SELECTED STUDIES ON AVIAN RNA VIRUSES

A Dissertation

by

ITAMAR DAVI SCHABERT VILLANUEVA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2010

Major Subject: Veterinary Microbiology

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Approved by:

Chair of Committee,	Ian Tizard
Committee Members,	Yawei Ni
	Luc Berghman
	John Quarles
Head of Department,	John August

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ABSTRACT

Selected Studies on Avian RNA Viruses. (2010)

Itamar Davi Schabert Villanueva, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Ian Tizard

There are many pathogens that infect birds and perhaps many more that researchers have not yet identified. Of all potential pathogens, the research presented in this manuscript focuses on two avian RNA viruses.

First, a serodiagnostic test for newly described Avian Borna Virus (ABV), which has been recently identified as the etiological agent of Proventricular Dilatation Disease (PDD), was developed. PDD is a deadly disease which affects many birds, but to this point, has mainly been a concern of psittacines. The need for a diagnostic test is imperative. An antigen associated with PDD was identified from the brains of affected birds by use of the Western blot assay. This antigen was subsequently purified using various protein purification protocols, including a modification of reverse-phase chromatography. The antigen was then identified as the ABV nucleoprotein according to tandem mass spectroscopy analysis and protein database search. A serodiagnostic assay was developed and standardized using infected cell culture as an antigen source. Over 100 avian serum samples were submitted by veterinarians to test for the presence of antibodies against ABV nucleoprotein. This serodiagnostic assay was found to have 90% sensitivity and 82% specificity for the diagnosis of ABV in infected birds.

Second, the ability of a carbohydrate epitope to enhance the humoral immune response to an influenza vaccine was tested in chickens. Influenza is a serious infection that causes 36,000 deaths annually in the United States. The need for a more efficacious is addressed by incorporating a carbohydrate antigen targeted by natural antibodies that are produced by chickens as well as humans. Therefore, chickens may be a suitable animal model to test this hypothesis. Influenza vaccines with alpha-gal antigen are prepared from cell culture. The antigen is then enzymatically removed from some vaccines and the nature of the ensuing humoral immune response to these vaccines in chickens is attempted.

Though ABV is not known to be zoonotic at this time, zoonotic infections pose the highest risk as new and emerging infectious diseases in the human population. The following research contains applications relative to challenges faced by researchers and clinicians in infectious disease containment.

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CHAPTER I

AVIAN BORNA VIRUS

1. Introduction

Borna disease is a condition which was most likely first characterized in the late 18th century in southeastern Germany. Published in a veterinary handbook, the author correctly described the clinical symptoms of Borna disease in horses and attributed the condition to unsatisfied sexual desire or overfeeding. It was recommended, among other things, to thread a rope covered in Spanish fly through the subcutaneous tissues of the patient (Kaprowski, 1995). A second account was recorded in 1885 in Borna, Saxony, Germany where horses in a cavalry regiment suffered deaths due to a neurologic disease. The horses presented with behaviors such as abnormal head movements, walking into walls and being unable to chew food (Keizo, 2002). Despite the long history of this disease, much remains to be understood about the nature, infectivity, and molecular pathogenesis of the etiologic agent, named Borna Disease Virus (BDV). The virus has been found to cause serious neurologic disease in some hosts, and yet not cause any clinical symptoms in others. The virus adds to this enigma by its slow growth in cell culture, noncytolytic property, and ability to establish lifelong chronic infection. Throughout the literature, Borna Disease Virus (BDV) is the name of the virus that, regardless of host, causes a neurological disease which affects behavior and coordination. The recent discovery of a new type of BDV, Avian Borna Virus (ABV), is currently known to be limited to avian species and has been associated with a disease that is specific to birds.

This dissertation follows the style of Veterinary Microbiology.

Proventricular Dilatation Disease (PDD) is a disease specific to the avian gastrointestinal system and therefore unable to be duplicated in a mammalian system due to anatomical differences. However, evidence indicates that the nervous system of both birds and mammals may be similarly affected by ABV and BDV, respectively. In this regard, both viruses cause similar diseases. For clarity, the term Borna virus (BV) in this manuscript will be given to the mammalian Borna virus known to cause Borna Disease in susceptible hosts. The term Avian Borna Virus (ABV) will be given to the virus that causes PDD in susceptible birds.

