIFNγ production.** Protein production was determined by western blotting. Briefly, pTriEX vector, pTriEX-ZJ1-F and pTriEX-IFNγ were individually transfected into DF-1 cells using Lipofectamine 2000 or Lipofectamine LTX (Invitrogen) by the manufacturer's protocol for transient expression. Cell monolayers were detached by incubation with 0.05% Trypsin with EDTA (Gibco). Cells were pelleted to remove the media and then re-suspended in 1X phosphate buffered saline (PBS) mixed with SIGMAFAST protease inhibitor cocktail tablets, EDTA-free (Sigma-Aldrich, San Luis, MO). Cells were lysed by multiple freeze-thaw cycles. Insoluble material was pelleted at 12000 x g for 10 minutes at 4°C. Supernatants were stored at -80°C for later analysis. Relative protein concentrations of cell lysates were determined with a BCA protein assay (Pierce). All samples were diluted with 1 x PBS with protease inhibitors to match the protein concentration of the most dilute sample. Cell lysates were boiled for 5 minutes after addition of 2 x laemmli buffer with 350 mM dithiothreitol (1:1) and analyzed through western blotting using Biorad Mini-PROTEAN TGX gels (BioRad), and anti-NDV-F-gene and anti-chIFN-γ (KingFisher Biotech) antibodies.

***Development and characterization of rZJ1*L/IFNγ***

**Construction of recombinant cDNA full-length clone ZJ1*L/IFNγ.** Plasmid pNDV/ZJ1, used as back bone to construct our cDNA full-length clone expressing chIFN-γ, was kindly donated by Dr. Lui and collaborators from the Animal Infectious Disease Laboratory, School of Veterinary Medicine, Yangzhou University, Yangzhou, PR China. This plasmid contains the whole genomic cDNA of the wild-type vNDV ZJ1,

and its development and characterization have been previously described [47]. The plasmid called pCRIFNγ containing the chIFN-γ gene with gene start and gene end (GS and GE, respectively) codons was previously developed in our laboratory [48] and was used as the source for chIFN-γ gene to be inserted into the ZJ1 genome. Development of the full-length cDNA was conducted as described by Dr. Susta and collaborators [48] with a few modifications to the protocol. Briefly, the F protein cleavage site from pNDV/ZJ1 was attenuated through site-directed mutagenesis using the Phusion Site-Directed Mutagenesis kit (New England Biolabs Inc., Ipswich, MA) according to the manufacturer's instructions, giving origin to pNDV/ZJ1*L. Thereafter, to insert the chIFN-γ gene into the ZJ1 backbone, the 2857-5637 region of the ZJ1 genome was amplified from pNDV/ZJ1 and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). This region was sub-cloned into the pUC19 vector (Invitrogen) using HindIII and XbaI restriction enzymes, resulting in the plasmid pUCZJ1. The chIFN-γ gene was then transferred from the pCRIFNγ plasmid into the pUCZJ1 plasmid through the ApaI restriction site, and the resulting intermediate plasmid was named pUCZJ1-IFNγ. Plasmid pUCZJ1-IFNγ was then digested with AgeI/PsiI restriction enzymes, and the region containing the chIFN-γ with GS, GE, and ApaI restriction sites was sub-cloned into the full-length pNDV/ZJ1*L between the P and M genes of the ZJ1 genome, within the untranslated regions (UTRs) of the P gene. The resultant plasmid was designated pNDV/ZJ1*L-IFNγ.

**Virus rescue.** The recombinant virus was rescued by reverse genetic techniques from pNDV/ZJ1*L-IFNγ as described elsewhere [12], using Hep-2 cells grown and maintained in Dulbeco's Modified Eagle Medium (DMEM) (Corning cellgro,

Invitrogen), supplemented with 5% Fetal Bovine Serum (FBS) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), at 37°C with a 5% CO2 atmosphere. The rescued virus was designated as rZJ1*L/IFNγ and further subjected to RNA extraction, RT-PCR, and sequencing to confirm its identity.

**Intracerebral pathogenicity index (ICPI).** One day-old SPF White Leghorn chickens were inoculated intracerebrally with 50 µL of a 1:10 dilution of allantoic fluid (AF) harvested from ECEs infected with either vZJ1, LS, rZJ1*L or rZJ1*L/IFNγ. Birds were monitored every 24 hours for 8 days and scored as follows: 0 = normal, 1 = sick, or 2 = dead. Any virus with an ICPI $\geq 0.7$ was considered as virulent NDV [49, 50].

**Mean death time (MDT).** Nine to eleven-day-old SPF ECEs were inoculated as preciously described [49, 50] with vZJ1, LS, rZJ1*L, or rZJ1*L/IFNγ. The MDT was expressed as the mean time in hours at which the highest dilution killed 100% of the embryos. Allantoic fluids were harvested after death or at the end of the experimental period (7 days post-inoculation) from chilled eggs and used to determine virus titers by HA test and using the Spearmann-Karber method to calculate the $EID_{50}$/mL [51].

**Expression of chIFN-γ from DF-1 cells and in ECEs infected with live virus.** DF-1 cells were maintained in DMEM media supplemented with 10% FBS, 100 U/mL of penicillin, and 100 mg/mL of streptomycin, at 37°C with a 5% CO2 atmosphere. Cells were seeded in 6-well plates at a density of $1 \times 10^6$ cells/well and left to incubate overnight. Thereafter, the cells were washed with 1X PBS three times and 500 µl of inoculum containing either rZJ1*L (10 MOI) or rZJ1*L/IFNγ (10 MOI) were added to the designated wells in triplicates. Inoculated cells were incubated at 37°C with a 5% CO2 atmosphere for 1 hour, rocking the plates every 15 minutes. Thereafter, the

inoculum was removed from each well and fresh DMEM supplemented with 10% FBS, 100U/mL of penicillin, and 100 mg/mL of streptomycin was added. Twenty four hours post infection, the cell layers and supernatants were mixed with 2x laemmli buffer, boiled for 5 minutes at 100°C, and stored at -80°C until processed. Cell lysates and supernatants were analyzed through western blotting using 8-16 % polyacrylamide gels and an anti-chIFN-γ polyclonal antibody (Cat.# PB0442C-100, KingFisher Biotech, Inc., Saint Paul, MN). In addition, 10-day-old ECEs were inoculated with ZJ1*L or rZJ1*L/IFNγ at an $EID_{50}$/egg of $10^3$. Allantoic fluids were collected 24, 48, 72, and 96 hrs post inoculation (3 eggs per time point, per virus) and analyzed through ELISA using a commercial antibody pair to detect chIFN-γ (Cat.# CAC1233, Invitrogen). Concentrations of chIFN-γ were also determined by ELISA from vaccine virus stocks (ZJ1*L and ZJ1*L/IFNγ) and uninfected AF treated with β-Propiolactone (BPL); these BPL-treated AFs were used to prepare the emulsified inactivated vaccines.

**Determination of chIFN-γ bio-activity from BPL-treated AFs.** In order to confirm bio-activity of the chIFN-γ present in AF infected with ZJ1*L/IFNγ after inactivation with BPL, HD11 cells were stimulated with various BPL-treated AFs to determine macrophage activation through quantification of nitrites (a sub-product of nitric oxide). The day prior to the assay, cells were seeded at a density of $4 \times 10^5$ cells/well in a 96-well plate and incubated overnight. Thereafter, the media was replaced with 100 µL of supplemented RPMI 1640 without phenol red per well. Then, 100 µL of a 1:10 dilution of either BPL-treated uninfected AF (BPL-AF), BPL-inactivated ZJ1*L (BPL-ZJ1*L), or BPL-inactivated ZJ1*L/IFNγ (BPL-ZJ1*L/IFNγ) were added per well in triplicates and incubated at 37°C under 5% $CO_2$ atmosphere. Forty eight hours post-

stimulation, 50 µL of each replicate per treatment were tested for nitrite concentration in duplicate using the Griess Reagent System (Cat.# G2930, Promega; Madison, WI).

***Immunization and challenge experiments***

**In ovo immunization with DNA vaccines.** Vaccines were prepared by dilution of recombinant plasmids in neutral TE buffer. One vaccine dose contained a total 150 µg of plasmid DNA in 200 µL of TE buffer except for the control group which contained 200 µL of TE buffer alone (Table 4.1). Eighteen-day-old SPF ECEs were split into seven groups (n=30 eggs/group). Every egg was inoculated by amniotic sac route with one dose of the corresponding DNA vaccine (TE buffer, pTriEX, pTriEX-ZJ1-F or pTriEX-ZJ1-F + pTriEX-IFNγ) as indicated in Table 4.1. Two weeks after hatch, birds were boosted intramuscularly (in the right pectoral muscle) with the corresponding vaccine using the same dose as before. Hatchability and survival after vaccination were evaluated. Two weeks after booster vaccination, survivors were transferred into an ABSL-3 facility and challenged with vZJ1 ($10^5$ $EID_{50}$/bird) by ocular and choanal instillation. Birds were monitored for 14 dpc for clinical sings and mortality. Oropharyngeal and cloacal swab samples were collected 3 dpc to measure challenge virus shedding. Blood samples were collected for serology at termination (14 dpc).

**In ovo immunization with live recombinant vaccines.** Nineteen-day old SPF ECEs were randomly assigned to either one of 4 vaccine groups and inoculated with brain heart infusion (BHI) (Sham-vaccinated), LS, rZJ1*L, or rZJ1*L/IFNγ at a dose of $10^{3.5}$ $EID_{50}$/egg. Regardless of the treatment, eggs were manually inoculated with 100 µL of the corresponding vaccine or uninfected inoculum through the amniotic route, using 1 mL syringes with 24 G x 1/2". After vaccination, each group of vaccinated eggs was

placed in a 2362E Turbofan Hova-Bator Incubator (by GQF). Each incubator was placed inside of a BSL-2 isolator and the temperature and humidity were monitored until 21 days of embryonation (doe). After hatch, chicks were monitored daily for survival and clinical signs until 14 days post-hatch (dph). At 14 dph, 12 chickens from each group were individually identified, and serum was collected for serology. Thereafter, these birds were challenged with $10^{4.9}$ $EID_{50}$/bird of vZJ1 by the ocular and choanal cleft instillation (100 µL/bird). Challenged birds were monitored daily for clinical signs and mortality for two weeks. Pre-challenge and post-challenge antibody titers were determined by hemagglutination inhibition (HI) assay [50].

**Immunization and challenge of 4-week-old, SPF chickens with live recombinant vaccines.** In order to study the effect of rZJ1*L/IFNγ on juvenile chickens upon challenge, forty four 4-week-old SPF White Leghorn chickens were vaccinated and challenged 2 weeks after vaccination. Birds were vaccinated with 100 µL of BHI, LS, rZJ1*L or rZJ1*L/IFNγ by ocular and choanal cleft instillation (50 µL each route) at 4 weeks of age. The intended dose for each vaccine was $10^{6.5}$ $EID_{50}$/bird. Two weeks after vaccination, all birds were challenged with vZJ1 at an $EID_{50}$/bird of $10^{6.5}$. Mortality was recorded until 14 dpc.

**Inactivated-vaccine preparation.** Allantoic fluid (AF) from uninfected ECEs and from ECEs infected with LS, rZJ1*L or rZJ1*L/IFNγ was titrated in 9 to 11-days-old ECEs and adjusted to the same titer ($EID_{50}$/mL). Thereafter, AFs were inactivated with BPL as follows: 0.11 % (v/v) of BLP was slowly added to each AF while rocking. Five minutes later, the fluids were transferred into new sterile flaks and incubated for 3.5 hours at room temperature while rocking. Thereafter, the lids of the flasks were opened

to allow air to come inside and the flasks were incubated at 4 °C overnight. The next day, the pH was adjusted to 7 using pH strips and sterile sodium bicarbonate. The BPL-treated AFs were used to prepare oil emulsion vaccines by mixing 36 mL of mineral oil (ce6vr or Drakeol 6VR) with 3 mL of Arlacel 80 and 1 mL of Tween 80 into a sterile container. A blender and a sterile metal mixing cup were assembled together, and the oil mix was poured inside the cup, followed by BPL treated-AF. The mixture was blended as follows: 1 minute low, one minute rest, one minute low, one minute rest, and last 30 seconds high. The blended vaccines were poured into new sterile vaccine bottles and were properly sealed. Four emulsified vaccine preparations (Sham, LS, ZJ1*L and ZJ1*L/IFNγ) were kept at 4°C until needed.

**Immunization with inactivated recombinant vaccines.** Two different vaccine doses were evaluated in two independent experiments to study the effect of ZJ1*L/IFNγ on AMI and CMI responses, and post-challenge viral shedding and survival. Seventy two, 2-week-old, SPF, White Leghorn chickens were randomly allocated into four groups (n=18) and thereafter vaccinated subcutaneously (SC) with 300 µL/bird of either Sham-vaccine (uninfected AF), LS, ZJ1*L, or ZJ1*L/IFNγ emulsions. Titers before BPL inactivation were $10^{8.1}$ (no-challenge experiment) and $10^{9.1}$ (challenge experiment) $EID_{50}$/mL. Three weeks after vaccination, blood was collected from the brachial vein without anticoagulant for serology, and each bird was boosted SC with 300 µL of the corresponding emulsified vaccine. One week after boost, blood samples were collected for serology the same as before; thereafter, 6 birds from each group vaccinated with the $10^{9.1}$ $EID_{50}$/mL-vaccine batches, were euthanized through cervical dislocation and the spleens were aseptically removed for lymphocyte isolation as described below. The

remaining birds (n=12/group) were transferred to an ABSL-3 facility and challenged with vZJ1 ($10^{9.5}$ $EID_{50}$/bird). Oropharyngeal and cloacal swab samples were collected 2 and 4 days post-challenge (dpc). Birds were monitored daily for up to 14 dpc for clinical signs and mortality. At termination (14 dpc), blood was collected from every survivor for serology. Birds vaccinated with $10^{8.1}$ $EID_{50}$/mL-vaccine batches were terminated after the bleeding procedure.

***Evaluation of the recall CMI response in birds immunized with inactivated vaccines***

**Lymphocyte isolation from spleen.** Six birds per vaccine group were euthanized through cervical dislocation one after boost (4 weeks after initial vaccination). Spleens were aseptically removed and placed into 50 mL conical tubes containing 15 mL of ice-cold 1X PBS (HyClone,) for their subsequent transportation to a BSL-2 laboratory. Thereafter, each spleen was gently passed through a 70 μm cell strainer (Fisher) into a sterile petri dish containing 6 mL of room temperature (RT) 1X PBS using the barrel of a 10 cc syringe. The strainer was then rinsed of RT 1X PBS to have a final cell suspension volume of approximately 10 mL. The cell suspensions were pipetted up and down a few times and then transferred into a 50 mL conical tube to be centrifuged at 450 x g for 5 minutes at RT. The supernatants were discarded and the cells pellets were re-suspended with 6 mL of RT 1X PBS. Then, 3 mL of cell suspension were overlaid onto 3 mL of Histopaque 1.077 (Sigma) in a 15 mL centrifuge tube and centrifuged at 450 x g for 30 minutes at 18ºC. Following centrifugation and using a glass Pasteur pipette, the opaque interface containing the lymphocytes was removed and washed 3 times in 10 mL RT 1X PBS, centrifuging at 450 x g for 10 min at 18ºC. Following the final wash, the cells were

re-suspended in RPMI-1640 (HyClone) supplemented with 10% FBS (Gibco), 1X penicillin-streptomycin mix (Gibco) and 2 mM of L-glutamine (Gibco).

**Lymphocyte proliferation assay.** Recall CMI response was evaluated implementing a lymphocyte proliferation assay. This assay was previously standardized to identify the best assay conditions, namely temperature and incubation time, addition of AlamarBlue®, cell concentration, and the amount of antigen to be used (data not shown). Briefly, cells were seeded into round-bottom 96-well plates (cat. # CLS3799-50EA); Corning Inc., Corning, NY) at 2.5 x $10^5$ cells/well in 100 µL of complete growth media, and stimulated with either 10 µg/mL of Con A (cat. # C5275-5MG; Sigma-Aldrich), inactivated NDV ZJ1*L ($EID_{50}$/0.1 mL of $10^{6.2}$, before BPL inactivation) or media only, adding 100 µL/well of each treatment to the to the corresponding wells, in triplicate. Cells were incubate at 41ºC in a 5% CO2 environment during 86 hours and then, 20 µL of alarmar blue were added to each well. Plates were read 120 hours post-stimulation in a micro-plate reader using wavelengths of 570 nm and 600 nm. The calculations were made as described elsewhere (19). Briefly, the readings at 600 nm were subtracted from the readings at 570 nm; then we averaged the triplicate readings and calculated the mean OD per treatment per vaccine group.

**Determination of lymphocyte subpopulations in spleen from vaccinated birds.** Cells (5.0 x $10^5$/sample) were stained with anti-chicken CD3, CD4, CD8 and IgM antibodies (Southern Biotech, Birmingham, AL) for 30 min at 4°C in the dark. The cells were washed with 1X concentrated phosphate-buffered saline (PBS) and centrifuged at 200 x g for 10 min at 4°C. The cells were re-suspended with 100 mL of PBS and fixed with 100 mL of 2% paraformaldehyde. Samples were evaluated on a BD-LSR II flow

cytometer measuring 10,000 events per sample. Values were reported as percent expression.

***Statistical analysis***

One-way or two-way ANOVA followed by a multiple comparisons Tukey's test were employed, when appropriate, to analyze HI, viral shedding, and cell proliferation assay results. Survival curves were analyzed using the Long-Rank test. Morbidity results were evaluated as proportions, using a two-tailed Z test. Statistical difference was considered with a $P<0.05$ and the significant differences were denoted by different letters.

***Animal use and care***

All experiments were conducted complying with protocols reviewed and approved by the SEPRL institutional biosafety committee and were conducted with appropriate measures to maintain biosecurity and biosafety. General care of chickens was provided in accordance with the procedures reviewed and approved by the SEPRL Institutional Animal Care and Use Committee, as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

## Results

***DNA in ovo vaccine system***

**Identity of pTriEX-NDVF and pTriEX-IFNγ.** The identity of the recombinant plasmids expressing F and IFN-γ genes was confirmed through sequencing analysis. The full insert plus specific regions of the pTriEX vector were sequenced with gene-specific and vector-specific primers. Sequencing analysis results showed that both the F and IFN-γ genes were inserted in the right region and orientation.

**Expression of F gene.** Results from western blot analysis revealed that pTriEX-NDVF was able to express the F gene and the fusion protein was detected from transfected DF-1 cell lysates by western blotting, while no fusion protein was detected from the pTriEX transfected cells (Figure 4.1-A).

**IFN-γ production.** The capability of pTriEX-IFNγ to produce chIFN-γ was confirmed *in vitro*. DF-1 cells were inoculated with pTriEX or pTriEX-IFNγ. Cell culture supernatants were assessed for the presence of chIFN-γ by western blotting, using polyclonal antibodies as described above. Chicken IFN-γ was detected from pTriEX-IFNγ transfected DF-1 cells, while there was no protein detected from pTriEX-transfected DF-1 cell (Figure 4.1-B).

**Effects of chIFN-γ delivered through an in ovo DNA vaccine system.** A second system to deliver IFN-γ during vaccination was evaluated. Eighteen-day-old, SPF ECEs were inoculated with plasmid DNA or TE buffer alone, boosted 2 weeks after hatch, and challenged with vZJ1 2 weeks after booster vaccination. The effects of IFN-γ on hatchability, challenge virus shedding, and morbidity and mortality after challenge were evaluated. Hatchability after *in ovo* vaccination ranged between 90% (Sham-, pTriEX- and pTriEX-ZJ1F+pTriEX-IFNγ-vaccinated groups) and 93% (pTriEX-ZJ1F-vaccinated group).

Evaluation of viral shedding after challenge did show differences between groups. Vaccination with pTriEX-ZJ1F alone significantly reduced the viral shedding from oropharynx and cloaca compared to the control groups at 3 dpc, and most importantly, it shed significantly less than the pTriEX-ZJ1F+pTriEX-IFNγ-vaccinated group (Figure 4.1-C and 4.1-D). Addition of pTriEX-IFNγ during vaccination did affect cloacal viral

shedding as it was observed that the amount of virus shed by the pTriEX-ZJ1F+pTriEX-IFNγ-vaccinated group was greater than by the pTriEX-ZJ1F-vaccinated group, while it was not significantly different than the shedding observed from the Sham- and pTriEX-vaccinated groups (Figure 4.1-C and 4.1-D).

Additionally, both morbidity and mortality after challenge were impacted by chIFN-γ. Birds were observed daily for 14 days after being challenged with vZJ1 for clinical signs and mortality. Co-administration of pTriEX-IFNγ and pTriEX-ZJ1F induced statistically significant higher morbidity (82%) upon challenge than the administration of pTriEX-ZJ1F alone (17%) (*P=0.0001*), but not significantly lower than the non-vaccinated control groups (100% both) (*P=0.068*), while the pTriEX-ZJ1F group showed lower morbidity than the non-vaccinated groups (*P<0.0001*). Mortality for Sham-, pTriEX-, pTriEX-ZJ1F+pTriEX-IFNγ- and pTriEX-ZJ1F-vaccinated groups were 100%, 100%, 41% and 11%, respectively. Mortality observed in the Sham-, pTriEX- and pTriEX-ZJ1F-vaccinated groups was as expected, while mortality was significantly higher than expected for the pTriEX-ZJ1F+pTriEX-IFNγ-vaccinated group as compared to the pTriEX-ZJ1F-vaccinated group (*P=0.049*) (Figure 4.1-E and 4.1-F).

***Live vaccine system***

**Identity and virulence of rZJ1*L/IFNγ.** An attenuated, recombinant NDV expressing the chIFN-γ gene was generated by reverse genetics and fully characterized. The attenuation of the F protein cleavage site was confirmed by nucleotide sequencing, determination of the ICPI in one-day-old chickens, and the MDT in ECEs. The ICPI results, MDT values and the F protein cleavage site amino acid sequences from LS, the recombinant vaccine and the parental virulent virus are shown in Table 4.2.

Recombinant ZJ1*L/IFNγ had an ICPI compatible with NDV strains of low virulence (0.00), while the parental virulent virus (vZJ1) exhibited high ICPI (1.83). The F protein cleavage site for rZJ1*L/IFNγ was confirmed to be identical to the low virulence cleavage site from LS ($_{112}$G R Q G R↓ L$_{117}$). Furthermore, the MDT value for rZJ1*L/IFNγ (>175 hrs.) also classified this virus as low virulence NDV.

**IFN-γ production by live rZJ1*L/IFNγ.** The ability of rZJ1*L/IFNγ to produce chIFN-γ was confirmed *in vivo* and *in vitro*. Nine to 10-day-old, SPF ECEs (*in vivo*) and DF-1 cells (*in vitro*) were inoculated with rZJ1*L or rZJ1*L/IFNγ. Cell culture supernatants and cell lysates were assessed for the presence of chIFN-γ by western blotting using polyclonal antibodies. Chicken IFN-γ was detected from both DF-1 cell culture supernatants and cell lysates of samples infected with rZJ1*L/IFNγ, while no protein was detected from rZJ1*L-infected DF-1 supernatants or cell lysates (Figure 4.2-A). In addition, chIFN-γ specific ELISA was employed to detect production of chIFN-γ *in vivo* from infected ECEs. The concentration of IFN-γ in rZJ1*L/IFNγ-infected AFs increased over time until reaching very high concentrations between 72 and 96 hrs post-infection that saturated the ELISA. Very low levels of IFN-γ were detected in the rZJ1*L-infected AFs (Figure 4.2-B).

**Effects of chIFN-γ delivered through an in ovo live vaccine system.** In order to determine the applicability of rZJ1*L/IFNγ as an *in ovo* vaccine and its effect on immune response modulation, 19-day-old SPF ECEs were vaccinated. One of the most important parameters when evaluating *in ovo* vaccines is the survival after hatch. Here we evaluated the effect of vaccinating with rZJ1*L/IFNγ on AMI response and protection enhancement compared to the standard LS vaccine strain and rZJ1*L; survival results are

summarized in Table 4.3. In summary, the best survival after 14 dph was achieved by the sham-vaccinated control, followed by rZJ1*L and rZJ1*L/IFNγ. The vaccine group with the lowest survival rate was LS as expected. According to or results, survival rates increased when ECEs were vaccinated at 19 doe. Both rZJ1*L and rZJ1*L/IFNγ conferred better protection than LS; however, the co-production of chIFN-γ along with the vaccine virus (rZJ1*L/IFNγ) did not provide any enhancement over the vaccine virus alone (rZJ1*L) (Table 4.3). In addition, our results showed that pre-challenge antibody titers for the Sham-vaccinated, ZJ1*L, and LS groups were significantly different from one another (P=0.003), while there was no significant difference between LS and rZJ1*L/IFNγ groups or between the rZJ1*L and rZJ1*L/IFNγ groups (Figure 4.2-C). In regard to post-challenge antibody titers, there was no significant difference between vaccinated groups (Figure 4.2-C). Although there were differences in the pre-challenge antibody response between vaccinated groups, 100% protection against mortality and clinical disease after challenge was achieved, whereas the Sham-vaccinated group reached 100% mortality by 5 days after challenge (Figure 4.2-D).

In summary, rZJ1*L/IFNγ administered *in ovo* did not seem to improve survival after hatch, when compared to rZJ1*L, or AMI response when compared to LS and rZJ1*L vaccines.

**Effects of chIFN-γ delivered through a live vaccine system in juvenile chickens.** The effect of rZJ1*L/IFNγ as live vaccine in juvenile (4-week-old) chickens was also evaluated. Vaccinated chickens were challenged with vZJ1. Survival analysis after challenge showed that 100% of the birds survived regardless of the given vaccine

treatment; however, 100% of the sham-vaccinated birds succumbed by day 5 after challenge (Figure 4.2-E).

***Inactivated vaccine system***

**Quantification of chIFN-γ from BPL-inactivated rZJ1*L/IFNγ AF.** Very high concentrations of chIFN-γ were detected in rZJ1*L/IFNγ-infected AF after treatment with BPL as well as in the untreated rZJ1*L/IFNγ-infected AF control (Figure 4.3-A).

**Chicken IFN-γ bio-activity after treatment with BPL.** Bio-activity of chIFN-γ contained in the BPL-treated AFs used for inactivated vaccine preparation was confirmed by a macrophage activation assay consisting in the measurement of nitrites released to the cell culture supernatant as an indirect way to measure nitric oxide production from activated macrophages. Our results showed that the BPL-rZJ1*L/IFNγ AF was able to induce nitrite production in HD11 cells, while very low levels of nitrites were detected in the BPL-AF- and BPL-ZJ1*L-treated cells (Figure 4.3-B). These results together with the quantification of chIFN-γ in BPL-rZJ1*L/IFNγ AF, confirmed that neither the concentration nor the bio-activity of chIFN-γ were affected by treatment with BPL.

**Effects of chIFN-γ delivered through an inactivated vaccine system.** The effects of chIFN-γ delivered by an inactivated vaccine virus were evaluated on AMI response, recall CMI response, and virus shedding and survival after challenge. Evaluation of the AMI response through antibody titer determination by HI test demonstrated that the inactivated rZJ1*L/IFNγ, given at an $EID_{50}$/mL of $10^{8.1}$, induced lower mean pre- (8.8) and post-boost (23.4) titers of antibodies specific to the vaccine virus rZJ1*L as compared to the control vaccines LS (37.4 and 92.85, respectively) and

rZJ1*L (47.2 and 115.6, respectively) (Figure 4.3-C). However when the vaccine dose was increased by one log ($EID_{50}$/mL of $10^{9.1}$), the pre- and post-boost mean HI titers for the rZJ1*L/IFNγ-vaccinated group increased considerably (81.8 and 234.7, respectively) compared to the previous vaccine dose (Figure 4.3-D), while there no significant change in the mean HI titer for the rZJ1*L-vaccinated group (72.5 and 177.8, respectively), or for the LS-vaccinated group at this dose (38.2 and 69.4, respectively (Figure 4.3-D). Additionally, the antibody response after challenge was also evaluated, but no significant differences were found between rZJ1*L/IFNγ-, rZJ1*L-, and LS-vaccinated groups, showing mean HI titers of 744.7, 810.7, and 907.6, respectively (Figure 4.3-E). These results together suggest that the rZJ1*L/IFNγ inactivated vaccine did not enhance the AMI response and that its effect on AMI response may be affected by the vaccine dose as compared to the LS and rZJ1*L vaccines, for which there was not much dose effect on their ability to induce an AMI response to the vaccine antigens.

Survival after challenge was affected by the addition of chIFN-γ, but no significant difference was found. Approximately 92% of the birds vaccinated with rZJ1*L/IFNγ survived, while there was 100% survival for both LS- and rZJ1*L-vaccinated birds. By day 6 after challenge, 100% sham-vaccinated controls had succumbed to the virulent challenge (Figure 4.3-F).

Inactivated rZJ1*L/IFNγ did not seem to have an effect on viral shedding after challenge. All vaccinated groups numerically decreased OP shedding compared to the sham-vaccinated control at 4 dpc, and the difference between Sham-vaccinated and rZJ1*L groups was significantly different. However, there was no significant difference between LS, rZJ1*L, and rZJ1*L/IFNγ vaccinated-groups (Figure 4.3-G). In addition, all

vaccinated groups significantly decreased cloacal shedding compared to the sham-vaccinated control, but no significant differences were found between vaccinated groups at 2 or 4 dpc (Figure 4.3-H). When we evaluated the recall CMI response, no significant difference in antigen-specific response was observed between groups (Figure 4.3-I). Lymphocyte populations from the spleens collected after booster vaccination were also monitored using flow cytometric analysis; however, no significant differences between groups were observed (Figure 4.3-J).

These results showed once more that delivering chIFN-γ, together with vaccine antigen, neither enhanced AMI nor protection against mortality, and in addition, it had no effect on CMI response or challenge virus shedding.

## Discussion

In order to generate a protective vaccine, it is important to understand the immune response to avian pathogens and vaccines, along with its modulators. Avian cytokines are crucial modulators of the immune response, and several studies have been conducted to characterize different avian cytokines including type I and type II interferons, IL-2, Il-4, IL-10, IL-12, IL-13, IL-15, IL-18, etc., and their effects on cells of the immune system, as well as their effect on modulating and enhancing the immune response during vaccination. In the present study, we studied three different ND vaccination systems to deliver chIFN-γ, along with their effect on immune response and protection upon challenge. These systems consisted of: 1) a DNA vaccine expressing NDV F gene co-administered *in ovo* with a plasmid expressing chIFN-γ, 2) a recombinant live vaccine expressing chIFN-γ administered *in ovo* and in 4-week-old, SPF chickens, and 3) the recombinant NDV vaccine expressing chIFN-γ gene used as an inactivated vaccine. Our

results showed that chIFN-γ, co-administered at the same time with the vaccine antigen, does not exert a significant effect on the immune response upon vaccination and challenge.

Co-delivery of chIFN-γ with a DNA vaccine system had a negative effect on viral shedding after challenge. Addition of chIFN-γ did impact the amount of challenge virus shed, while the group vaccinated only with vector carrying the F gene shed significantly less virus from cloaca (Figure 4.1-D); the group that received both F and chIFN-γ vectors shed significantly more virus from cloaca, comparable with the shedding amounts detected in the sham-vaccinated and pTriEX controls. Moreover, morbidity and mortality increased significantly when chIFN-γ was co-administered with the F gene (Figure 4.1-E and 4.1-F). Our results are in disagreement with what was reported by Yin and collaborators [39]. Their results showed increased survival after challenge (40%) and decreased challenge virus load from multiple organs and cloaca, as demonstrated by qRRT-PCR, when chIFN-γ was co-administered. However, after 4 rounds of vaccination, their vaccination protocol with plasmids expressing F and HN genes from NDV conferred no protection against mortality at all (100% mortality), while our DNA vaccine expressing only the F gene prevented mortality in 83% of experimental subjects after one *in ovo* administration followed by an IM booster vaccination 2 weeks later (Figure 4.1-F). On the contrary, Sawant and collaborators found no effect of the addition of the co-administration of chIFN-γ on AMI response or on survival of vaccinated birds upon challenge [38]. Similarly, Park and collaborators did find that the co-administration of chIFN-γ along with their infectious bursal disease virus (IBDV) DNA vaccine system, induced lower survival rates after challenge with vvIBDV than the

control vaccine without cytokine [52]. Determination of AMI response was not possible in our case due to the lack of an appropriate assay to detect antibodies elicited after vaccination and challenge.

Results obtained with the live vaccine systems co-delivering chIFN-γ showed no significant effect on protection upon challenge when the cytokine was delivered along with the vaccine antigen. Although there was improvement on survival after hatch with the administration of rZJ1*L/IFNγ compared to the standard LaSota vaccine, the mortality rates remained very high and were no better compared to rZJ1*L not expressing the cytokine (Table 4.3), making this vaccine candidate not suitable for *in ovo* vaccination. Additionally, insertion of chIFN-γ into the viral genome did not induce a significant effect on the AMI response compared to the non-cytokine virus rZJ1*L (Figure 4.2-C). Moreover, chIFN-γ had no effect on survival of vaccinated juvenile chickens after challenge with vZJ1, since all the vaccinated groups showed 100% survival (Figure 4.2-D). In a previous study performed with live vaccines, the authors reported increased NDV-specific antibody HI titers after vaccination and boost with live LaSota and $R_2B$ vaccines, with co-administration of chIFN-γ either during vaccination or 6 hrs after vaccination. The HI antibody titers increased when chIFN-γ was co-administered during vaccination; however, the highest titers were induced with the cytokine was administered 6 hours after vaccination. Although the study reported increased HI titers, those were still below the protective limit (4 $Log_2$) and not comparable to our HI results for the *in ovo* vaccine system. One of the downsides of that study is that the boost was administered eight weeks after initial vaccination [41]. The authors stressed an important factor to be considered during cytokine-adjuvant

administration, which is timing. They made clear that adding chIFN-γ a few hours after vaccination may improve the outcome.

Supporting some of our results obtained with the live vaccine system, our recombinant inactivated vaccine rZJ1*L/IFNγ system was not able to exert any significant effect. No significant effects were noted regarding antigen-specific T cell memory response, challenge virus shedding, or mortality after challenge. Of note, the AMI response after vaccination was proportionally influenced by the vaccine dose. Results from our no-challenge experiment showed that the rZJ1*L/IFNγ-vaccinated group had significantly lower HI titers compared to the control vaccinated groups (Figure 4.3-C), but when the vaccine dose was increased by one log (challenge experiment), the antibody response showed a significant increment compared to the previous experiment. Both pre- and post-boost HI titers were even significantly higher than those for the LS-vaccinated group, although the HI titers were not significantly different than the rZJ1*L-vaccinated group (Figure 3-D). In agreement with our results, Schijns reported no significant effect of co-administration of chIFN-γ together with an IBDV inactivated vaccine. On the contrary, their results of co-administration of chIFN-γ with tetanus toxoid (TT) showed increased TT-specific antibody titers at 2, 6, 9, and 12 weeks post immunization [53]. This suggests that the effects of chIFN-γ on immune response modulation may depend on the vaccine antigen co-administered with the cytokine as well.

Results concerning challenge virus shedding, mortality and antigen-specific memory response were not significantly affected by the co-delivery of chIFN-γ with inactivated rZJ1*L/IFNγ. Addition of chIFN-γ did not provide any advantage on

controlling viral shedding and mortality compared to the rZJ1*L group. There are no available reports about the effects of chIFN-γ as adjuvant for avian oil-emulsion vaccines on viral shedding and antigen-specific memory response, but there a few reporting the use of chIFN-γ along with DNA vaccines showing decreased challenge virus load [39] and/or increased antigen-specific memory response [38, 39]. Another study in mammals using a DNA vaccine system co-delivering gp120 HIV protein with IFN-γ induced increased antigen-specific T cell proliferative response [36]. Unfortunately, due to the discrepancies in approach, none of those results can be fairly compared to ours.

There have been several reports that disagree with our results, showing IFN-γ can increase AMI response when co-administered with diverse antigens such as sheep red blood cells (SRBC), tetanus toxoid, NDV, CAV-VP1 protein, and E. tenella SO7 protein [37, 41, 44, 46, 53, 54]. However, negative or no effect on AMI response has also been observed when IFN-γ was co-administered with IBDV DNA or inactivated vaccine, respectively [52, 53]. All of this suggests that the effects of chIFN-γ used as a vaccine adjuvant are very variable and may depend on the antigen and the timing of administration of chIFN-γ. In addition to the results from our lab and from others, previously discussed in the above paragraphs, there are many other reports referring to the effects of co-administration of chIFN-γ with other vaccines antigens (mainly DNA vaccines) showing positive or negative modulation of the immune response. For example, when co-administered with DNA vaccines against 3-1E protein from *E. acervulina*, no effect or decreased body weight gain were observed along with reduction in number of oocysts shed [42, 43]; on the contrary, co-administration of chIFN-γ with a

S07 (*E. tenella*) subunit vaccine increased body weight gain, antibody response, and antigen-mediated lymphocyte proliferative response [46].

## Conclusions

Our results provide evidence that we were able to deliver chIFN-γ through DNA, live and inactivated vaccine systems. However, none of our systems was able to enhance the immune response or improve protection and survival upon NDV vaccination and challenge. Our results were in disagreement with previous publications, but certainly have provided important information that helps us understand the importance of timing during cytokine delivery and the variable effects that may be elicited by chIFN-γ when used as a vaccine adjuvant. Our results, gathered together with what has been published on the effects of chIFN-γ as a vaccine adjuvant, show that the use of this cytokine needs to be evaluated in the context of antigen, expression, and delivery systems, and the timing of co-administration during vaccination. Evaluating the effect of timing in cytokine administration will not be an easy task using our live or inactivated vaccine systems, but it is certainly a factor that can be further investigated with our DNA vaccine system.