2. Host Range and Epidemiology

BV has a broad host range and presumably just as broad a geographical range. The presence of borna virus infection, or exposure, is confirmed by presence of viral ribonucleic acid (RNA) and/or antibodies to BV antigens. Antibodies and/or RNA to BV have been detected in psychiatric patients in Japan, as well as clinically healthy Japanese individuals (Haga et al., 1997a; Haga et al., 1997b; Kishi et al., 1995; Matsunaga et al., 2008). Evidence of BV infection has also been demonstrated in sheep, horses, cats, cattle, and recently raccoons (Hagiwara et al., 1997; Hagiwara et al., 2009; Hagiwara et al., 1996; Nakamura et al., 1996; Nakamura et al., 1995). Australia has reported evidence of Borna virus infections in humans as well as cats, but not in horses (Flower et al., 2008; Kamhieh and Flower, 2006; Kamhieh et al., 2006; Kamhieh et al., 2008). Investigators have demonstrated evidence of borna virus infection in ostriches and farm workers in ostrich farms in South Africa and Israel (Verwoerd, 2000; Weisman et al., 1994; Wormser et al., 1996). Individuals with psychiatric disorders and clinically healthy individuals were found to have antibodies against BV phosphoprotein in Brazil (Miranda et al., 2006). In the United States, patients with neurological disease were found to be seropositive for BV by indirect immunofluorescent assay in a study conducted in the early 1980s (Amsterdam et al.,

1985; Rott et al., 1985). Additionally, the presence of Borna virus was also determined to be in such varied hosts as deer, primates, sloths, alpacas, llamas and hippopotami (Ernst, 1927; Ikuta, 2002; Zwick, 1939). Chickens have been shown to be susceptible to experimental infection with borna virus (Ludwig et al., 1973). In light of these findings, it seems appropriate to conclude that Borna virus has a world-wide presence and has an extremely broad host range in warm blooded animals. It seems as if BV has been found almost wherever its presence was sought. Because the disease has been found in such a diverse group of hosts, it seems difficult to speculate about the natural host of this virus. However, researchers have suggested that the reservoir host may be the shrew, *Crocidura leucodon*. The shrews were found in an area where BV was endemic and harbored the virus without any detectable histopathologic signs of infection (Hilbe, et al., 2006). One controversial topic is BV's ability to cause disease in humans. The literature suggests that humans have been infected with BV because of the detection of antibodies and RNA specific for BV. However, BV infection in humans has been associated with varied neurologic conditions thereby making it harder to establish this virus as the etiologic agent of these diseases. The fact that BV has also been detected in clinically normal human patients adds to the mystery. Detection of BV in human patients by PCR has been put in question because the technique falls victim to its own strength. As the sensitivity of the PCR assay increases, so does the probability that the sample tested was contaminated. False positive results are always of great concern given the virus' remarkable genetic conservation.

3. Molecular Biology of Borna Virus

Borna virus is an enveloped, nonsegmented, negative-sense, single stranded RNA virus and the prototype member of the family Bornaviridae in the order Mononegavirales. Other members of this order include Filoviridae, Paramyxoviridae, and Rhabdoviridae. Borna virus

distinguishes itself from other members in the order Mononegavirales because it replicates in the nucleus, specifically, in the nucleolus (Cubitt et al., 1994; Pyper et al., 1998). Because of its intranuclear replication, BV has the ability to utilize cellular splicing proteins in order to alternatively splice viral mRNA and is able to use different initiation codons to transcribe overlapping open reading frames that allows for the production of a wide variety of proteins (Briese et al., 1994; Schneider et al., 1994; Tomonaga et al., 2000). The BV genome codes for at least six proteins of which the function has been at least partly characterized. These include the L-polymerase (L, 190 kDa), the glycoprotein (G, 57 kDa) two isoforms of the nucleoprotein (N, 38kDa and 40kDa), the phosphoprotein (P, 23kDa), the matrix protein (M, 16 kDa), and the more recently characterized X protein (X, 10 kDa) (Hornig et al., 2003). Expression of the L protein, the BV polymerase, provides the prototypical example of alternative splicing. In the third transcription unit of the BV genome, fusion of a small upstream open reading frame with a larger downstream continuous open reading frame produces the polymerase protein of BV(Keizo, 2002). The L protein has a nuclear localization sequence, is phosphorylated, and interacts with P protein in a fully functional ribonucleoprotein (RNP) complex (Walker et al., 2000; Walker and Lipkin, 2002). The glycoprotein (G) of BV is thought to mediate viral entry into the host cell by clathrin mediated endocytosis (Clemente and de la Torre, 2009). The G protein is heavily glycosylated and is cleaved into GP1 and GP2 by the cellular enzyme furin (Bajramovic et al., 2003; Clemente and de la Torre, 2007). Though the molecular weight of the non-glycosylated G protein is about 57 kDa, extensive glycosylation causes the molecular weight to increase to 84-94 kDa (Clemente and de la Torre, 2009). GP1 has been shown to be sufficient for viral entry in to the host cell, whereas GP2 is thought to mediate pH dependent fusion between the virus and cellular membranes (Gonzalez-Dunia et al., 1998; Perez et al., 2001).