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**Table 4.1 DNA-vaccine groups and plasmid combinations**

| Groups | Plasmids combinations |
|---|---|
| pTriEX-ZJ1F | 100 µg pTri-ZJ1F plus 50 µg empty plasmid |
| pTriEX-ZJ1F + pTri-INFγ | 100 µg pTri-ZJ1F plus 50 µg pTri-INFγ plasmid |
| pTriEx | 150 µg empty vector plasmid |
| Sham-vaccinated | TE buffer, no plasmid DNA |

**Table 4.2 ICPI values, MDTs and amino acid sequence of the Fusion protein cleavage site.**

| Virus Strain | ICPI[a] Value | MDT[b] (hrs.) | Fusion Protein Cleavage Site |
|---|---|---|---|
| PBS | 0.00 | ------ | ------ |
| LS-wt | 0.30 | 153.25 | $_{112}$G R Q G R↓ L$_{117}$ |
| rZJ1*L | 0.43 | >175 | $_{112}$G R Q G R↓L$_{117}$ |
| rZJ1*L/ IFNγ | 0.00 | >175 | $_{112}$G R Q G R↓ L$_{117}$ |
| vZJ1 | 1.83 | 54.5 | $_{112}$R R Q K R↓ F$_{117}$ |

[a]Intracerebral pathogenicity index

[b]Mean death time

**Table 4.3 Effect of chIFN-γ on hatchability and survival after *in ovo* vaccination with rZJ1*L/IFNγ and challenge with vZJ1**

| Vaccine Group | Vaccinated at 19 doe | | |
|---|---|---|---|
| | Hatchability (%) | Survival after hatch (%) | Survival after challenge (%) |
| Sham-vaccinated | 92.3[a] | 100[a] | 0[b] |
| LS $10^{3.5}$ | 92[a] | 60.87[b] | 100[a] |
| rZJ1*L $10^{3.5}$ | 92.3[a] | 80[b] | 100[a] |
| rZJ1*L/IFNy $10^{3.5}$ | 92.5[a] | 72.97[b] | 100[a] |

* doe = days of embryonation

------ Not tested

**Fig. 4.1** Plasmids expressing NDV F and chIFN-γ genes were developed and characterized for their use as DNA vaccines and adjuvant, respectively. DF-1 cells were transfected with pTriEX, pTriEX-ZJ1-F, and pTriEX-IFNγ. Cell culture supernatants were tested by western blotting for the presence of F protein **(A)** and chIFN-γ **(B)**, respectively. Eighteen-day-old SPF ECEs, were inoculated with TE buffer, pTriEX, pTriEX-ZJ1-F, or pTriEX-ZJ1-F plus pTriEX-IFNγ and boosted 2 weeks after hatched. Two weeks after booster vaccination, birds were challenged with vZJ1. Oropharyngeal **(C)** and cloacal **(D)** swab samples were collected 3 days after challenge to measure the amount of challenge virus shed into the environment. Viral titers were determined by qRRT-PCR. A standard was prepared with a vZJ1 virus stock of know concentration, this was included in every plate and was used to obtain viral titers expressed as $EID_{50}$/mL. Morbidity **(E)** and mortality **(F)** were also evaluated. Viral shedding results were analyzed with One-way ANOVA followed by a multiple comparisons Tukey's test. Differences in morbidity between groups were evaluated using a two-tailed Z test for comparison of sample proportions. Survival curves were analyzed using the Long-Rank test. Statistical difference was considered with a $P<0.05$. Significant differences are denoted by different letters.

**Fig. 4.2** DF-1 cells and 10-day-old ECEs **(B)** were infected with live rZJ1*L and rZJ1*L/IFNγ to confirm *in vitro* and *in vivo* expression of chIFN-γ. Cell culture supernatants were collected 24hrs. after infection and tested for chIFN-γ by western bloting **(A)**. Infected AFs were collected 24 hrs., 48 hrs, 72hrs, and 96 hrs. after infection; chIFN-γ concentrations from infected AFs were determined by ELISA **(B).**

Nineteen-day old SPF ECEs were inoculated with BHI, LS, rZJ1*L or rZJ1*L/IFNγ and challenged two weeks after hatch with vZJ1. Sera were collected before and after the challenge for HI antibody titer determination **(C)**. Survival after challenge was recorded **(D)**. Four-week-old SPF chickens were also vaccinated with BHI, LS, rZJ1*L or rZJ1*L/IFNγ and challenged two weeks later with vZJ1 to record mortality **(E)**. HI antibody titres were analyzed with One-way ANOVA followed by a multiple comparisons Tukey's test. Survival curves were analyzed using the Long-Rank test. Statistical difference was considered with a $P<0.05$. Significant differences are denoted by different letters.

**Fig. 4.3** Uninfected AF, rZJ1*L- and rZJ1*L/IFNγ-infected AFs were inactivated with BPL for inactivated vaccine preparation and tested for chIFN-γ concentration by ELISA **(A)**. BPL-treated AFswere used to stimulate HD11 cells and confirm chIFN-γ bio-activity through determination of nitrites as a sub-product of nitric oxide induced upon macrophage activation. Stimulated cells were incubated for 48 hrs. at 37°C under a 5% $CO_2$ atmosphere. Cell culture was used to determine nitrite concentration using the Griesse's method **(B)**. Two-week-old SPF birds were vaccinated and boosted with inactivated Sham-vaccine, rZJ1*L or rZJ1*L/IFNγ. Two different vaccine doses were tested and serum samples were collected before and after challenge for antibody titer determination by HI test. Pre-challenge HI titers after vaccination with an $EID_{50}$/mL of $10^{8.1}$ **(C)** and $10^{9.1}$ **(D)**, and post-challenge titers **(E)** are shown. Mortality was recorded daily for 2 weeks **(F)**. Oropharyngeal **(G)** and cloacal **(H)** swab samples were collected 2 and 4 dpc, viral titers were determined in 10-day-old ECEs and are expressed as

$EID_{50}$/mL. One week after booster vaccination, 6 birds from each vaccinated group were euthanized and the spleens were collected for the isolation of lymphocytes to be used in a proliferation assay to measure antigen-specific memory T cell response **(I)** and to determine T and B cell subpopulation by flow cytometric analysis **(J)**. HI, viral shedding and cell proliferation assay results were analyzed with One-way ANOVA followed by a multiple comparisons Tukey's test. Survival curves were analyzed using the Long-Rank test. Statistical difference was considered with a $P<0.05$. Significant differences are denoted by different letters.

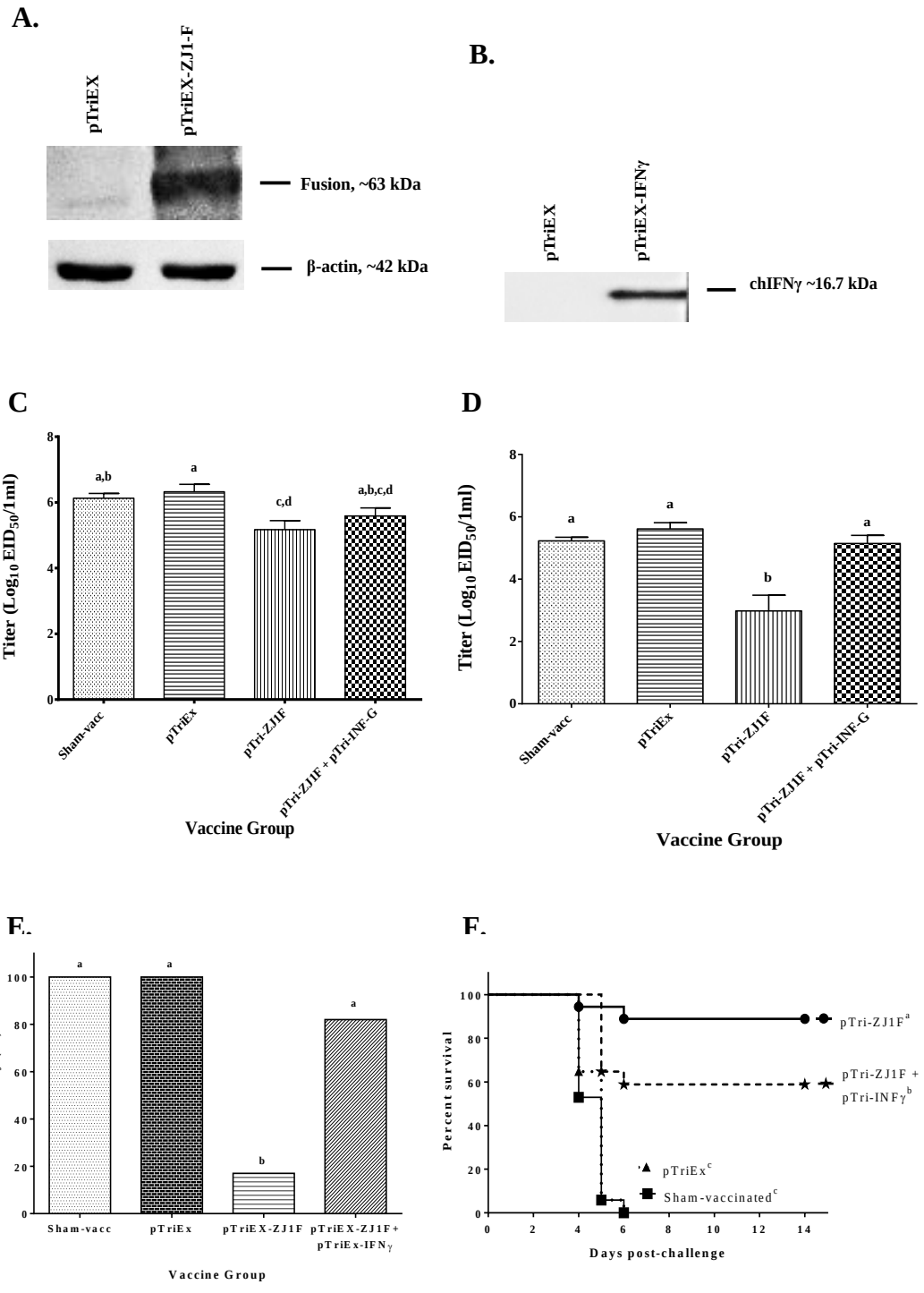
A.
pTriEX
pTriEX-ZJ1-F
Fusion, ~63 kDa
β-actin, ~42 kDa
B.
pTriEX
pTriEX-IFNγ
chIFNγ ~16.7 kDa
C
Titer (Log10 EID50/1ml)
a,b
a
c,d
a,b,c,d
Sham-vacc
pTriEx
pTri-ZJ1F
pTri-ZJ1F + pTri-INF-G
Vaccine Group
D
Titer (Log10 EID50/1ml)
a
a
b
a
Sham-vacc
pTriEx
pTri-ZJ1F
pTri-ZJ1F + pTri-INF-G
Vaccine Group
E.
Morbidity (%)
a
a
b
a
Sham-vacc
pTriEx
pTriEX-ZJ1F
pTriEX-ZJ1F+ pTriEx-IFNγ
Vaccine Group
F.
Percent survival
pTri-ZJ1F[a]
pTri-ZJ1F + pTri-INFγ[b]
pTriEx[c]
Sham-vaccinated[c]
Days post-challenge

**Fig. 4.1**

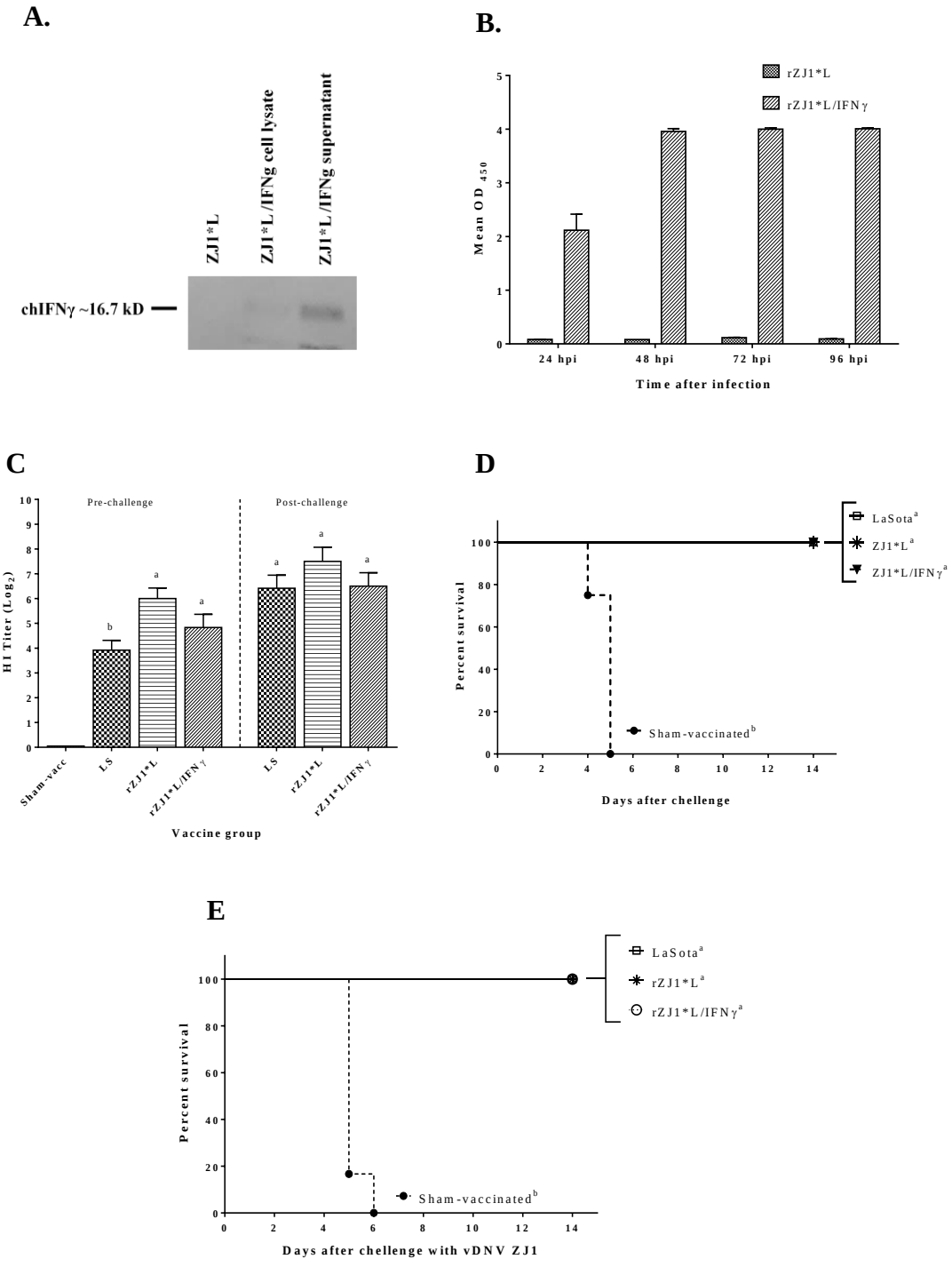
A.
ZJ1*L
ZJ1*L/IFNg cell lysate
ZJ1*L/IFNg supernatant
chIFNγ ~16.7 kD
B.
rZJ1*L
rZJ1*L/IFNγ
Mean OD 450
24 hpi
48 hpi
72 hpi
96 hpi
Time after infection
C
Pre-challenge
Post-challenge
HI Titer (Log2)
Sham-vacc
LS
rZJ1*L
rZJ1*L/IFNγ
Vaccine group
D
LaSota[a]
ZJ1*L[a]
ZJ1*L/IFNγ[a]
Sham-vaccinated[b]
Percent survival
Days after chellenge
E
LaSota[a]
rZJ1*L[a]
rZJ1*L/IFNγ[a]
Sham-vaccinated[b]
Percent survival
Days after chellenge with vDNV ZJ1

**Fig. 4.2**

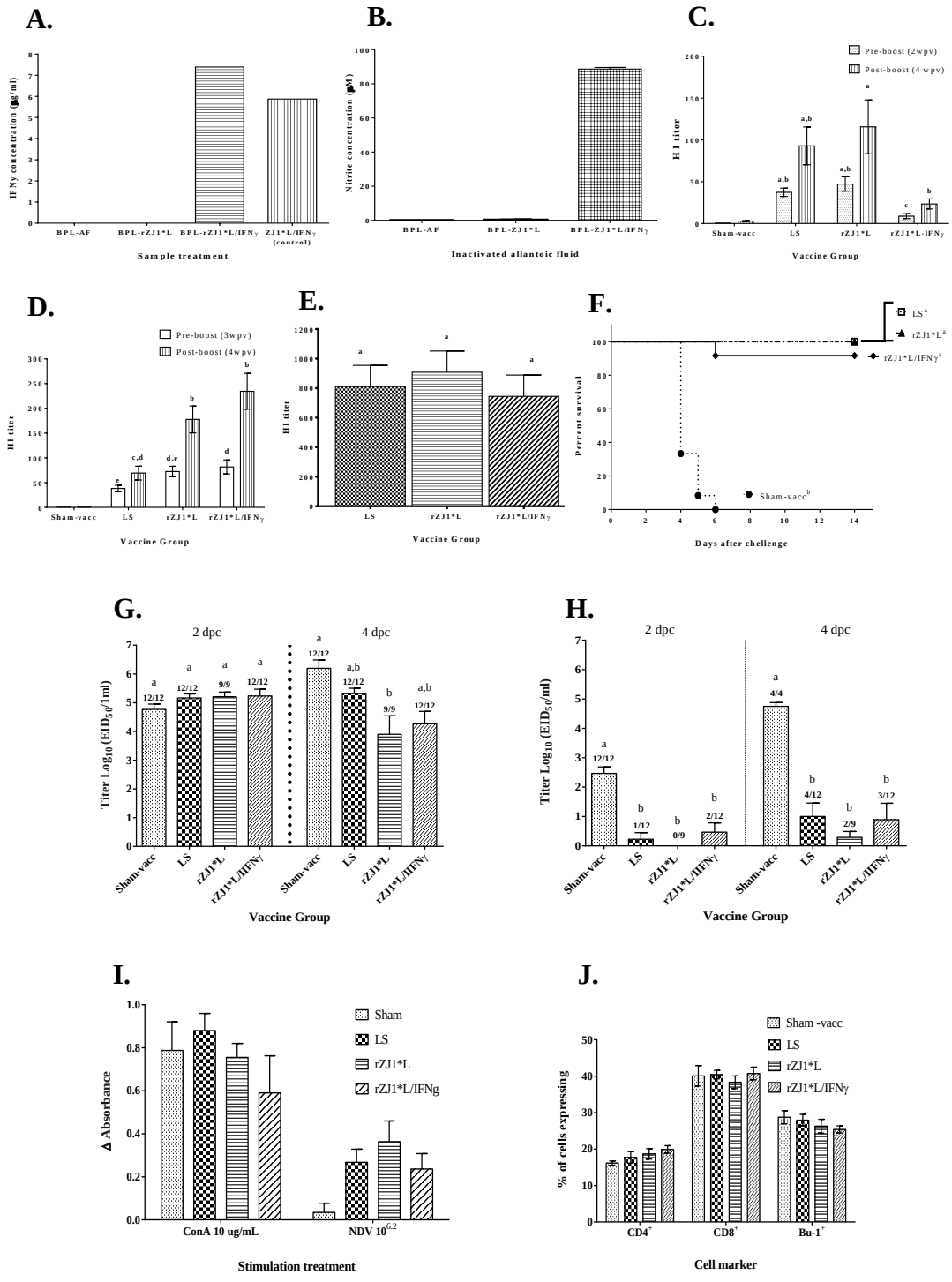
A.
IFNγ concentration (pg/ml)
BPL-AF
BPL-rZJ1*L
BPL-rZJ1*L/IFNγ
ZJ1*L/IFNγ (control)
Sample treatment
B.
Nitrite concentration (μM)
BPL-AF
BPL-ZJ1*L
BPL-ZJ1*L/IFNγ
Inactivated allantoic fluid
C.
Pre-boost (2wpv)
Post-boost (4 wpv)
HI titer
Sham-vacc
LS
rZJ1*L
rZJ1*L-IFNγ
Vaccine Group
D.
Pre-boost (3wpv)
Post-boost (4wpv)
HI titer
Sham-vacc
LS
rZJ1*L
rZJ1*L/IFNγ
Vaccine Group
E.
HI titer
LS
rZJ1*L
rZJ1*L/IFNγ
Vaccine Group
F.
LSa
rZJ1*La
rZJ1*L/IFNγa
Sham-vaccb
Percent survival
Days after chellenge
G.
2 dpc
4 dpc
Titer Log10 (EID50/1ml)
Sham-vacc
LS
rZJ1*L
rZJ1*L/IFNγ
Vaccine Group
H.
2 dpc
4 dpc
Titer Log10 (EID50/ml)
Sham-vacc
LS
rZJ1*L
rZJ1*L/IFNγ
Vaccine Group
I.
Sham
LS
rZJ1*L
rZJ1*L/IFNg
Δ Absorbance
ConA 10 ug/mL
NDV 10^6.2
Stimulation treatment
J.
Sham -vacc
LS
rZJ1*L
rZJ1*L/IFNγ
% of cells expressing
CD4+
CD8+
Bu-1+
Cell marker

**Fig. 4.3**

# CHAPTER 5

# ATTENUATED NEWCASTLE DISEASE VIRUS EXPRESSING CHICKEN IL-10 INDUCES HIGHER ANTIBODY RESPONSE AND LOWER ANTIGEN-SPECIFIC CELLULAR RESPONSE AFTER VACCINATION[1]

[1] Cardenas-Garcia, S., Dunwoody, R.P., Diel, G.D., Marcano, V., Williams, R.J., Gogal Jr., R.M., Brown, C.C., Miller, P.J. and Afonso, C.L. 2015. To be submitted to *Clinical and Vaccine Immunology*.

## Abstract

Newcastle disease is one of the most important diseases that affect the poultry industry. Vaccination is one of the main methods used to control the disease, but it does not prevent virus replication. Interleukin-10 (IL-10) is a cytokine involved in activation of the humoral immune response (Th2) with the ability to suppress cellular responses (Th1). There is a need for more effective vaccines that are able to boost the humoral immune response without compromising the health and productivity of the chicken. In the present study, a recombinant Newcastle disease virus expressing chicken IL-10 (rZJ1*L/IL-10) was developed in order to evaluate the effectiveness of chicken IL-10 (chIL-10) in modulating the avian immune response and its effects on protection upon challenge. The inactivated rZJ1*L/IL-10 delivering chIL-10 was able to induce higher levels of NDV-specific antibodies, when compared with two control vaccines (LaSota and the non-cytokine expressing rZJ1*L), and significantly decreased challenge virus shedding when compared to a sham-vaccinated control. In addition, it induced lower antigen-specific cellular memory response, and no clinical signs or mortality were observed after challenge. Our work demonstrates that it is possible to produce, secrete, and cleave active chIL-10 in chickens using a vaccine virus. Most importantly, these findings may confirm that the effects of chIL-10 on antibody-mediated and cell-mediated immune responses are conserved in chickens with respect to the mammalian IL-10.



**Key words:** Newcastle disease, chicken IL-10, gene expression, vaccines, immune response, protection, Newcastle disease virus, cytokine.

## Introduction

Over the past years, the study of the immune response in mammals has revealed that cytokines are crucial regulators of the immune response. More recently, a growing interest in understanding the avian immune response to pathogens and vaccines has allowed for the identification and characterization of certain chicken cytokines that have conserved functions with their mammalian orthologues. It has been suggested that the avian adaptive immune response can be driven towards a Th1 or Th2 response, as in mammals, depending on the key cytokines being secreted during infection [1, 2]. Th1 responses are driven mostly by IL-12, IFN-γ, and IL-2, while Th2 responses can be driven by IL-4, IL-5, IL-13, and IL-10 [2-4]. The ability to clone and express chicken cytokines has facilitated their use as vaccine adjuvants and on immunotherapy for chickens [5-8].

Chicken IL-10 (chIL-10) is considered to be one of those cytokines with a conserved function in the chicken [2, 9]. In 2004, chIL-10 was cloned and initially characterized by Rothwell and collaborators [9]. According to their findings, chIL-10 was produced by mitogen-stimulated thymocytes, macrophages, and HD11 cells. Furthermore, it was able to inhibit expression chIFN-γ from mitogen-activated splenocytes at both the mRNA and protein levels similar to its mammalian orthologue [9]. IL-10 has been identified and/or cloned for other avian species such as turkey [10], duck [11], and quail [12]. Turkey IL-10 (tuIL-10) shares 92% amino acid identity with the chIL-10. In addition, tuIL-10 also inhibited expression of both chIFN-γ and tuIFN-γ by mitogen-stimulated splenocytes and vice versa [10]. The chIL-10 receptor (chIL-10R)

is expressed mainly on macrophages, dendritic cells, T cells, and NK cells. It is composed of two chains (chIL-10R1 and chIL-10R2).

It is expected that chIL-10 would function as an anti-inflammatory molecule as does the mammalian IL-10 (mIL-10). Mammalian IL-10 is secreted primarily by T cells (especially regulatory T cells), APCs, NK, and B cells [13-15]. It is a pleiotropic, anti-inflammatory molecule that down-regulates the expression of IL-1β, TNF-α, IL-12, co-stimulatory molecules (B7, CD28, CD40 and CD40L), and MHC II in dendritic cells and macrophages in order to inhibit T cell activation [4, 16-18]. It also promotes the development of Th2 responses by inhibiting Th1 cytokines (IL-12 and IFN-γ) [18]. However, only the ability of chIL-10 to inhibit chIFN-γ and the activation of macrophages has been proven to be true in chickens [9].

Other effects of IL-10 have only been studied in mammals (mIL-10). It has been demonstrated that mIL-10 is able to increase the antibody response to a variety of antigens *in vitro* [19-22]. Studies on the effect of mIL-10 on cell-mediate immune (CMI) response have revealed that mIL-10 was able to downregulate the development of antigen-specific CMI from vaccinated mice in response to fungal infections [23, 24]. In addition, it has been shown that mIL-10 directly hampers accumulation of antigen-specific $CD4^+$ T cells [25]. These effects have not yet been studied in chickens *in vivo* or *in vitro*. An increased antibody-mediated immune (AMI) response after vaccination would be beneficial to protect against Newcastle disease viral infection and to increase pathogen neutralization.

Newcastle disease is caused by virulent strains of Newcastle disease virus (NDV) and is one of the most devastating diseases that affect the poultry industry around the

world [26, 27]. The primary measures to control ND are vaccination with live and/or inactivated vaccines, and the implementation of effective biosecurity programs. However, current vaccines have not been able to completely eradicate ND from countries with endemic disease, nor have they prevented infection and shedding of the virulent virus [26, 28]. Therefore, the use of chIL-10 as a vaccine adjuvant to enhance the AMI response would provide a new tool to improve the chicken immune response to vaccination and could provide a new method of vaccine enhancement.

In the present study, we focused on the development and characterization of a system to deliver chIL-10 during vaccination, in order to study its effects on modulating both AMI and CMI responses. This system was contingent on the development of a recombinant NDV expressing chIL-10, which was used as an inactivated vaccine to evaluate its effect on CMI and AMI upon vaccination and/or challenge with virulent NDV (vNDV). Due to previous reports showing the capability of mIL-10 to increase disease susceptibility by downregulating the CMI response, the effects of this recombinant virus on viral shedding, morbidity, and mortality were also evaluated.

## Materials and Methods

### *Viruses*

Virulent NDV ZJ1 (vZJ1) (Goose/China/ZJ1/2000) (GB AF431744.3) was used as a challenge virus in the vaccination experiments. NDV strain LaSota (LS) is used worldwide as a live or inactivated vaccine and was used here as a control vaccine in the immunization-challenge experiments. Recombinant ZJ1*L (rZJ1*L) is an attenuated version of vZJ1 that was previously generated in our laboratory through reverse genetics; this virus was also included as control vaccine virus for all of the characterization and

immunization experiments reported here. All three viruses were obtained from the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA) repository, and viral stocks were propagated in 10-day-old, specific-pathogen-free (SPF) embryonated chicken eggs (ECEs). The recombinant modified vaccinia virus Ankara expressing the T7 RNA polymerase (MVA/T7) (a gift from Bernard Moss, National Institute of Health) was propagated in primary chicken embryo fibroblast cells (CEF); this virus was used to rescue the recombinant virus described in this study.

***Chickens and eggs***

All ECEs and White leghorn SPF chickens were obtained from the Southeast Poultry Research Laboratory (SEPRL, USDA-ARS, Athens, GA) SPF flocks. Birds were housed in negative pressure isolators in an animal biosecurity level 2 enhanced facility (ABL-2E) during vaccination. Thereafter, birds were transferred to an ABSL-3E facility and allocated into negative pressure isolators to be challenged with vZJ1. Birds received food and water *ad libitum* throughout the duration of the experiment.

***Cloning of the chicken IL-10 gene***

Total RNA was extracted from chicken spleen using Trizol-LS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols. Chicken IL-10 (chIL-10) cDNA was transcribed from total RNA using the SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase kit (Invitrogen, Carlsbad, CA). Amplicons were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA), and the correct sequence was confirmed by sequencing. The "gene start" (GS), "gene end" (GE), and the ApaI restriction sites sequences were added to the IL-10 gene by PCR amplification (High Fidelity PCR kit, Promega, Madison, WI). The amplicon was cloned

into the pCR2.1 vector. An additional stop codon was inserted at the end of the IL-10 ORF to maintain the number of nucleotides between the two ApaI sites as a multiple of six. The resulting plasmid was named pCRIL-10.

***Development and characterization of recombinant virus expressing chIL-10***

**cDNA full length clone construction.** Plasmid pNDV/ZJ1, containing the whole genomic cDNA of the wild-type vZJ1 previously described elsewhere [29], was used as backbone to construct the NDV vaccine expressing chicken IL-10. The Fusion protein cleavage site from pNDV/ZJ1 was attenuated through site-directed mutagenesis using the Phusion Site-Directed Mutagenesis kit (New England Biolabs Inc., Ipswich, MA) according to the manufacturer's instructions, giving origin to pNDV/ZJ1*L. Thereafter, to insert the chIL-10 gene into the ZJ1 backbone, the 2857-5637 region of the ZJ1 genome was amplified from pNDV/ZJ1 and cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA). This region was sub-cloned into the pUC19 vector using HindIII and XbaI restriction enzymes, resulting in the plasmid pUCZJ1. The chIL-10 gene was then transferred from the pCRIL-10 plasmid into the pUCZJ1 plasmid through the ApaI restriction site, and the resulting intermediate plasmid was named pUCZJ1-IL-10. The pUCZJ1-IL-10 plasmid was then digested with AgeI/PsiI restriction enzymes, and the region containing the chIL-10 (GS, GE and ApaI restriction sites) was sub-cloned into the full-length pNDV/ZJ1*L between the P and M genes of the ZJ1 genome, within the untranslated regions of the P gene. The final construct was designated as pNDV/ZJ1*L-IL-10.

**Virus rescue.** The recombinant virus was rescued by reverse genetic techniques from pNDV/ZJ1*L-IL-10, using Hep-2 cells grown and maintained in Dulbecco's

Modified Eagle Medium (DMEM) (Corning cellgro, Invitrogen), supplemented with 5% Fetal Bovine Serum (FBS) and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin), at 37°C with a 5% CO2 atmosphere, following our standard protocol [30]. The rescued virus was designated as rZJ1*L/IL-10 and further subjected to RNA extraction, RT-PCR and sequencing to confirm its identity.

**Intracerebral pathogenicity index (ICPI) assay.** One day-old SPF chicks were inoculated intracerebrally with 50 µL of a 1:10 dilution of allantoic fluid (AF) harvested from ECEs infected with vZJ1, LS, rZJ1*L, and rZJ1*L/IL-10. Birds were monitored every 24 hours for 8 days and scored as follows: 0 = normal, 1 = sick, or 2 = dead [31, 32].

**Mean death time (MDT) and titration in ECEs.** Ten-day-old SPF ECEs were inoculated as preciously described [31, 32] with vZJ1, LS, rZJ1*L, or rZJ1*L/IL-10. The MDT was expressed as the mean time in hours at which the highest dilution killed 100% of the embryos. Allantoic fluids (AFs) were harvested after death or at the end of the experimental period (6 days post-inoculation) from chilled eggs and used to determine virus titers by hemagglutination assay (HA) and using the Spearmann-Karber method to calculate the EID50/mL [33].

**Expression of chIL-10 by rZJ1*L/IL-10.** Ten-day-old ECEs were inoculated with a 1:1000 dilution of AF infected with rZJ1*L or rZJ1*L/IL-10, and incubated for 6 days at 37°C. Fluids were collected and HA tested to confirm infection. Thereafter, uninfected AF, AF infected with rZJ1*L, and AF infected with rZJ1*L/IL-10 were analyzed for chIL-10 expression through western blotting using 8-16 % polyacrylamide gels (Cat. #4568105, Bio-Rad, Hercules, CA) and anti-chIL-10 monoclonal antibodies

(donated by Dr. Peter Kaiser, Roslin Institute, University of Edinburgh). In addition, DF-1 cells, maintained in DMEM media supplemented with 10% FBS, 10 U/mL of penicillin and 100 mg/mL of streptomycin, at 37°C with a 5% CO2 atmosphere, were seeded in 6-well plates at a density of 1 x $10^6$ cells/well and left to incubate overnight. Thereafter, cells were washed with 1X PBS three times and 500 μL of inoculum containing either plain DMEM, ZJ1*L (10 MOI or 50 MOI) or ZJ1*L/ IL-10 (10 MOI or 50 MOI) were added to the designated wells in triplicates. Inoculated cells were incubated at 37°C with a 5% CO2 atmosphere for 1 hour, rocking the plates every 15 minutes. Then, the inoculum was removed from each well, and fresh DMEM supplemented with 10% FBS, 100 U/mL of penicillin and 100 mg/mL of streptomycin was added. Twenty four hours post-infection, the cell layers and supernatants were mixed with 2x Laemmli buffer, boiled for 5 minutes at 100°C, and stored at -80°C until processed. Cell lysates and supernatants were analyzed by western blotting using 8-16 % polyacrylamide gels (Bio-Rad) and anti-chIL-10 monoclonal antibody.

***Vaccine preparation***

Allantoic fluid (AF) from uninfected ECEs and from ECEs infected with either LS, rZJ1*L, or rZJ1*L/IL-10 were titrated in 10-day-old ECEs and adjusted to the same titer ($EID_{50}$/mL). Thereafter, the AFs were inactivated with beta-propiolactone (BPL) as follows: 0.11 % (vv) of BLP was slowly added to each AF while rocking. Five minutes later, the fluids were transferred into new sterile flaks and incubated for 3.5 hours at room temperature while rocking. Thereafter, the lids of the flasks were opened to allow air to enter, and the flasks were incubated at 4°C overnight. The next day, the pH was adjusted to 7 using pH strips and sterile sodium bicarbonate. The BPL-treated AFs were used to

prepare oil emulsion vaccines by mixing 36 mL of mineral oil (ce6vr or Drakeol 6VR) with 3 mL of Arlacel 80 and 1 mL of Tween 80 into a sterile container. A blender and a sterile metal mixing cup were assembled together, and the oil mix was poured inside the cup, followed by BPL-treated AF. The mixture was blended as follows: 1 minute low, one minute rest, one minute low, one minute rest, and last 30 seconds high. The blended vaccines were poured into new sterile vaccine bottles and were properly sealed and allowed to de-gas with an 18 gauge needle overnight at 4 ° C [34].

***Vaccination and challenge***

Two separate experiments, using two different vaccine doses, were performed to evaluate the effects of chIL-10 on AMI and CMI, and protection upon challenge with vNDV. Two-week-old, White Leghorn, SPF chickens were vaccinated subcutaneously with 300 µL of Sham vaccine, LS, rZJ1*L, or rZJ1*L/IL-10 vaccine emulsion using an 18 gauge needle. The vaccine emulsions had a titer of $10^{8.1}$ and $10^{9.1}$ $EID_{50}$/mL before inactivation. Blood samples were collected 3 weeks after vaccination for ZJ1-specific antibody titer determination by HI test. Immediately after, all birds were boosted with the corresponding vaccine following the same protocol as for the initial vaccination. One week after booster vaccination, all birds were bled to collect samples for serology. Thereafter, 12 birds per group were transferred to a BSL-3E facility to be challenged with vZJ1 at a dose of $10^5$ EID50/bird by ocular and choanal instillation. At 2, 3, and 4 days after challenge, oropharyngeal and cloacal swabs were taken to assess viral shedding. Mortality and clinical signs were monitored for up to 14 days after challenge, and blood was collected at termination. Antibody titers specific to vZJ1 were determined through

HI test for all collection time points as previously described [32]. Virus isolation and titration from swab samples were performed in 10-day-old, SPF ECEs.

***Evaluation of antigen-specific memory response by T cell proliferation assays***

**Lymphocyte isolation.** Six birds per vaccine group were euthanized through cervical dislocation after booster vaccination (4 weeks after initial vaccination). Spleens were aseptically removed and placed into 50 mL conical tubes containing 15 mL of ice-cold 1X PBS (HyClone) for their subsequent transport to a BSL-2 laboratory. Thereafter, each spleen was gently passed through a 70 μm cell strainer (Fisher) into a sterile petri dish containing 6 mL of room temperature (RT) 1X PBS using the barrel of a 20 cc syringe; the strainer was then rinsed with RT 1X PBS to have a final cell suspension volume of approximately 10 mL. The cell suspensions transferred into a 50 mL conical tube to be centrifuged at 450 x G for 5 minutes at room temperature. The supernatants were discarded, and the cells pellets were re-suspended with 6 mL of RT 1X PBS. Then, 3 mL of cell suspension were overlaid onto 3 mL of Histopaque 1.077 (Sigma) in a 15 mL centrifuge tube and centrifuged at 450 x G for 30 minutes at 18ºC. Following centrifugation and using a glass Pasteur pipette, the opaque interface containing the lymphocytes was removed and washed 3 times in 10 mL RT 1X PBS, centrifuging at 450 x G for 10 min at 18 ºC. Following the final wash, the cells were re-suspended in RPMI-1640 (HyClone) supplemented with 10% FBS (Gibco), 100 U/mL of penicillin, 100 μg/mL of streptomycin (Gibco), and 2 mM of L-glutamine (Gibco).

**Lymphocyte proliferation assay.** The cells were seeded into round bottom 96-well plates at a density of 2.5 x $10^5$ cells/well in 100 μL of supplemented growth media,

and stimulated with Con A (10 µg/mL) (Sigma), $10^{6.2}$ $EID_{50}$/well (titer before inactivation) of purified BPL-inactivated rZJ1*L, or media only. One hundred µL of each treatment were added to the desired wells in triplicates. Cells were incubated at 41ºC in a 5% $CO_2$ atmosphere. Eighty six hours later, 20 µL of AlamarBlue® (BioRad) were added to each well. Plates were read 120 hours post-stimulation (36 hours after addition of AlamarBlue®) in a micro-plate reader using wavelengths of 570 nm and 600 nm. The calculations were made as described previously [35]. Briefly, the readings at 600 nm were subtracted from the readings at 570 nm; the triplicate readings were averaged, and the mean OD per treatment per vaccine group was calculated.