Interaction between the G protein and a cellular receptor for viral entry into the host cell has not been demonstrated, but it is speculated that the G protein may interact with a member of the glutamate receptor family (Hornig et al., 2002). The N protein is the main target of both the humoral and cellular immune response. Nucleoprotein is also the most abundant protein in BV infected cell culture and in experimentally infected animals (Keizo, 2002) . The two isoforms of BV nucleoprotein are 38 kDa and 40 kDa. The production of these two isoforms may result from two different mRNA species, or from differential usage of inframe initiation codons in the BV N gene (Pyper and Gartner, 1997). The N protein is responsible for binding viral RNA to form the ribonucleoprotein (RNP). The 38kDa isoform of the BV N protein is able to transport the infectious RNP into the cytoplasm of the infected cell. The 40 kDa isoform not only contains a nuclear export signal, it also contains a nuclear localization signal so that it is transported back into the nucleus (Kobayashi et al., 2001). The 40kDa is also the only isoform of the N protein that is able to support replication and transcription in studies undertaken with a BV minigenome (Schneider et al., 2004b). Crystal structures of the BV N protein show oligomerization of the protein and RNA binding domains that are similar to other negative-sense RNA viral nucleoproteins of influenza and two rhabdoviruses (Luo et al., 2007; Rudolph et al., 2003). The BV phosphoprotein is responsible for regulation of the active viral nuclear polymerase by interaction with the N, L, and X proteins (Poenisch et al., 2004; Schneider, 2005). P protein is also involved in encapsidation of viral RNA (Schneider, 2005). Like the N protein, the P protein has an isoform termed P' that is the result of differential usage of initiation codons in BV infected cell cultures. However, the P' isoform does not seem to play an active role in supporting viral replication (Schneider et al., 2004a). More recently, P protein was found to interact with gamma-aminobutyric acid receptor-associated protein (GABARAP) in infected cell

culture. GABARAP plays an important part in regulation of gamma-aminobutyric acid, an important neurotransmitter. The interaction between GABARAP and P protein may shed light on the manipulation of the neuronal network by BV proteins (Peng et al., 2008) . The matrix protein has been reported to be part of the RNP complex, but its role in viral RNA transcription is under investigation (Mayer et al., 2005). BV M protein has been shown to interact with the P protein, but not other proteins in the RNP complex. Unlike the matrix proteins of other viruses such as influenza and respiratory synctial virus that serve to negatively regulate viral transcription, the BV M protein seems to enhance viral transcription without interference of the viral polymerase (Chase et al., 2007). The matrix protein of a similar virus, measles virus, was shown to regulate viral transcription via interaction with the measles nucleoprotein (Iwasaki et al., 2009). Single-strand RNA binding properties have been described in the crystal structure of the BV M protein. With a structure similar to Ebola virus matrix protein, the BV M protein is able to bind single stranded RNA and lipid structures, presumable lipid membranes (Neumann et al., 2009). Lastly, the non-structural X protein has been shown to negatively regulate viral polymerase function through interaction with P protein and perhaps indirect contact with N protein via the P protein (Perez et al., 2003; Poenisch et al., 2004; Schneider et al., 2003). The X protein has been shown to serve as a buffer against the effects of P protein interaction with N protein during viral replication (Poenisch et al., 2008). As will be shown later, the ratio of N protein to P protein in the nucleus regulates the rate of viral replication. Other possible functions of the X protein include interaction with heat shock cognate protein 70 as a means to translocate in and out of the nucleus and ability to inhibit cellular apoptosis (Hayashi et al., 2009; Poenisch et al., 2009).