**Determination of T lymphocyte subpopulations in spleen from vaccinated birds.** Cells ($5.0 \times 10^5$/sample) were stained with anti-chicken T cell (CD3, CD4, CD8) antibodies (Southern Biotech, Birmingham, AL) for 30 min at 4°C in the dark. The cells were washed with 1X concentrated phosphate-buffered saline (PBS) and centrifuging at 200 x G for 10 min at 4°C. The cells were re-suspended with 100 mL of PBS and fixed with 100 mL of 2% paraformaldehyde. Samples were evaluated on a BD-LSR II flow cytometer measuring 10,000 events per sample. Values were reported as percent expression.

***Statistical analysis***

HI and virus titers are expressed as arithmetic means plus or minus the standard error of the mean for each vaccine group. Animals negative for virus isolation were also included in the group mean. Group means were analyzed by ANOVA and Tukey's test for multiple comparisons, and using Student's t-test when comparing only two groups at a time. Survival curves were analyzed using the Log-rank test. The level of significance

used to determine statistical differences among groups was 5% ($\alpha = 0.05$). The data was analyzed using Prism software version 6.0.

***Animal use and care***

All experiments were conducted complying with protocols reviewed and approved by the SEPRL institutional biosafety committee and were conducted with appropriate measures to maintain biosecurity and biosafety. General care of chickens was provided in accordance with the procedures reviewed and approved by the SEPRL Institutional Animal Care and Use Committee, as outlined in the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching.

## Results

An attenuated, recombinant ND virus expressing chIL-10 (rZJ1*L-IL-10) was generated by reverse genetics and fully characterized. The identity and attenuation of the fusion protein cleavage site from rZJ1*L-IL-10 was confirmed by nucleotide sequencing, determination of the ICPI in one-day-old chickens, and the MDT in ECEs (Table 5.1). Recombinant rZJ1*l/IL-10 had an ICPI value compatible with NDV strains of low virulence (loNDV) (0.06), while the parental virulent virus (vZJ1) exhibited high ICPI (1.83). The cleavage site amino acid sequence from rZJ1*lL/IL-10 was confirmed to be identical to the low virulence cleavage site from LS ($_{112}$G R Q G R↓ L$_{117}$). Furthermore, the MDT value for rZJ1*L/IL-10 (>168 hrs) classified this virus as loNDV.

The ability of rZJ1*L/IL-10 to express chIL-10 was confirmed using *in vivo* and *in vitro* systems. Ten-day-old SPF ECEs (*in vivo*) and DF-1 cells (*in vitro*) were inoculated with uninfected allantoic fluid (AF), media, rZJ1*L, or rZJ1*L/IL-10. Harvested AFs, cell culture supernatants, and cell lysates were assessed for the presence

of chIL-10 by western blotting using monoclonal antibodies. Chicken IL-10 was detected in both DF-1 cell culture supernatants and cell lysates of samples infected with rZJ1*L/IL-10 at 10 and 50 MOI, while protein was not detected in the mock-infected or the rZJ1*L-infected supernatants or cell lysates. Notably, the protein was secreted into the media and cleaved as expected. In addition, chIL-10 was detected in the rZJ1*L/IL-10-infected AF (Fig. 5.1).

***Delivery of chIL-10 during vaccination with inactivated rZJ1*L/IL-10 induced enhancement of the AMI response.***

In order to investigate the effects of chIL-10 delivery during vaccination, the antibody response was evaluated in two different vaccination experiments using inactivated vaccines. Two-week-old, SPF chickens were vaccinated with a sham vaccine formulated with uninfected AF from SPF ECE, LS, rZJ1*L, or rZJ1*L/IL-10 vaccine emulsions. As determined by the HI test, the antibody (Ab) titers were significantly higher in birds vaccinated with rZJ1*L/IL-10 at the lower dose ($10^{8.1}$ $EID_{50}$/mL before inactivation) than those in birds vaccinated with the sham (*P<0.0001*), LS (*P=0.0075*), or rZJ1*L (*P=0.03*) vaccines at 3 weeks post-vaccination (wpv), but only higher than sham- (*P<0.0001*) and LS-vaccinated (*P=0.0136*) birds at 4 wpv. Significant differences were also noted between time points within groups; LS-, rZJ1*L-, and rZJ1*L/IL-10-vaccinated groups showed a significant increment in HI Ab titers after challenge compared to the post-vaccination HI ab titers (*P<0.001*) (Fig. 5.2-A). When the vaccine dose was increased by one log, the HI Ab titers increased between 1.5 and 2.7 logs compared to the titers observed with the lower vaccine dose. However, even with numerical differences between the groups, there were no significant differences (*P>0.05*)

between LS-, rZJ1*L, and rZJ1*L/IL-10 at 3 wpv, 4 wpv, nor at 2 weeks post challenge (6 wpv); although, all had HI Ab titers significantly higher than the sham-vaccinated control group ($P<0.0001$) at all time points. A significant increase in HI titer after boost (4 wpv) and after challenge compared to the titers at 3 wpv ($P<0.0001$, $P<0.0001$, respectively) was observed in the three vaccinated groups. Only the LS-vaccinated group showed a significant difference between the 4 wpv and post-challenge titer ($P=0.006$) (Fig. 5.2-B). These results suggest that there was an enhancement of the AMI response in response to vaccination with rZJ1*L/IL-10, but such advantage was not observed when the vaccine dose was increased by one log, suggesting that the effect of delivering chIL-10 during vaccination may be more notable when lower vaccine doses are used.

***Effect of rZJ1*L/IL-10 on challenge virus shedding***

After detecting differences in the AMI response, we wanted to evaluate if challenge virus shedding was also affected by delivering chIL-10 with rZJ1*L/IL-10 during vaccination. Oropharyngeal and cloacal swab samples from the lower vaccine dose experiment were analyzed through virus isolation and titration in ECEs. The results of such analysis, showed that all of the vaccinated groups significantly reduced the amount of challenge virus shed from oropharyngeal secretions on day 3 after challenge ($P<0.001$), when compared to the sham-vaccinated control. However, no significant difference in virus shedding was observed between vaccinated groups ($P>0.05$) (Fig. 5.2-C). Furthermore, the number of vaccinated birds shedding challenge virus from cloacal was significantly reduced compared to the sham-vaccinated control; the LS- and rZJ1*L-vaccinated groups had no birds shedding virus, and the rZJ1*L/IL-10-vaccinated group had only one bird shedding. In addition, the titers were significantly lower for all of the

vaccinated groups in comparison to the sham-vaccinated control (*P<0.001*). No significant differences were detected in cloacal shedding between vaccinated groups (*P>0.05*) (Fig. 5.2-D). These results showed that there may not be disadvantage in the utilization of rZJ1*L/IL-10 over the control vaccines LS and rZJ1*L, with respect to viral shedding after challenge.

***Clinical signs and mortality***

Vaccination with rZJ1*L/IL-10 offered 100% protection against mortality and morbidity. Clinical signs and mortality were evaluated after vaccination and after challenge in two experiments. When evaluating survival after challenge, all vaccinated groups, for both vaccine doses, showed 100% survival after challenged with vZJ1; the sham-vaccinated birds succumbed by day 5 or 6 after challenge (Fig. 5.2-E and 5.2-F). There were no signs of disease after challenge in the LS-, rZJ1*L nor rZJ1*L/IL-10-vaccinated groups after challenge. Appearance of clinical signs was limited to the sham-vaccinated controls and consisted of unilateral or bilateral conjunctivitis, ranging from moderate to severe in intensity, and severe depression before death.

***Effect of rZJ1*L/IL-10 on antigen-specific T cell memory response***

Vaccination with rZJ1*L/IL-10 induced a lower antigen-specific proliferative response in lymphocytes isolated from spleen. Spleens from birds vaccinated with sham vaccine, LS, rZJ1*L, or rZJ1*L/IL-10 were collected and processed for lymphocyte isolation. The isolated lymphocytes were plated in 96-well plates and stimulated either with media, BPL-inactivated rZJ1*L, or ConA. This experiment was performed twice with spleens from two independent experiments. The results showed that, the cells obtained from rZJ1*L/IL-10-vaccinated birds always had the lowest proliferative

response as measured by absorbance readings after addition of AlamarBlue® (Fig. 5.3-A and 5.3-B). When analyzing distribution of T cells from the spleen, $CD4^+$ T cells were slightly lower than in the sham-, LS-, and rZJ1*L-vaccinated groups (3.4%, 2.7% and 2% respectively), consistent with the observations made with the proliferation assays (Figure 5.3-C). These results suggest that the chIL-10-expressing virus affected the formation of an antigen-specific memory response.

## Discussion

In the present study, we were able to demonstrate that chIL-10 can be successfully co-expressed by a recombinant Newcastle disease virus with a decrease in the virulence demonstrated by an ICP1 of 0.06 (down from 0.43 observed with the rZJ1*L). The protein can be delivered in the form of an inactivated oil emulsion vaccine prepared using rZJ1*L/IL-10 and was able to modulate the AMI and CMI responses *in vivo* and *ex vivo*, respectively. It has been assumed that the chIL-10 will behave has its mammalian orthologue, but limited studies on the effect of this cytokine have been performed. To our knowledge, the only effects of chIL-10 confirmed to be the same as those of the mIL-10 are its ability to directly inhibit IFN-γ and indirectly inhibit macrophages [9]. Its effects on AMI and CMI responses in chickens remained to be studied.

Our results on the effect of chIL-10 delivered by inactivated rZJ1*L/IL-10 during vaccination showed an effect on the AMI response. When chickens were vaccinated with the lower-dose vaccine set, the group that received rZJ1*L/IL-10 had an enhancement of the HI Ab titers after the administration of a primary and booster vaccination (Fig. 5.2-A). However, when the vaccine dose was increased by one log, the differences were not significant any more (Fig. 5.2-A). These results suggest that the effect of chIL-10

delivered using this particular system may be dose dependent. Chicken IL-10 was able to increase antigen-specific Ab production in response to NDV vaccination, which is consistent with some studies performed in mammals. This suggests that the chicken cytokine may have a role in stimulating B cells, and therefore, stimulating antibody production as previously reported by Xu and collaborators; they studied the *in vitro* effect of stimulating heterogeneous populations of peripheral blood lymphocytes with IL-10 in mice after priming with mite extract. Their findings showed that IL-10 was able to increase IgM and IgG production compared to the cells that did not receive the cytokine and the antigen. In addition, they were able to demonstrate that IL-10 induced B cell maturation by measuring size and the expression of CD38 (cell maturation marker) on sorted, primed, and stimulated B cells [19]. Another study employing lymphocytes isolated from children revealed that mIL-10 induced an increased production of Abs specific to *Pneumococcus*, when cells were primed with pneumococcal antigen and stimulated with the cytokine [21]. In addition, mIL-10 was also able to increase production of IgG specific for systemic lupus erythematosus (SLE) in peripheral blood monocytes (PBMCs) from SLE patients; on the contrary, no effect was observed in PBMCs from healthy patients. When anti-mIL-10 antibodies were added, the IgG levels decreased, proving the direct involvement of mIL-10 on Ig production [22]. These are some of the studies that have demonstrated the involvement of IL-10 on the enhancement of Ab production.

When we evaluated viral shedding after challenge, no effect of chIL-10 was observed. The analysis of the amount of virus present in oropharyngeal and cloacal secretions collected 3 days after challenge showed that all of the vaccinated groups

decreased viral shedding compared to the sham-vaccinated control groups, but no difference was found between the groups vaccinated with rZJ1*L/IL-10 and those vaccinated with LS and rZJ1*L (Fig. 5.2-C and 5.2-D). This may suggest that chIL-10 did not increase viral burden, which also correlates with the HI Ab titers observed 2 weeks after challenge, for which there was no significant difference between groups (Fig. 5.2-A).

Evaluation of the antigen-specific CMI response revealed that chIL-10 caused down-regulation as previously described in mammals. Our results from two independent experiments showed that rZJ1*L/IL-10 virus expressing chIL-10 induced lower cellular memory response when the cells were stimulated with NDV antigen. The response intensity was different between experiments, but both showed the same trend for the cells from birds that received chIL-10 during vaccination (Fig. 5.3-A and 5.3-B) Additionally, the group vaccinated with rZJ1*L/IL-10 had the lowest $CD4^+$ T cell percentage (Fig. 5.3-C). These results are in agreement with previous studies performed in mammals. Hung and collaborators showed that knockout mice lacking expression of IL-10 ($IL\text{-}10^{-/-}$) developed a better *Coccidioides*-specific memory response than the wild-type (WT) mice; additionally, vaccinated $IL\text{-}10^{-/-}$ mice showed increased $CD4^+$ T cell responsiveness when stimulated with *Coccidioides* antigens than the WT mice, showing the negative impact of IL-10 on CMI response [23]. Furthermore, another study performed in WT and knockout mice, infected with lymphocytic choriomeningitis virus (LCMV), showed that lack of IL-10 induced higher numbers of IFN-γ-producing $CD4^+$ T cells and LCMV-specific effector $CD4^+$ T cells; when the memory response was evaluated, the authors

also found increased LCMV-specific memory $CD4^+$ T cells in the IL-$10^{-/-}$ mice compared to the WT [25].

When evaluating morbidity and mortality after challenge, no negative effect of chIL-10 was observed. Due to the concern about the IL-10-related increased susceptibility to disease with certain pathogens, we wanted to evaluate the effect of rZJ1*L/IL-10 on morbidity and mortality. Surprisingly, in spite of observing decreased antigen-specific CMI response in chickens vaccinated with rZJ1*L/IL-10, no clinical signs or mortality were observed in that group as well as in the LS- and rZJ1*L-vaccinated groups (Fig. 5.2-E and 5.2-F). These may have to do with the levels of Abs induced during vaccination.

IL-10-related disease exacerbation raises concerns and has prevented its use as a vaccine adjuvant in mammals, but the use of chIL-10 as a vaccine adjuvant in chickens may be plausible. As shown by our results utilizing a inactivated vaccine system to deliver chIL-10, this cytokine was able to modulate the AMI response in a dose-dependent manner and protect against morbidity and mortality, even that it induced reduced antigen-specific CMI response. Most importantly, our results showed that the effects of chIL-10 on AMI and CMI responses are in agreement with the reported effects for the IL-10 in mammals, providing a new insight on the effects of chIL-10 on immune response modulation in chickens.

Mammalian IL-10 has been proven to be a potent growth and maturation factor for B cells. Therefore, our next step would be to study in more depth the effects of chIL-10 on B cell maturation and stimulation in order to corroborate our findings and provide a deeper explanation of the effects seen in our *in vivo* system.

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**Table 5.1** ICPI values, MDTs and amino acid sequence of the Fusion protein cleavage site.

| Virus Strain | ICPI[a] Value | MDT[b] (hrs) | Fusion Protein Cleavage Site |
|---|---|---|---|
| PBS | 0.00 | ------ | ------ |
| LS-wt | 0.30 | 153.25 | $_{112}$G R Q G R↓L$_{117}$ |
| rZJ1*L | 0.43 | >168 | $_{112}$G R Q G R↓L$_{117}$ |
| rZJ1*L/IL-10 | 0.06 | >168 | $_{112}$G R Q G R↓L$_{117}$ |
| vZJ1 | 1.83 | 54.5 | $_{112}$R R Q K R↓F$_{117}$ |

[a] Intracerebral pathogenicity index

[b] Mean death time in eggs

**Fig. 5.1 *In vivo* and *in vitro* expression of chIL-10 by rZJ1*L/IL-10.** DF-1 cells and 10-day-old ECEs were infected with live rZJ1*L and rZJ1*L/IL-10 to confirm *in vitro* and *in vivo* expression of chIL-10. Cell culture supernatants (SN) and cell lysates (CL) were collected 24 hrs after infection; allantoic fluids were collected 144 hrs after inoculation. Cell culture supernatants, cell lysates, and allantoic fluids were tested for chIL-10 by western blotting. A β-actin antibody was used as loading control for cell lysates.

**Fig. 5.2 Effects of chIL-10 co-delivery during vaccination on AMI response and protection.** The effects of chIL-10, delivered by inactivated rZJ1*L/IL-10, on antibody response, viral shedding and morbidity and mortality after challenge were evaluated. Serum samples were collected 3 wpv, 4 wpv and 2 wpc to measure antibody titers by HI test after immunization and challenge; significant differences within groups are denoted with different letters, significant differences between groups are denoted with Greek characters **(A and B)**. Oropharyngeal and cloacal swab samples were collected 3 days post challenge (dpc), and challenge virus titers were determined by virus isolation and titration in 10-day-old ECEs. Titers are expressed as $EID_{50}$/mL. The stars represent significant differences compared to the sham-vaccinated control **(C and D)**. Morbidity and mortality were recorded for up to 2 weeks after birds were challenged with vZJ1 **(E and F)**. HI and viral shedding results were analyzed with One-way ANOVA followed by a multiple comparisons Tukey's test. Survival curves were analyzed using the Long-Rank test. Statistical difference was considered with a $P<0.05$.

**Fig. 5.3 Effect of rZJ1*L/IL-10 on CMI.** One week after booster vaccination, 6 birds from each vaccinated group were euthanized by cervical dislocation, and the spleens were collected for the isolation of lymphocytes to be used in a proliferation assay to measure antigen-specific memory T cell response **(A**=assay 1 and **B=**assay 2**)** and to determine T cell subpopulation by flow cytometric analysis **(C).** Cell proliferation assay and flow cytometric analysis results were analyzed with One-way ANOVA followed by a multiple comparisons Tukey's test. Statistical difference was considered with a $P<0.05$.

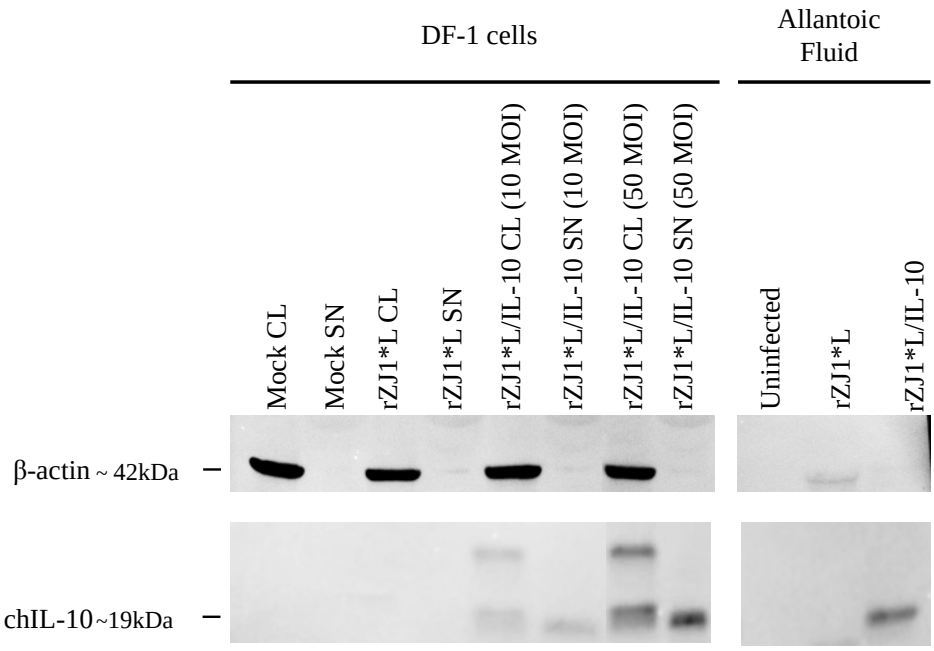
DF-1 cells
Allantoic Fluid
Mock CL
Mock SN
rZJ1*L CL
rZJ1*L SN
rZJ1*L/IL-10 CL (10 MOI)
rZJ1*L/IL-10 SN (10 MOI)
rZJ1*L/IL-10 CL (50 MOI)
rZJ1*L/IL-10 SN (50 MOI)
Uninfected
rZJ1*L
rZJ1*L/IL-10
β-actin ~ 42kDa
chIL-10 ~19kDa

**Fig. 5.1**

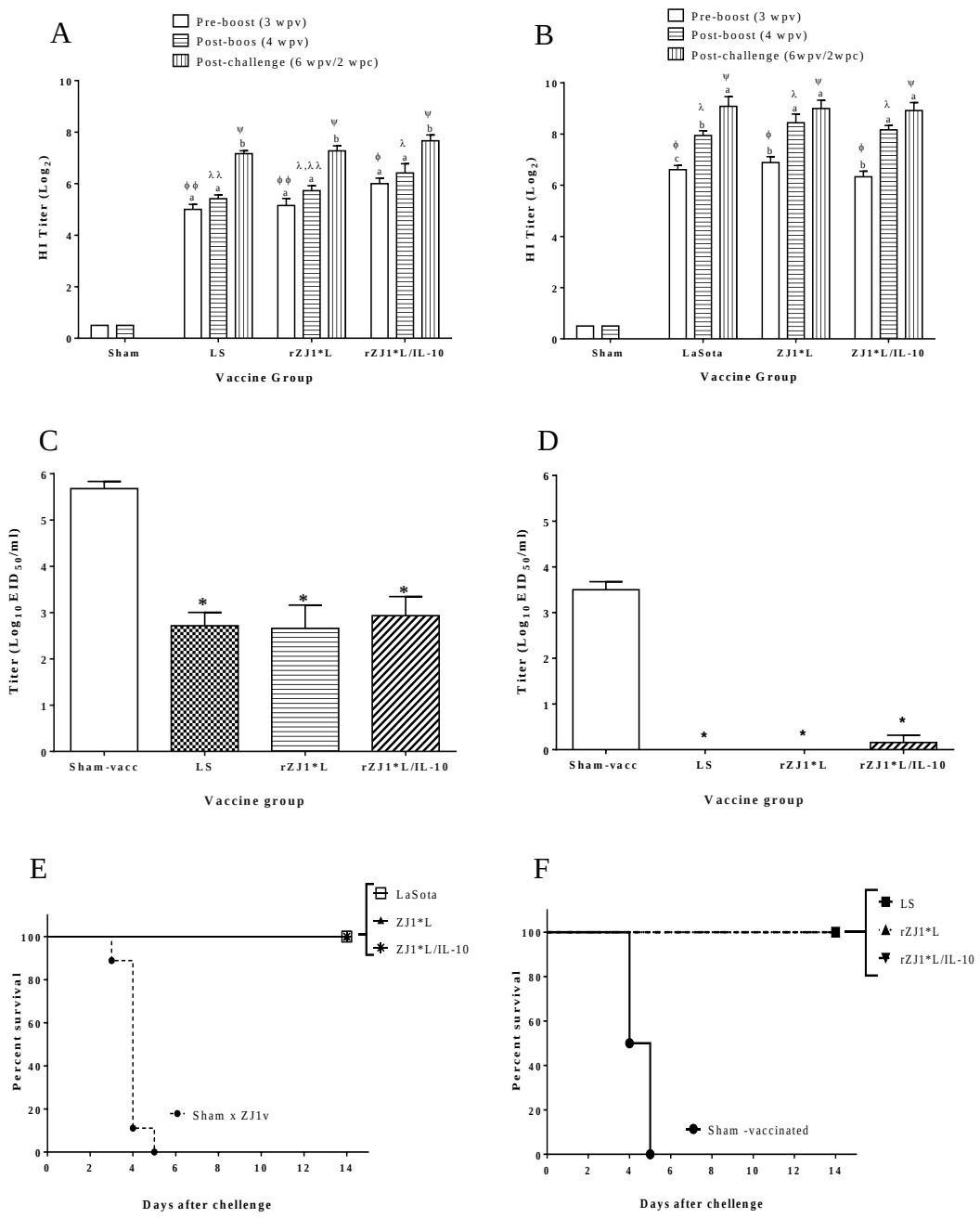
A
Pre-boost (3 wpv)
Post-boos (4 wpv)
Post-challenge (6 wpv/2 wpc)
HI Titer (Log2)
Sham
LS
rZJ1*L
rZJ1*L/IL-10
Vaccine Group
B
Pre-boost (3 wpv)
Post-boost (4 wpv)
Post-challenge (6wpv/2wpc)
HI Titer (Log2)
Sham
LaSota
ZJ1*L
ZJ1*L/IL-10
Vaccine Group
C
Titer (Log10EID50/ml)
Sham-vacc
LS
rZJ1*L
rZJ1*L/IL-10
Vaccine group
D
Titer (Log10EID50/ml)
Sham-vacc
LS
rZJ1*L
rZJ1*L/IL-10
Vaccine group
E
LaSota
ZJ1*L
ZJ1*L/IL-10
Percent survival
Sham x ZJ1v
Days after chellenge
F
LS
rZJ1*L
rZJ1*L/IL-10
Percent survival
Sham -vaccinated
Days after chellenge

**Fig. 5.2**

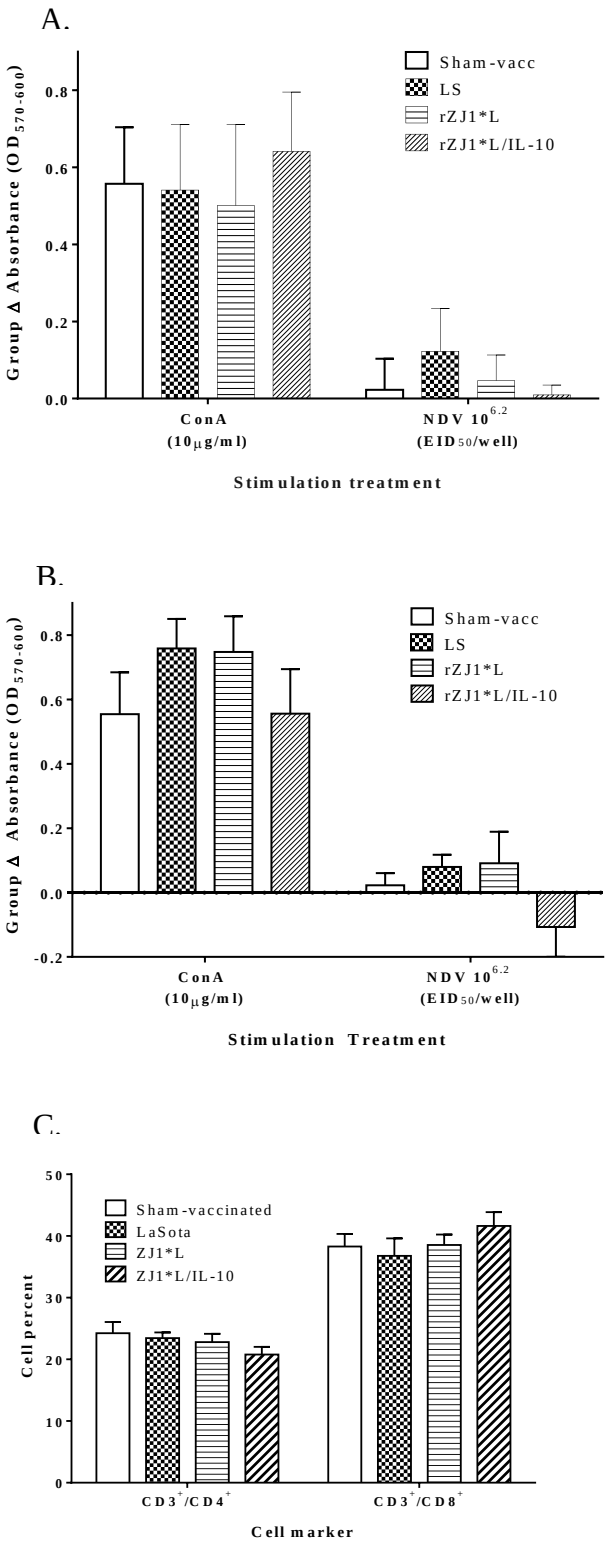
A.
Sham-vacc
LS
rZJ1*L
rZJ1*L/IL-10
Group Δ Absorbance (OD570-600)
0.0
0.2
0.4
0.6
0.8
ConA
(10μg/ml)
NDV 10^6.2
(EID50/well)
Stimulation treatment
B.
Sham-vacc
LS
rZJ1*L
rZJ1*L/IL-10
Group Δ Absorbance (OD570-600)
-0.2
0.0
0.2
0.4
0.6
0.8
ConA
(10μg/ml)
NDV 10^6.2
(EID50/well)
Stimulation Treatment
C.
Sham-vaccinated
LaSota
ZJ1*L
ZJ1*L/IL-10
Cell percent
0
10
20
30
40
50
CD3+/CD4+
CD3+/CD8+
Cell marker

**Fig. 5.3**

# CHAPTER 6

# CONCLUSIONS

The major objective of this research work was to evaluate two different strategies to improve ND vaccines. First, it was hypothesized that the F and HN genes matching the virulent challenge virus into a vaccine backbone would be enough to reduce challenge virus shedding more efficiently than the standard LaSota vaccine. The data contained in Chapter 3 confirmed the stated hypothesis about genotype-specific vaccines. The immune response induced by a live attenuated vaccine NDV with the F and HN genes from PK33 successfully decreased virus shedding after challenge with vNDV PK33 more efficiently that the LaSota vaccine, which did not match the virulent challenge virus. Additionally, the effect of the genotype-specific vaccine on survival was studied by vaccinating birds with optimal and suboptimal vaccine doses ($10^3$ through $10^6$ $EID_{50}$/bird), which may be representative of what happens in the field during mass vaccination, and by challenging with an unusually high dose of PK33. The results showed that the genotype-specific vaccine (rLS-PK) significantly increased survival compared to a heterologous vaccine (LaSota) when lowered doses of vaccine were administered ($10^3$), and induced higher antibody response than LaSota regardless of the vaccine dose.

Higher survival rates induced by genotype matching vaccines at suboptimal doses may suggest that rLS-PK vaccine is a better option to protect suboptimally-vaccinated birds against a field challenge than the current LaSota vaccine. More importantly, this shows the importance of the implementation of more sensitive methods to evaluate

vaccine efficacy, such as the use of different vaccine doses, high titers of challenge virus, different challenge times, and measurement of virus shedding following challenge to better predict the efficacy of newly-developed vaccines under field conditions. The implementation of an improved evaluation system for NDV vaccines together with the use of NDV vaccines that decrease the amount of vNDV shed from vaccinated birds may offer an opportunity to decrease the number and severity of ND field outbreaks.

Second, it was hypothesized that delivery of chIFN-γ and chIL-10 using inactivated vaccine viruses will enhance cell-mediated and antibody-mediated immune responses, respectively, leading to better protection after challenge and to more efficient reduction in the amount of challenge virus shed than vaccine viruses not expressing cytokines. Results shown in Chapter 4 demonstrate that the hypothesis was disproved. Regardless of the cytokine delivery system, chIFN-γ failed to enhance AMI or CMI responses under the conditions tested here. Especially when delivered with a DNA vaccine system, there was a detrimental effect, with higher rates of morbidity and mortality, and higher amounts of challenge virus shed when compared to vaccination without cytokine. Comparing our methods with previously published research, there may have been some factors that led to those results. Specifically, the amount of cytokine delivered (especially for the inactivated vaccine system) and the administration timing may have affected the outcome.

Studies performed to evaluate the effect of chIL-10 on AMI response did support the hypothesis (Chapter 5). Delivery of chIL-10 by an inactivated vaccine system efficiently increased AMI (in a dose-dependent manner) compared to the vaccinated groups that did not received the cytokine. In addition, it induced lower antigen-specific

CMI memory response. These findings are also in agreement with previous studies performed in mammals.

There is very limited information on the effects of chIL-10 in the literature. In addition to showing potential for its use as a vaccine adjuvant, these results also demonstrate, for the first time, that chIL-10 is able to modulate AMI and CMI as reported for its mammalian orthologue (mIL-10). The present work provides a new insight on the immunomodulatory effects of chIL-10.

The present work has provided confirming, innovating, and contradicting information about methods to enhance ND vaccines and the effects of chicken cytokines in immune response modulation. Further work would be required to test the limits of the proposed enhancement systems. For the genotype-specific vaccine system, a transmission experiment showing the capacity of the rLS-PK vaccine to decrease transmission in comparison with the LaSota vaccine would clarify if the differences in challenge virus shedding observed between vaccines would really impact transmission to susceptible contact birds. In addition, it would also be appropriate to test the rLS-PK and the LaSota vaccine under certain conditions that are closer to a commercial setting, including birds with maternal antibodies. These settings would confirm if the protection advantages conferred by the genotype-specific vaccine are applicable to real field conditions.

Study of chIFN-γ delivery timing would be the next step to further explore the use of this cytokine as a vaccine adjuvant. It would be interesting to investigate the effects of combining chIFN-γ delivery vaccine systems with standard vaccines. For example, applying primary immunization with a non-cytokine expressing vaccine, then boosting

with a vaccine system that delivers the cytokine at different time points to find the best combination that enhances the immune response.

Mammalian IL-10 has been proven to be a potent stimulation and maturation factor for B cells. In order to further investigate the effect of chIL-10 in AMI response enhancement, the next step would be to study in more depth the effects of chIL-10 on B cell maturation and stimulation in order to corroborate these findings and provide a deeper explanation of the effects seen in the present *in vivo* system. It also would be interesting to study the use of chIL-10 delivered by a live vaccine system.

# APENDICES

## A. MOLECULAR EPIDEMIOLOGY OF NEWCASTLE DISEASE IN MEXICO AND THE POTENTIAL SPILLOVER OF VIRUSES FROM POULTRY INTO WILD BIRD SPECIES

[1] Cardenas-Garcia, S., Navarro-Lopez, R., Morales, R., Olvera, M.A., Marquez, M.A., Merino, R., Miller, P.J. and Afonso, C.L. 2015. *Applied and Environmental Microbiology. 79(16):4985-92. doi: 10.1128/AEM.00993-13*

## Abstract

Newcastle disease, one of the most important health problems that affect the poultry industry around the world, is caused by virulent strains of Newcastle disease virus. Newcastle disease virus is considered to be endemic in several countries in the Americas, including Mexico. In order to control Newcastle disease outbreaks and spread, intensive vaccination programs, which include vaccines formulated with strains isolated at least 60 years ago, have been established. These vaccines are dissimilar in genotype to the virulent Newcastle disease viruses that had been circulating in Mexico until 2008. Here, 28 isolates obtained between 2008 and 2011 from different regions of Mexico from free-living wild birds, captive wild birds, and poultry were phylogenetically and biologically characterized in order to study the recent epidemiology of Newcastle disease viruses in Mexico. Here we demonstrate that, until recently, virulent viruses from genotype V continued to circulate and evolve in the country. All of the Newcastle disease viruses of low virulence, mostly isolated from nonvaccinated free-living wild birds and captive wild birds, were highly similar to LaSota (genotype II) and PHY-LMV42 (genotype I) vaccine strains. These findings, together with the discovery of two virulent viruses at the Mexican zoo, suggest that Newcastle disease viruses may be escaping from poultry into the environment.

## Introduction

Newcastle disease (ND) is one of the most important health problems that affect the poultry industry around the world [1]. It is caused by virulent strains of Newcastle disease virus (NDV), also known as avian paramyxovirus type 1 (APMV-1), a nonsegmented, negative, single-stranded RNA virus that is part of the genus Avulavirus

[2], family Paramyxoviridae. The NDV genome contains six genes which encode at least seven proteins: nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN), RNA-dependent RNA polymerase or large protein (L), and the V protein produced through editing of the phosphoprotein mRNA (1). NDV has been classified into two major classes, I and II; class II contains most of the virulent viruses circulating worldwide, subclassified into at least 16 genotypes (I to XVI) [3, 4]. According to the clinical manifestations and the tropism of the virus, Newcastle disease virus has been classified into the following five pathotypes: subclinical enteric, lentogenic, mesogenic, viscerotropic velogenic, and neurotropic velogenic. Lentogenic NDV have low virulence, mesogenic NDV have middle virulence, and velogenic strains are highly virulent. Both mesogenic and velogenic NDV are considered virulent by the World Organization for Animal Health (OIE), and any isolate from birds infected with these strains is reportable [1, 5].

In order to control ND outbreaks and spread, intensive vaccination programs have been established in different countries around the world. The most widely used vaccine strains during the last 60 years have been LaSota and B1, which are class II, genotype II viruses [1, 6]. Some other vaccines used commercially, such as the Ulster and PHY-LMV42 strains, form part of genotype I. Vaccination prevents disease in chickens but does not prevent viral infection and replication; therefore, virulent NDV (vNDV) continues to circulate on vaccinated animals [7].

NDV is considered to be endemic in several countries of the Americas, including Mexico [5, 8]. In 1946, velogenic Newcastle disease virus was first reported in Mexico and it was detected in 1-day-old chicks imported from the United States of America [8–

10]. Most of the recent NDV isolates from Mexico belong to class II, genotype V [8, 10, 11], and have a divergence of approximately 16% in the amino acid sequence compared with those of the genotype II vaccines. The dissimilarity among virulent genotype V viruses and the vaccine strains that facilitate viral shedding, besides the persistence of NDV in backyard poultry and free-living wild birds, may explain why vNDV caused sporadic outbreaks in the Mexican poultry industry until recently [7, 12].

In the present study, NDV isolated from captive wild birds, free-living wild birds, and poultry in Mexico were analyzed in order to better understand the current epidemiology of NDV in the country and the relation of these viruses to older isolates.

## Materials and methods

### *Isolates*

The viruses described here were isolated from different species of free-living wild birds, wild birds kept in captivity as part of a zoo exhibition, and commercial poultry in different regions of Mexico from 2008 to 2011. Table 1 contains detailed information about the GenBank accession number, year of isolation, affected avian species, and region of isolation for each isolate. In the text, the isolates are referred to by a shortened name described by the GenBank accession number, the species, and the year of isolation.

### *Virus isolation*

Virus isolation was performed from oropharyngeal and cloacal swabs or from tissue samples by inoculating 9- to 11-day-old specific-pathogen-free (SPF) embryonating chicken eggs (ECEs) into the allantoic cavity as has been described before [5, 13]. After the incubation was completed or after the embryos died, the allantoic fluids were collected from chilled eggs and tested for hemagglutination activity with chicken

red blood cells as previously described [5, 13]. The hemagglutinin (HA)-positive samples were tested for hemagglutination inhibition (HI) with specific NDV antibodies [5, 13].

***Characterization***

The intracerebral pathogenicity index (ICPI) was determined as previously described [5, 13]. Briefly, 1-day-old SPF chicks were inoculated with 50 μl of a 1:10 dilution of infected allantoic fluid by an intracerebral route. The chicks were monitored every 24 h for 8 days, scoring the birds as 0 if normal, 1 if sick, or 2 if dead [5, 13]. The intravenous pathogenicity index (IVPI) was also determined in 6-week-old SPF chickens which were inoculated with 100 μl of a 1:10 dilution of infectious allantoic fluid; birds were examined every 24 h for 10 days and scored as 0 if normal, 1 if sick, 2 if paralyzed, or 3 if dead [14]. Mean death time (MDT) in eggs was determined by inoculating 9- to 11-day-old SPF ECEs as previously described [5, 13].