4. Viral Life Cycle

The life cycle of BV is one that has seen significant advances in recent years, but details remain to be elucidated. The cycle of infection is suspected to occur in a similar manner as other members of the order Mononegaviradae with a few important exceptions, most notably replication in the nucleus. Borna virus is noncytopathic and able to infect primarily neuronal cells including astrocytes, oligodendrocytes, and Schwann cells as well as peripheral blood mononuclear cells (Hatalski et al., 1997; Koprowski, 1995; Richt et al., 1997; Sierra-Honigmann et al., 1993). Cell culture lines susceptible to BV infection include MDCK, Vero cells and L929 cells (Koprowski, 1995). Viral entry into the host cell is mediated by G protein attachment to an unknown cellular receptor followed by endocytosis into the host cell via a clathrin mediated, caveola-independent pathway (Clemente and de la Torre, 2009). The receptor for BV entry into the host cell is thought to belong to the glutamate family, although this has been disputed (Hornig, et al., 2002; Richter, et al., 2009). The viral G protein attaches to its receptor and is cleaved into gp56 and gp43. It is thought that gp56 mediates the attachment to the cellular receptor and gp43 mediates pH dependent fusion to the cellular membrane in order to release the ribonucleic protein (RNP) complex into the cytoplasm (Gonzalez-Dunia, et al., 1998). The genomic and anti-genomic RNA are neither capped, nor polyadenylated, and therefore exist as infectious RNP complexes (de la Torre, 1994). The RNP complex is targeted for import into the nucleus by the nuclear localization sequences encoded in the N, P, and L proteins. The X and M proteins are also targeted to the nucleus via nuclear localization sequences. However, it is not known whether these proteins enter the nucleus as part of the RNP, or if they bind to the RNP complex once in the nucleus. Transcription of the polycistronic genome is carried out from the 3’ to the 5’ end by the polymerase complex, consisting of the N, P, L, X, proteins and perhaps

the M protein. One proposed mechanism for viral transcription is "cartwheeling" where the P protein provides the "legs" upon which the polymerase (L) protein rests. The P protein then "walks" on the N proteins with simultaneous making and breaking of bonds with the N protein while the L protein transcribes the viral RNA (Curran, 1998; Schneider, 2005). Transcription is regulated stoicheometrically by the ratio of N to P. It is proposed that the optimal ratio of N to P for efficient transcription is 1:20; a ratio of 1:1 completely abolishes viral transcription (Schneider et al., 2003). However, it was shown that addition of the 38 kDa isoform of the N protein to an infected cell could reactivate transcription in unfavorable N to P ratios (Schneider et al., 2004b). The P protein is able to inhibit N protein from exiting the nucleus by masking its nuclear exit signal and serves as another method of viral transcriptional regulation. The N protein, and perhaps the RNP complex, exits the nucleus through the CMR1-dependent pathway (Kobayashi et al., 2001). CMR-1 is a nuclear export receptor that carries proteins, such as the HIV Rev protein, through nuclear pores and into the cytoplasm (Stutz and Rosbash, 1998). Though both isoforms of the N protein contain a nuclear export sequence, only the 40 kDa isoform contains a nuclear localization sequence and is thus targeted back to the nucleus for further rounds of transcription (Kobayashi et al., 2001). The process of BV virion assembly is currently unknown, but researchers reported cytoplasmic enveloped viral particles 50-100 nm in infected cells that were morphologically similar to partially purified BV virions (Compans et al., 1994). Borna virus is thought to infect cells in a manner similar to that of rabies virus in that the RNP complexes spread along the neuronal networks inside axons. However, unlike rabies virus, fully formed BV particles have not been visualized along neuronal networks (Carbone et al., 1987; Gosztonyi et al., 1993). Several investigators have been successful in visualizing virus-like particles from cell cultures infected with BV by adding sodium butyrate to the cell culture

media. The addition of sodium n-butyrate (6-10mM) is known to inhibit cell division and is thought to produce conditions in which viral RNA transcription is favored over cellular RNA transcription (Kohno et al., 1999; Pauli and Ludwig, 1985). Amantadine has been proposed as a drug that could be used as treatment for BV infection. However, the mechanism of the drug and its efficacy are still a topic of debate (Cubitt and de la Torre, 1997; Gonzalez-Dunia et al., 1998; Ludwig and Bode, 2000; Stitz et al., 1998b).