***RNA extraction and sequencing***

Total RNA was extracted by mixing 250 μl of allantoic fluid with 750 μl of TRIzol LS reagent (Invitrogen, Carlsbad, CA) by following the manufacturer's instructions or from FTA cards as previously described [15]. The fusion (F) gene was amplified by reverse transcription-PCR (RT-PCR) using the SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). Amplicons were sequenced with fluorescence dideoxynucleotide terminators in an ABI 3700 automated sequencer (Applied Biosystems, Inc., Fosters City, CA). Assembly and editing of sequencing data were performed using the DNAStar LaserGene software package, version 10.0.

### *Phylogenetic analysis*

The F gene sequences from the received samples and from samples published in GenBank were used to construct the phylogenetic trees; a total of 93 sequences were aligned using the 374-bp region, and 70 sequences were used to construct the full fusion gene tree. Additionally, 11 sequences of the full fusion were used to estimate the evolutionary divergence. The analyses were performed on January 2013 and May 2013 by using the MEGA software, version 5.0 [16].

### *Nucleotide sequence accession numbers*

All the sequences from the isolates characterized in this study are available in GenBank under the accession numbers KC808487 to KC808512 (Table A-1).

## Results

In February 2008, Newcastle disease was diagnosed in a captive barn owl (Tyto alba) from a zoo located in Tuxtla Gutierrez, Chiapas, Mexico (lat 16.723957, long −93.093362), which presented no clinical signs before death and from which a vNDV with an ICPI of 1.36 was isolated, but no more samples from this isolate were received for further characterization. This diagnosis obliged the veterinarians from the zoo and the animal health authorities to start an epidemiological surveillance in that region and to temporarily close the zoo to the public.

As a result of the epidemiological surveillance in Chiapas, the NDV isolates KC808489-Hawk/2008 (*Buteo brachyurus*), KC808490-Yellow-naped Parrot/2008 (Amazona auropalliata), KC808493-Red-lored Parrot/2009 (*Amazona autumnalis*), KC808510-Scarlet Macaw/2009 (*Ara macao*), KC808498-Highland-Guan/2009 (*Penelopina nigra*), and KC808511-Tree-Duck/2009 (*Dendrocygna autumnalis*) were

obtained from captive wild birds in the zoo, and the majority of those isolates were lentogenic, except the isolates KC808510-Scarlet Macaw/2009 and KC808511-Tree-Duck/2009, which were identified as vNDV. The isolates KC808487-Oropendola/2008 (*Psarocolius montezuma*), KC808488-Flycatcher/2008 (*Myiarchus sp.*), KC808491-Robin/2008 (*Turdus grayi*), KC808492-Woodpecker/2008 (*Melanerpes sp.*), KC808494-Chachalaca/2009, KC808495-Chachalaca/2009, and KC808496-Chachalaca/2009 (*Ortalis vetula*), and KC808497-Great-Egret/2009 (*Ardea alba*), all lentogenic NDV, were obtained from free-living wild birds found within the territory of the zoo or in the surrounding areas (Tables A-1 and A-2).

The NDV isolates KC808508-Gamefowl/2008, KC808509-Gamefowl/2008, KC808512-Quail/2009, KC808499-Chicken/2009, KC808500-Chicken/2009, JQ697743-Chicken/2010, JQ697744-Broiler/2010, KC808501-Rooster/2010, KC808502-Chicken/2010, KC808503-Chicken/2010, KC808504-Chicken/2010, KC808505-Chicken/2010, KC808506-Chicken/2011, and KC808507-Chicken/2011 were obtained from commercial or backyard poultry as part of epidemiological surveillance programs in the states of Mexico, Puebla, Aguascalientes, Guanajuato, Hidalgo, Queretaro, and Mexico City (Table A-1). Most of these isolates were identified as vNDV, but isolate KC808501-Rooster/2010 was characterized as lentogenic NDV (Table A-2).

***Characterization***

The ICPI, IVPI, and MDT were determined on those samples collected in Chiapas, Mexico, from free-living wild birds and captive wild birds. Most of the isolated viruses (KC808487-Oropendola/2008, KC808488-Flycatcher/2008, KC808489-Hawk/2008, KC808490-Yellow-naped Parrot/2008, KC808491-Robin/2008, KC808492-

Woodpecker/2008, KC808493-Red-lored Parrot/2009, KC808494-Chachalaca/2009, KC808495-Chachalaca/2009, KC808496-Chachalaca/2009, KC808497-Great-Egret/2009, and KC808498-Highland-Guan) were identified as lentogenic NDV, with ICPIs ranging between 0.0 and 0.61 and IVPIs equal to 0.0 (Table A-2) [5, 13]; the MDT also classified those isolates as lentogenic. However, the isolates KC808510-Scarlet Macaw/2009 and KC808511-Tree-Duck/2009 showed ICPIs (1.65 and 1.63, respectively), MDT values (36 h and 56 h, respectively), and IVPI values (greater than 2.0) compatible with vNDV (Table A-1) [5, 13].

***Sequencing***

Sequencing data from the F gene showed that most of the isolates from free-living wild birds and captive wild birds (KC808487-Oropendola/2008, KC808488-Flycatcher/2008, KC808489-Hawk/2008, KC808490-Yellow-naped Parrot/2008, KC808491-Robin/2008, KC808492-Woodpecker/2008, KC808493-Red-lored Parrot/2009, KC808494-Chachalaca/2009, KC808495-Chachalaca/2009, KC808496-Chachalaca/2009, KC808497-Great-Egret/2009, and KC808498-Highland-Guan) presented fusion protein cleavage sites with an amino acid sequence compatible with lentogenic NDV strains (112 G R Q G R L117 or 112G K Q G R L117) [5, 17]. KC808510-Scarlet Macaw/2009 and KC808511-Tree-Duck/2009 fusion proteins had virulent cleavage sites (112 R R Q K R F117), with multiple basic amino acids between positions 112 and 116 and a phenylalanine at position 117 (Table A-2) [5, 17]. However, the isolates obtained from poultry had virulent fusion protein cleavage sites (112 R R Q K R F117), except the isolate KC808501-Rooster/2010, which presented a lentogenic cleavage site (112 G R Q G R L117) (Table A-3).

***Phylogenetic analysis***

In order to localize the recent isolates into the phylogenetic trees and to compare them with older viruses isolated from Mexico in the past years, two analyses were performed. One tree was done using the 374-bp N-terminal region of the F gene, and the other was done with the full F gene sequence. These genomic regions have previously been used for NDV phylogenetic characterizations [10, 18]. The 374-bp N-terminal region was used due to the greater number of sequences available in GenBank for comparison; the full fusion region was used to confirm the classification according to the method proposed by Diel [3]. These analyses helped to classify all the isolates presented here within the major class II genotypes V, I, and II (Fig. A-1 and A-2). Also, the full fusion region was used to confirm the identity between representative lentogenic isolates and the LaSota and PHY-LMV42 vaccine strains (Table A-4).

All the virulent isolates were clustered into the new clade that emerged with NDV isolated after the intensive vaccination campaigns were established in Mexico in 2002 (10). The lentogenic isolates KC808487-Oropendola/2008, KC808488-Flycatcher/2008, KC808489-Hawk/2008, KC808490-Yellow-naped Parrot/2008, KC808491-Robin/2008, KC808492-Woodpecker/2008, KC808497-Great-Egret/2009, and KC808501-Rooster/2010 were highly similar to the NDV LaSota vaccine strain (genotype II) and were clustered together. Moreover, the isolates KC808493-Red-lored Parrot/2009, KC808494-Chachalaca/2009, KC808495-Chachalaca/2009, KC808496-Chachalaca/2009, and KC808498-Highland-Guan/2009 were highly similar to the PHY-LMV42 vaccine strain (genotype I) (Fig. A-1 and Table A-4).

The full fusion gene sequence analysis confirmed that all the virulent isolates continue to be clustered within the subgenotype Vb together with other NDV isolates that have been obtained since 2004 in poultry. These isolates are clearly different from those vNDV that have been isolated since 1992 in the United States and Canada from poultry and free-living wild birds (Fig. A-2).

## Discussion

Virus characterization tests and phylogenetic analysis of sequences have demonstrated that vNDV subgenotype Vb continues to circulate in Mexico [3, 8, 10]. Our phylogenetic analysis shows that all the vNDV isolated from poultry and from captive wild birds from 2008 and 2011 continued to be part of subgenotype Vb, with slight divergences that indicate that these viruses continue evolving. After virulent outbreaks affected the country in 2000, intensive vaccination campaigns were established in 2002 as part of the efforts to control ND in Mexico [10]. Presumably, this campaign worked, since no vNDV were reported in Mexico until 2004, when vNDV reappeared, forming a new clade within the subgenotype Vb group. These virulent viruses clustered into the new clade were only 93% to 94% similar to the virulent viruses isolated prior to 2001, demonstrating phylogenetic divergence between earlier (prior to 2001) and recent (after 2001) vNDV isolates [10] (Fig. A-2).

Highly related vNDV are present in different geographic regions of Mexico. The isolates KC808510-Scarlet Macaw/2009 and KC808511-Tree-Duck/2009, obtained in Chiapas from captive wild birds in April 2009 and May 2009, respectively, were identical to each other. Also, those two isolates were closely related to the isolate KC808512-Quail/2009, which was isolated in the state of Mexico from poultry, but the geographical

distance between the places of isolation made it unlikely that the outbreak in the zoo was related to the occurrence in the state of Mexico.

The origin of the vNDV isolated at the Chiapas zoo is unknown; however, the geographic and temporal proximity suggest that they are related to viruses present in poultry. In January 2009, ND was simultaneously identified in Tecpatan, Chiapas (lat 17.149305, long −93.418948), where clinical disease was detected in 10 out of 20 backyard birds (poultry), causing 8 birds to die, and in Cintalapa, Chiapas (lat 16.591961, long −93.869374), where 72 out of 90 backyard hens died; the circulating viruses from both municipalities were identified as vNDV, with ICPIs ranging from 1.64 to 1.70. By the end of March 2009, a new episode with high mortality in backyard hens and turkeys was reported at the north of Tuxtla Gutierrez, 9.76 km from the zoo. Unfortunately, it was not possible to further analyze those samples, but considering when the outbreaks occurred and the time when the viruses KC808510-Scarlet Macaw/2009 and KC808511-Tree-Duck/2009 were isolated, it seems to be more likely that the occurrence in the zoo may be related to those outbreak strains.

Both non-vaccinated free-living wild birds and captive wild birds were carrying ND vaccine viruses. The phylogenetic analysis of the 374-bp N-terminal region demonstrated high similarity between the isolates KC808487-Oropendola/2008, KC808488-Flycatcher/2008, KC808489-Hawk/2008, KC808490-Yellow-naped Parrot/2008, KC808491-Robin/2008, KC808492-Woodpecker/2008, KC808497-Great-Egret/2009, and KC808501-Rooster/2010 and the LaSota vaccine strain (Fig. A-1). In addition, when the sequence of the full fusion gene from representative isolates (KC808487-Oropendola/2008, KC808489-Hawk/2008, KC808490-Yellow-naped

Parrot/2008, and KC808497-Great-Egret/2009) was analyzed, the identities between the LaSota vaccine strain and those isolates were 99.8% (Table A-4). Moreover, the identities between KC808493-Red-lored Parrot/2009, KC808494-Chachalaca/2009, KC808495-Chachalaca/2009, KC808496-Chachalaca/2009, and KC808498-Highland-Guan/2009 and the PHY-LMV42 vaccine strain were 99.8% (Fig. A-1 and Table A-4). The identities between isolates of the same genotype were equal to 100% no matter the ICPI value for both LaSota-like and PHY-LMV42-like isolates (Table A-4), suggesting that the observed differences in ICPI may be due to the existence of additional changes elsewhere in the genome. This identity and the known ability of NDV to rapidly evolve [19, 20] suggest that these isolates are not lentogenic viruses that have been circulating in free-living birds since 1947 (for the viruses similar to the LaSota vaccine) or 1966 (for the viruses similar to the vaccine PHY-LMV42). Most likely, these isolates are spillover from recent infections with live vaccines utilized in poultry vaccination programs. Our findings suggest that vaccine viruses may be escaping from poultry and being carried by free-living wild birds, which may be playing a role in their dissemination. A similar situation was observed in Luxembourg, where lentogenic viruses highly similar to the LaSota strain were isolated from waterfowl [21]. In a previous study conducted in our lab to evaluate the presence of lentogenic NDV in waterfowl in the United States, we have found no evidence of spillover from vaccinated animals into wild birds; however, an isolate similar to viruses of genotype Ia from wild birds was identified in a commercial turkey along with several cases of NDV genotypes from wild birds identified in live bird markets [22], suggesting that viruses from wild birds may spill over into poultry.

The results presented here may be explained by differences in biosecurity practices between Mexico and the United States, widespread presence of backyard chickens in the proximity of poultry farms in Mexico, presence of other avian species that are more susceptible to infection in Mexico, or a combination of these and other factors. Since vaccination of poultry with live NDV is the accepted standard against ND, with billons of doses used worldwide, there is a need to perform additional epidemiological surveillance in free-living wild birds in the vicinity of poultry farms to determine the role of wild birds in the spread of NDV and to identify possible weaknesses in the poultry industry that may allow this escape to happen. As new live NDV recombinant vaccines enter the market around the world, it is important to identify the risks of spillover of lentogenic and virulent NDV from poultry into the environment. In addition, since previous studies performed by our lab and others have demonstrated that vaccines homologous to the circulating virulent viruses reduce viral shedding and may potentially reduce transmission and spillover from poultry into the environment, the development of vaccines that prevent virus replication should be explored [7, 12].

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**Table A-1** Isolates per year, species and region of the country.

| Isolate Name | GenBank Accession No. | Isolation Date/Year | Common Name | Scientific Name | Place of Isolation | Living Environment |
|---|---|---|---|---|---|---|
| Oropendola/Mex(CH)/661-ZM01/2008 | KC808487 | July 2008 | Oropendola | *Zacua sp.* | Chiapas | Wildlife |
| Flycatcher/Mex(CH)/662-ZM02/2008 | KC808488 | July 2008 | Mexican Crested Flycatcher | *Myiarchus sp.* | Chiapas | Wildlife |
| Short-tailed Hawk/Mex(CH)/663-ZM03/2008 | KC808489 | August 2008 | Short-tailed Hawk | *Buteo brachyurus* | Chiapas | Zoo |
| Yellow-napped Parrot/Mex(CH)/664-ZM04/2008 | KC808490 | August 2008 | Yellow-napped Parrot | *Amazona auropalliata* | Chiapas | Zoo |
| Robin/Mex(CH)/665-ZM05/2008 | KC808491 | August 2008 | Robin | *Turdus grayi* | Chiapas | Wildlife |
| Woodpecker/Mex(CH)/666-ZM06/2008 | KC808492 | August 2008 | Woodpecker | *Melanerpes sp.* | Chiapas | Wildlife |
| Gamefowl/Mex(Mex)/616/2008 | KC808508 | 2008 | Gamefowl | Not specified | State of Mexico | Unknown |
| Gamefowl/Mex(D.F)/619/2008 | KC808509 | 2008 | Gamefowl | Not specified | Mexico City | Unknown |
| Red-lored Parrot/Mex(CH)/667-ZM07/2009 | KC808493 | March 2009 | Red-lored Amazon Parrot | *Amazona autumnalis* | Chiapas | Pet |
| Chachalaca/Mex(CH)/668-ZM08/2009 | KC808494 | April 2009 | Chachalaca | *Ortalis vetula* | Chiapas | Wildlife |
| Chachalaca/Mex(CH)/669-ZM09/2009 | KC808495 | April 2009 | Chachalaca | *Ortalis vetula* | Chiapas | Wildlife |
| Chachalaca/Mex(CH)/670-ZM10/2009 | KC808496 | April 2009 | Chachalaca | *Ortalis vetula* | Chiapas | Wildlife |
| Great-Egret/Mex(CH)/671-ZM11/2009 | KC808497 | April 2009 | Great Egret | *Ardea alba* | Chiapas | Wildlife |
| Scarlet macaw/Mex(CH)/672-ZM12/2009 | KC808510 | April 2009 | Scarlet macaw | *Ara macao* | Chiapas | Zoo |
| Highland-Guan/Mex(CH)/674-ZM14/2009 | KC808498 | April 2009 | Highland Guan | *Penelopina nigra* | Chiapas | Zoo |
| Tree-Duck/Mex(CH)/675-ZM15/2009 | KC808511 | May 2009 | Tree-duck | *Dendrocygna autumnalis* | Chiapas | Zoo |
| Quail/Mex(Mex)/615/2009 | KC808512 | 2009 | Quail | *Corurnix coturnix* | State of Mexico | Poultry |
| Chicken/Mexico/612/2009 | KC808499 | 2009 | Chicken | *Gallus gallus* | State of Mexico | Poultry |
| Chicken/Mexico/613/2009 | KC808500 | 2009 | Chicken | *Gallus gallus* | State of Mexico | Poultry |
| Chicken/Mex(PU)/634/2010 | JQ697743 | 2010 | Chicken | *Gallus gallus* | Puebla. | Backyard |
| Broiler/Mex(AC)/635/2010 | JQ697744 | 2010 | Broiler chicken | *Gallus gallus* | Aguascalientes | Poultry |
| Rooster/Mex(HO)/676/2010 | KC808501 | September 2010 | Chicken | *Gallus gallus* | Hidalgo | Poultry |
| Chicken/Mex(QT)/678/2010 | KC808502 | November 2010 | Broiler chicken | *Gallus gallus* | Queretaro | Poultry |
| Chicken/Mex(GT)/679/2010 | KC808503 | December 2010 | Layer hen | *Gallus gallus* | Guanajuato | Poultry |
| Chicken/Mex(HG)/682/2010 | KC808504 | December 2010 | Broiler chicken | *Gallus gallus* | Hidalgo | Poultry |
| Chicken/Mex(PU)/684/2010 | KC808505 | 2010 | Layer hen | *Gallus gallus* | Puebla | Poultry |
| Chicken/Mex(AG)/685/2011 | KC808506 | 2011 | Broiler breeder | *Gallus gallus* | Aguascalientes | Poultry |
| Chicken/Mex(AG)/686/2011 | KC808507 | 2011 | Broiler breeder | *Gallus gallus* | Aguascalientes | Poultry |