5. Animal Models of Borna Virus Disease

Borna virus has a broad host range and therefore has allowed investigators to use several animal models for experimental infection. Some animal models used to study BV pathogenesis include chickens, mice, rabbits, rhesus monkeys, and shrews with the rat serving as the most common animal model in BV research (Keizo, 2002). Borna disease has an acute phase in most animal models which include hyperactivity, aggression, large appetite, priapism, and incoordination. The chronic phase is characterized by a docile and passive attitude in infected animals that may turn abruptly, but temporarily, aggressive when disturbed (Pletnikov, 2002). These clinical symptoms correspond with the pathology of the acute and chronic phases of Borna disease. Namely, the acute phase is characterized by a high degree of encephalomyelitis followed by a chronic phase with reduced inflammation without a decrease in viral antigen (Gosztonyi and Ludwig, 1995). As with most viruses, the clinical and pathological manifestation of BV infection depends on such variables as strain type, immune status of the host, and age among other things. For example, adult rats that are infected with BV tend to have an acute and sometimes fatal encephalitis. Rats infected with BV at 5 to 6 weeks of age are found to demonstrate aggressive and neurotic behavior such as biting of cagemates, and have "ravenous appetites" at about 20 to 30 days post-infection. After the initial symptoms of disease

subside at about 50-60 days, rats became subdued and depressed as neuronal cell death occurs. The pathology of these rats corresponds with their clinical behavior in that there is a significant mononuclear inflammatory immune response primarily in the cerebral cortex, hypothalamus, and thalamus up to 20-30 days post infection (Narayan et al., 1983a). During the chronic phase of the disease, progressive cellular death is observed as the virus spreads from the hippocampus and frontal cortex to the hypothalamus, particularly the dentate gyrus, and all over the central nervous system and the spinal cord, including the ganglia of the spinal cord (Carbone et al., 1987; Jordan and Lipkin, 2001). Infection of neonatal rats results in chronic viral presence in the brain. Neonatal rats infected with BV exhibit a limited pro-inflammatory response, abnormal development of the cerebellum and hippocampus, retarded growth and antisocial behavior that is similar to autism spectrum disorder (Hornig et al., 2001; Hornig et al., 1999; Rubin et al., 1999) Because of the similarity that Borna disease in rats shares with autism, BV infection of neonatal rats is often used to study the possibility that autism may be virally induced, resulting in disruption of proper neuronal development, including imbalances of neurotransmitters such as serotonin and dopamine (Heimrich et al., 2009; Hornig et al., 2002). Immunosuppressed rats that are infected with BV have the same outcome as those that are infected neonatally with one important difference: neonatally infected rats are still able to mount a humoral response to BV antigens (Narayan et al., 1983b). The humoral immune response in hosts is thought to limit viral replication from spreading to non-neural tissues (Stitz et al., 1998a). Borna infection in rhesus monkeys tends to have the same pathology and clinical symptoms as BV infected rats. Just as in rats, immunopathology seems to be driven by T cells, more specifically cytotoxic CD8 T cells, infection is restricted to neural cells, and splenectomized test subjects tend to have a milder form of encephalomyelitis (Baur et al., 2008; Bilzer et al., 1995; Stitz et al., 1981). Of 13 day-old

chicks infected intracerebrally with rabbit brain homogenate containing BV, all suffered paralysis of the wings and feet and became ataxic. Four of the 13 chickens were able to recover from the disease after suffering mild paralysis (Ludwig et al., 1973). Borna virus is uncommon in that it is able to establish a persistent chronic infection if the host survives the acute phase of the disease. The ability of BV to establish a persistent neurological infection makes it similar to agents such as lymphocytic choriomeningitis virus, herpesvirus, and the transmissible spongiform encephalopathies. How an RNA virus is able to do this is of interest. One hypothesis is that BV is able to trim its genome in such a manner that the 5' termini of viral RNA contain monophosphate instead of triphosphate motifs so as to avoid detection by the host pattern-recognition receptor retinoic acid inducible gene (RIG-1), and thus triggering an immune response against the virus (Schneider et al., 2007). Recently, it has been shown that coxsakievirus, an RNA enterovirus, is able to establish chronic infection in the central nervous system by perhaps trimming its genome into a non-virulent form of the virus. Researchers were also able to show that coxsakievirus RNA from mouse brain homogenates was able to infect cell cultures (Feuer et al., 2009). Perhaps this may offer a clue as to how BV is able to establish a chronic infection in the nervous system.

6. Immune Response to Borna Virus

The adaptive immune response plays the dominant role in the host response to BV infection. More specifically, BV infection causes T-cell mediated immunopathology on the host nervous system due to the inability of the host to clear BV and due to inefficient major histocompatability class (MHC) presentation by neurons (Baur et al., 2008; Hart and Fabry, 1995; Joly et al., 1991). However, some researchers argue that astrocytes, in addition to microglial cells, are competent antigen presenting cells (Fontana et al., 1984) In animal

experimental models, the virus has a propensity to replicate in cells from the neural crest such as neurons, astrocytes and ependymal cells and tends to spread throughout the central nervous system (Carbone et al., 1991; Stitz et al., 1995). Histopathology of BV infection shows perivascular cuffing consisting of T cells, macrophages and B cells (Deschl et al., 1990). During the acute phase of Borna disease, inflammatory cells infiltrate neural vessels to form perivascular cuffs. During the chronic phase of Borna disease, the numbers of these inflammatory cells decrease and the histopathology resembles a "burned out" encephalitis. Cytotoxic CD8 T cells play a substantial role in immunopathology by causing lesions due to cell death in the brain of hosts infected with BV (Bilzer and Stitz, 1994; Planz et al., 1995; Stitz et al., 1993). These lesions appear to be IFN-γ mediated and may not require antigen presentation by MHC (Baur et al., 2008; Hausmann et al., 2005). Though cell death may be driven by cytotoxic T cells, evidence exists that IFN-γ may have a protective effect on bystander cells (Richter et al., 2009). BV, and/or the resulting immune response, in concert with proinflammatory cytokines is somehow able to enhance T cell trafficking across the brain paremchyma (Baur et al., 2008). CD4 cells play a less prominent role in BV immunopathology than do CD8 T cells. An exception is noted in immunosuppressed rats experimentally infected with BV. Rats died without neurological symptoms and drastic weight loss as early as 5 days after injection of effecter CD 4 T cells (Planz et al., 1995). Antibodies designed to minimalize CD4 T cell activity during BV infection have minimal effects on the resulting encephalitis and merely delay the disease process (Noske et al., 1998; Pletnikov, 2002). Research into the role of T cells in the immunopathology of BV has mainly focused on CD4 and CD8 Tcells and has not addressed the role of other T cell subsets. Some papers did not account for the fact that T helper 1 cells have cytotoxic activity. Furthermore, little research has been performed on the role of regulatory T

cells. It is unquestioned that BV neurodegeneration is mediated by T cells. However, the fact that the virus is able to replicate in chronic Borna disease with a reduction of inflammatory cells suggests that immunoregulation is at work. In HIV-mediated neurodegeneration and coronavirus-induced acute encephalitis, regulatory T cells have a protective effect against superfluous inflammation and cell-mediated cytotoxicity (Anghelina et al., 2009; Liu et al., 2009). Addressing these questions may explain the observation of Planz, et al that "The characteristics [of some helper T cells] suggest the presence of an intermediate or unusual phenotype of T-helper cells." (Planz et al., 1995).

7. Proventricular Dilatation Disease

Proventricular Dilatation Disease (PDD) is a fatal disease of mainly psittacid birds that is of great concern to aviculturalists. PDD was first described in the 1970's in a group of birds brought to the United States from Peru (Tizard, personal communication). Also termed macaw wasting disease, neuropathic gastric dilatation, and lymphoplasmacytic ganglioneuritis, PDD classically results in malabsorption of nutrients by its host evidenced by weight loss, diarrhea, and undigested food present in the feces. PDD is highly suspect when in addition to these clinical signs, there are neurological symptoms such as ataxia, abnormal head movements, and paralysis (Gregory, 1994). Further confirmation of PDD is determined by radiographical evidence of dilatation of the proventriculus (Dennison et al., 2009; Dennison et al., 2008; Gregory et al., 1996). Histopathologic examination reveals peripheral neuritis of the autonomic nerves and ganglia connected to the digestive tract. In a PDD affected bird, the nerves and tissues connected to the digestive tract contain lymphoplasmacytic infiltrates consisting of lymphocytes, plasma cells, macrophages, and heterophils (Berhane et al., 2001). Chickens that experience a similar disease resulting in proventriculitis experience a massive infiltration of T