**Table A-2** Pathotyping and characterization of free-living wild birds and captive exotic bird isolates.

| Isolate Name | MDT[a] (Hours) | ICPI[b] | IVPI[c] | Fusion Protein Cleavage Site | Pathotype |
|---|---|---|---|---|---|
| Oropendola/Mex(CH)/661-ZM01/2008 | N/D[d] | 0.0 | 0.0 | G R Q G R L | Lentogenic |
| Flycatcher/Mex(CH)/662-ZM02/2008 | N/D | 0.0 | 0.0 | G R Q G R L | Lentogenic |
| Short-tailed Hawk/Mex(CH)/663-ZM03/2008 | N/D | 0.0 | 0.0 | G R Q G R L | Lentogenic |
| Yelow-napped Parrot/Mex(CH)/664-ZM04/2008 | N/D | 0.0 | 0.0 | G R Q G R L | Lentogenic |
| Robin/Mex(CH)/665-ZM05/2008 | N/D | 0.0 | 0.0 | G R Q G R L | Lentogenic |
| Woodpecker/Mex(CH)/666-ZM06/2008 | N/D | 0.0 | 0.0 | G R Q G R L | Lentogenic |
| Red-lored Parrot/Mex(CH)/667-ZM07/2009 | 124 | 0.0 | 0.0 | G K Q G R L | Lentogenic |
| Chachalaca/Mex(CH)/668-ZM08/2009 | 124 | 0.0 | 0.0 | G K Q G R L | Lentogenic |
| Chachalaca/Mex(CH)/669-ZM09/2009 | 124 | 0.61 | 0.0 | G K Q G R L | Lentogenic |
| Chachalaca/Mex(CH)/670-ZM10/2009 | 124 | 0.60 | 0.0 | G K Q G R L | Lentogenic |
| Great-Egret/Mex(CH)/671-ZM11/2009 | 124 | 0.60 | 0.0 | G R Q G R L | Lentogenic |
| Scarlet macaw/Mex(CH)/672-ZM12/2009 | 36 | 1.65 | 2.31 | R R Q K R F | Velogenic |
| Highland-Guan/Mex(CH)/674-ZM14/2009 | N/D | 0.31 | N/D | G K Q G R L | Lentogenic |
| Tree-Duck/Mex(CH)/675-ZM15/2009 | 56 | 1.63 | 2.11 | R R Q K R F | Velogenic |

a: Mean Death Time; b: Intracerebalr Pathogenicity Index; c: Intravenous Pathogenicity Index; d: not determined

**Table A-3** Characterization of isolates from poultry.

| Isolate Name | Fusion Protein Cleavage Site | Virulence Classification |
|---|---|---|
| Gamefowl/Mex(Mex)/616/2008 | R R Q K R F | High |
| Gamefowl/Mex(D.F)/619/2008 | R R Q K R F | High |
| Quail/Mex(Mex)/615/2009 | R R Q K R F | High |
| Chicken/Mexico/612/2009 | R R Q K R F | High |
| Chicken/Mexico/613/2009 | R R Q K R F | High |
| Chicken/Mex(PU)/634/2010 | R R Q K R F | High |
| Broiler/Mex(AC)/635/2010 | R R Q K R F | High |
| Rooster/Mex(HO)/676/2010 | G R Q G R L | Low |
| Broiler/Mex(QT)/678/2010 | R R Q K R F | High |
| Layer hen/Mex(GT)/679/2010 | R R Q K R F | High |
| Broiler/Mex(HG)/682/2010 | R R Q K R F | High |
| Layer hen/Mex(PU)/684/2010 | R R Q K R F | High |
| Broiler/Mex(AG)/685/2011 | R R Q K R F | High |
| Broiler/Mex(AG)/686/2011 | R R Q K R F | High |

**Table A-4** Estimates of Evolutionary Divergence between Lentogenic Full Fusion Sequences from genotypes I and II.

| | PHY-LMV42 | KC808493 | KC808494 | KC808495 | KC808498 | KC808496 | LaSota | KC808487 | KC808489 | KC808490 | KC808497 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Ia-PHY-LMV42 | | 0.001 | 0.001 | 0.001 | 0.001 | 0.001 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 |
| Ia-KC808493-Red-lored_Amazon_parrot/2009 | **0.2%** | | 0.000 | 0.000 | 0.000 | 0.000 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 |
| Ia-KC808494-Chachalaca/2009 | **0.2%** | 0.0% | | 0.000 | 0.000 | 0.000 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 |
| Ia-KC808495-Chachalaca/2009 | **0.2%** | 0.0% | 0.0% | | 0.000 | 0.000 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 |
| Ia-KC808498-Highland_Guan/2009 | **0.2%** | 0.0% | 0.0% | 0.0% | | 0.000 | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 |
| Ia-KC808496-Chachalaca/2009 | **0.2%** | 0.0% | 0.0% | 0.0% | 0.0% | | 0.008 | 0.008 | 0.008 | 0.008 | 0.008 |
| II-LaSota | 10.5% | 10.4% | 10.4% | 10.4% | 10.4% | 10.4% | | 0.001 | 0.001 | 0.001 | 0.001 |
| II-KC808487-Oropendola/2008 | 10.4% | 10.3% | 10.3% | 10.3% | 10.3% | 10.3% | **0.2%** | | 0.000 | 0.000 | 0.000 |
| II-KC808489-Short-tailed_Hawk/2008 | 10.4% | 10.3% | 10.3% | 10.3% | 10.3% | 10.3% | **0.2%** | 0.0% | | 0.000 | 0.000 |
| II-KC808490-Yellow-napped_Parrot/2008 | 10.4% | 10.3% | 10.3% | 10.3% | 10.3% | 10.3% | **0.2%** | 0.0% | 0.0% | | 0.000 |
| II-KC808497-Great_Egret/2009 | 10.4% | 10.3% | 10.3% | 10.3% | 10.3% | 10.3% | **0.2%** | 0.0% | 0.0% | 0.0% | |

The analysis involved 11 nucleotide sequences of the full Fusion gene from genotypes I and II. The percentages of divergence between sequences are shown. The divergences between the vaccines strains and their related isolates are in bold. The divergences between isolates from the same genotype are underlined. Standard error estimate(s) are shown above the diagonal. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1662 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 [16].

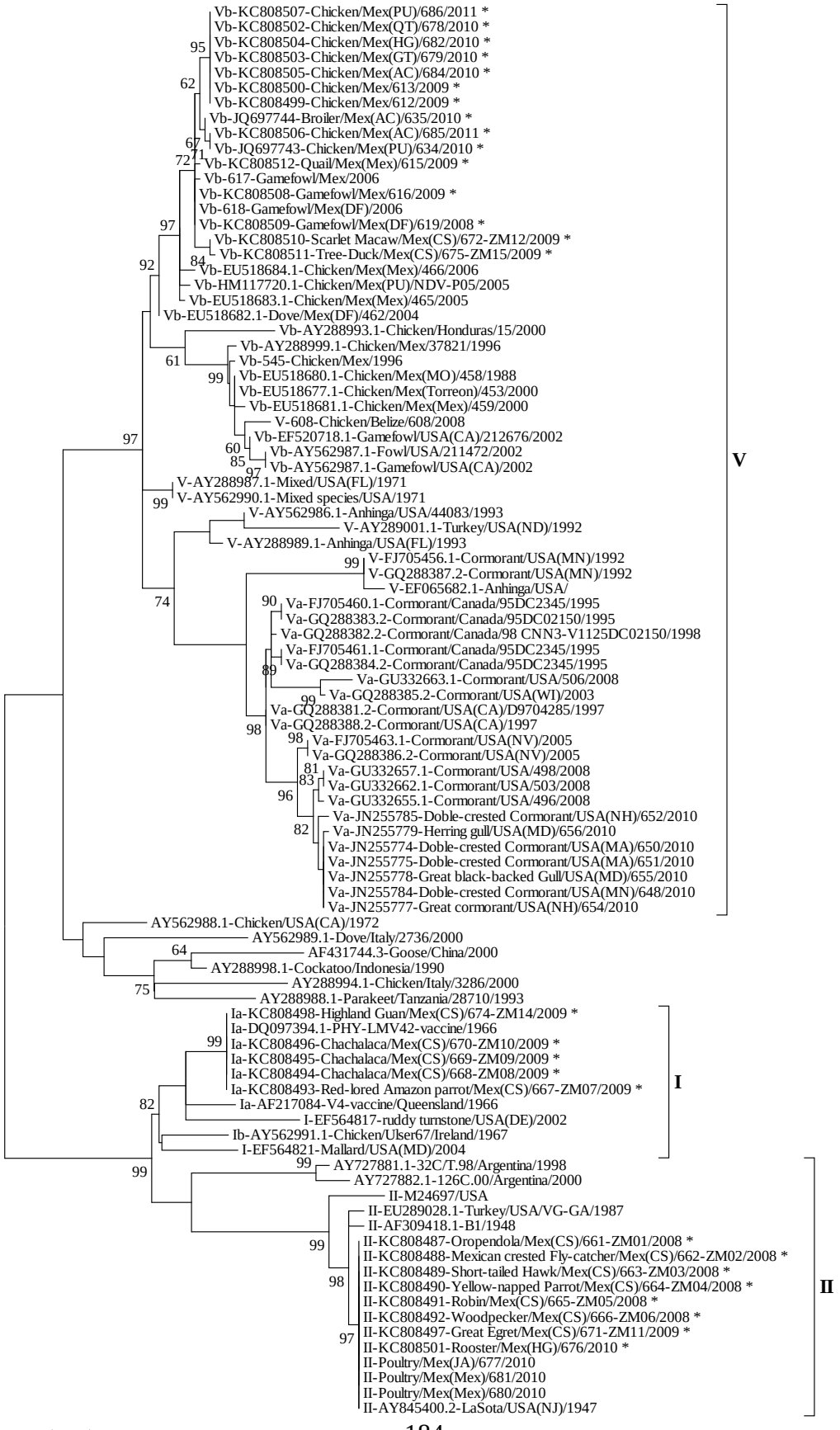
**Fig. A-1 Molecular Phylogenetic analysis by Maximum Likelihood method using the 374 bp N-terminal region of the fusion gene.**

Partial nucleotide sequences from 93 genotypes I, II and V were used in the analysis. There were a total of 374 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (16). The new isolates are denoted with a star (*). The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model [23]. The tree with the highest log likelihood (-3026.7428) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.7762)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.0000% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 94 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated.

**Fig. A-2 Molecular Phylogenetic analysis by Maximum Likelihood method using the full F gene region.**

Two sequences representing each NDV genotype were used and a total of 70 nucleotide sequences of the full Fusion gene were involved in the analysis. There were a total of

1651 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (16). The new isolates are denoted with a star (*). The evolutionary history was inferred by using the Maximum Likelihood method based on the General Time Reversible model (23). The tree with the highest log likelihood (-15015.6528) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.6623)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 37.2721% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. The analysis involved 70 nucleotide sequences. All positions containing gaps and missing data were eliminated.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Vb-KC808500-Chicken/Mex/613/2009 *
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Vb-KC808506-Chicken/Mex(AC)/685/2011 *
Vb-JQ697743-Chicken/Mex(PU)/634/2010 *
Vb-KC808512-Quail/Mex(Mex)/615/2009 *
Vb-617-Gamefowl/Mex/2006
Vb-KC808508-Gamefowl/Mex/616/2009 *
Vb-618-Gamefowl/Mex(DF)/2006
Vb-KC808509-Gamefowl/Mex(DF)/619/2008 *
Vb-KC808510-Scarlet Macaw/Mex(CS)/672-ZM12/2009 *
Vb-KC808511-Tree-Duck/Mex(CS)/675-ZM15/2009 *
Vb-EU518684.1-Chicken/Mex(Mex)/466/2006
Vb-HM117720.1-Chicken/Mex(PU)/NDV-P05/2005
Vb-EU518683.1-Chicken/Mex(Mex)/465/2005
Vb-EU518682.1-Dove/Mex(DF)/462/2004
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Vb-AY288999.1-Chicken/Mex/37821/1996
Vb-545-Chicken/Mex/1996
Vb-EU518680.1-Chicken/Mex(MO)/458/1988
Vb-EU518677.1-Chicken/Mex(Torreon)/453/2000
Vb-EU518681.1-Chicken/Mex(Mex)/459/2000
V-608-Chicken/Belize/608/2008
Vb-EF520718.1-Gamefowl/USA(CA)/212676/2002
Vb-AY562987.1-Fowl/USA/211472/2002
Vb-AY562987.1-Gamefowl/USA(CA)/2002
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V-AY562990.1-Mixed species/USA/1971
V-AY562986.1-Anhinga/USA/44083/1993
V-AY289001.1-Turkey/USA(ND)/1992
V-AY288989.1-Anhinga/USA(FL)/1993
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V-GQ288387.2-Cormorant/USA(MN)/1992
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Va-FJ705461.1-Cormorant/Canada/95DC2345/1995
Va-GQ288384.2-Cormorant/Canada/95DC2345/1995
Va-GU332663.1-Cormorant/USA/506/2008
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Va-GQ288381.2-Cormorant/USA(CA)/D9704285/1997
Va-GQ288388.2-Cormorant/USA(CA)/1997
Va-FJ705463.1-Cormorant/USA(NV)/2005
Va-GQ288386.2-Cormorant/USA(NV)/2005
Va-GU332657.1-Cormorant/USA/498/2008
Va-GU332662.1-Cormorant/USA/503/2008
Va-GU332655.1-Cormorant/USA/496/2008
Va-JN255785-Doble-crested Cormorant/USA(NH)/652/2010
Va-JN255779-Herring gull/USA(MD)/656/2010
Va-JN255774-Doble-crested Cormorant/USA(MA)/650/2010
Va-JN255775-Doble-crested Cormorant/USA(MA)/651/2010
Va-JN255778-Great black-backed Gull/USA(MD)/655/2010
Va-JN255784-Doble-crested Cormorant/USA(MN)/648/2010
Va-JN255777-Great cormorant/USA(NH)/654/2010
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AY562989.1-Dove/Italy/2736/2000
AF431744.3-Goose/China/2000
AY288998.1-Cockatoo/Indonesia/1990
AY288994.1-Chicken/Italy/3286/2000
AY288988.1-Parakeet/Tanzania/28710/1993
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AY727881.1-32C/T.98/Argentina/1998
AY727882.1-126C.00/Argentina/2000
II-M24697/USA
II-EU289028.1-Turkey/USA/VG-GA/1987
II-AF309418.1-B1/1948
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II-KC808489-Short-tailed Hawk/Mex(CS)/663-ZM03/2008 *
II-KC808490-Yellow-napped Parrot/Mex(CS)/664-ZM04/2008 *
II-KC808491-Robin/Mex(CS)/665-ZM05/2008 *
II-KC808492-Woodpecker/Mex(CS)/666-ZM06/2008 *
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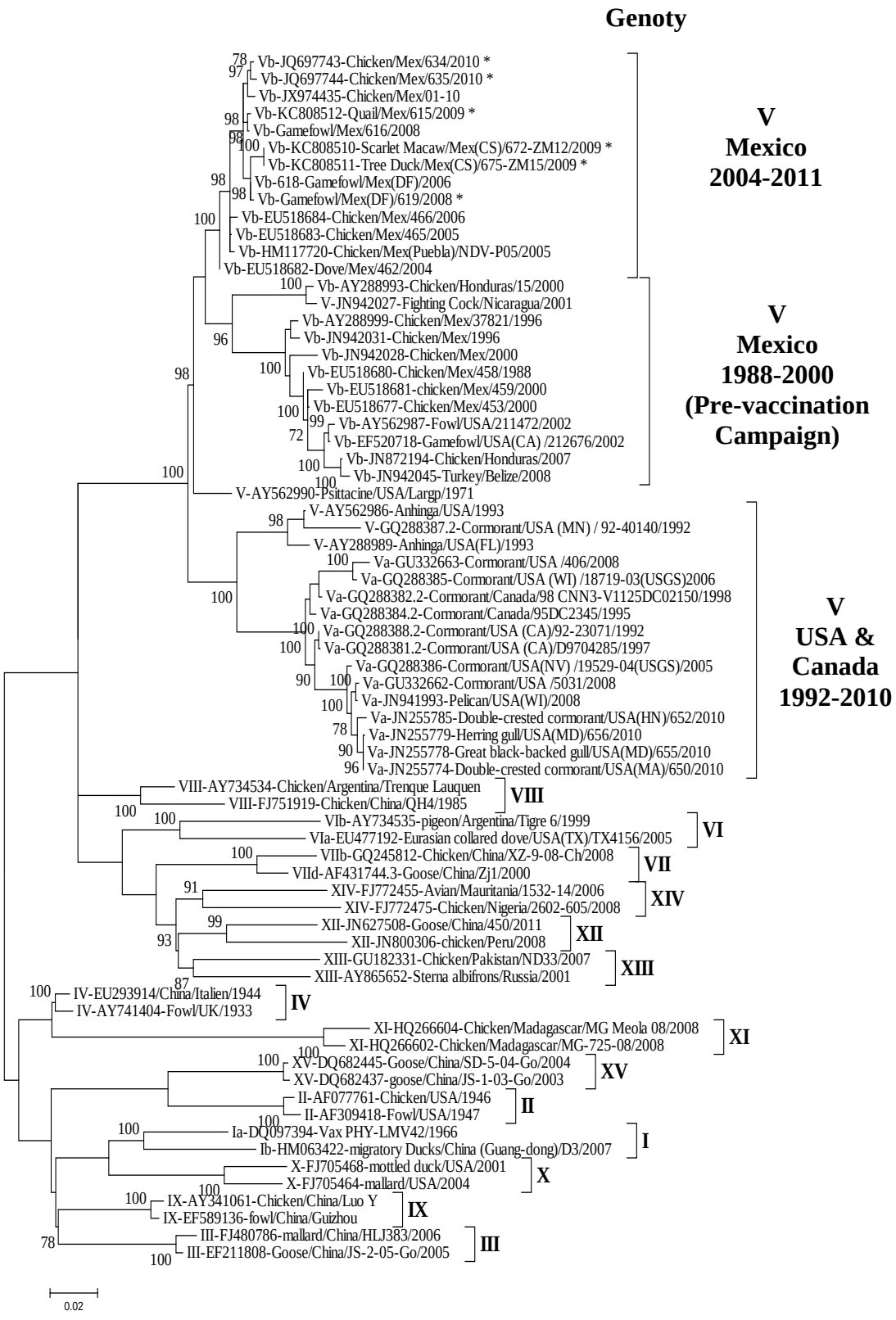
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Vb-KC808511-Tree Duck/Mex(CS)/675-ZM15/2009 *
Vb-618-Gamefowl/Mex(DF)/2006
Vb-Gamefowl/Mex(DF)/619/2008 *
Vb-EU518684-Chicken/Mex/466/2006
Vb-EU518683-Chicken/Mex/465/2005
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Vb-JN942028-Chicken/Mex/2000
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Vb-EU518681-chicken/Mex/459/2000
Vb-EU518677-Chicken/Mex/453/2000
Vb-AY562987-Fowl/USA/211472/2002
Vb-EF520718-Gamefowl/USA(CA) /212676/2002
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Vb-JN942045-Turkey/Belize/2008
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V-AY562986-Anhinga/USA/1993
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V-AY288989-Anhinga/USA(FL)/1993
Va-GU332663-Cormorant/USA /406/2008
Va-GQ288385-Cormorant/USA (WI) /18719-03(USGS)2006
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Va-GQ288384.2-Cormorant/Canada/95DC2345/1995
Va-GQ288388.2-Cormorant/USA (CA)/92-23071/1992
Va-GQ288381.2-Cormorant/USA (CA)/D9704285/1997
Va-GQ288386-Cormorant/USA(NV) /19529-04(USGS)/2005
Va-GU332662-Cormorant/USA /5031/2008
Va-JN941993-Pelican/USA(WI)/2008
Va-JN255785-Double-crested cormorant/USA(HN)/652/2010
Va-JN255779-Herring gull/USA(MD)/656/2010
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Va-JN255774-Double-crested cormorant/USA(MA)/650/2010
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Canada
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XIII-AY865652-Sterna albifrons/Russia/2001
XIII
IV-EU293914/China/Italien/1944
IV-AY741404-Fowl/UK/1933
IV
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II
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Ib-HM063422-migratory Ducks/China (Guang-dong)/D3/2007
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X-FJ705464-mallard/USA/2004
X
IX-AY341061-Chicken/China/Luo Y
IX-EF589136-fowl/China/Guizhou
IX
III-FJ480786-mallard/China/HLJ383/2006
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0.02