cells, with CD8 T cells outnumbering CD4 T cells in the proventricular glands of infected chickens (Pantin-Jackwood et al., 2004). Lymphoplasmacytic infiltrates are also found in central and peripheral nervous tissue of PDD infected birds that include the brain and spinal cord in the form of perivascular cuffing. In addition to the nervous and digestive systems, lymphoplasmacytic infiltrates have been described in the myenteric plexuses and connective tissue of the heart, adrenal glands, and other tissues in varying severity in birds diagnosed with PDD (Perpinan et al., 2007; Vice, 1992). These non-suppurative inflammatory infiltrates, especially in the crop, are the hallmark histopathologic diagnosis of PDD. A viral agent has long been suspect as the cause of this disease. Viruses thus far implicated include adenovirus, avian encephalitis virus, avian herpesvirus, coronavirus, eastern equine encephalitis virus, enterovirus, paramyxovirus, and polyomavirus, among others (Gough et al., 2006; Gough et al., 1996; Gregory, 1995, 1997a, b; Grund, 1999; Grund et al., 2002)

Accurate diagnosis of PDD has presented a challenge for avian medicine practitioners and researchers. Crop biopsy has been the standard method for PDD diagnosis with reported success rates of about 60 to 70% (Gregory et al., 1996). However, a significant number of PDD cases have been presented as not having any lymphoplasmacytic infiltrates in the crop (Doolen, 1994a). In another study, different sections of crops from birds histologically diagnosed with PDD were examined to determine if there was any tendency for lymphoplamacytic infiltration to accumulate in a specific section. The investigators concluded there was no correlation between location of crop biopsy and improved accuracy of PDD diagnosis (Gregory et al., 1996). Other methods for PDD diagnosis include radiography of the proventriculus to check for enlargement. However, proventricular dilatation may also be caused by metabolic and gastrointestinal problems due to proventricular stasis. General gastrointestinal stasis may be caused by lead

toxicosis, internal papillomas, proventriculitis, tumors and various metabolic disorders (Bond et al., 1993). In addition, the orientation of the proventriculus relative to the ventriculus varies between psittacine species, thus making it more difficult to determine proventricular distention. Likewise, certain species of parrots have a naturally distended proventriculus making diagnosis of PDD in juvenile birds more complicated (Hoppes, personal communication). These complications make radiographs often unreliable for PDD diagnosis. Serological diagnosis of PDD is an alternative option. Because virus isolation and identification has until recently been largely unsuccessful, PCR and ELISA techniques have not been employed in diagnosing PDD. Grund and colleagues proposed that paramyxovirus infection is associated with PDD and therefore may serve as a diagnostic test (Grund et al., 1999). This theory was put to practice. A population of 50 Spix's macaws, one out of three left in the world, at Al Whabra suffered an outbreak of PDD in 2004. Since that time, screening for PDD by both crop biopsy and detection of antibodies to avian paramyxovirus (APMV) was implemented. In 2008, Deb and colleagues presented results that suggested crop biopsies were only 30% sensitive for diagnosing PDD that was later confirmed by histopathology. Furthermore, APMV-1 seropositivity was only 22.3% sensitive in diagnosing cases of PDD that were later confirmed by crop biopsy (Deb et al., 2008). In their opinion, Deb and colleagues found that antibodies to avian paramyxovirus were less accurate than crop biopsy for diagnosis of PDD.

The summer of 2008 ushered in significant advances in PDD research. One group of researchers from the University of California, San Francisco, used a pan-viral microarray to detect BV hybridization in 5 of 8 PDD cases and none in 8 PDD negative cases, all verified by histopathology. Using ultra high throughput sequencing, the group partially sequenced 16 BV isolates and characterized 5 different strains of the virus. The group dubbed this virus Avian

Borna Virus (ABV) since this newly described virus shared around 64% nucleic acid homology with mammalian BV (Kistler et al., 2008). A group at the Schubot Center for Exotic Bird Health at Texas A&M University, College of Veterinary Medicine published the discovery of a 38 kDa antigen in the brains of 5 of 5 PDD cases and none in 3 PDD negative cases, all confirmed by histopathology. This research involved the use of a western blot technique with brain homogenate of PDD infected and uninfected birds as the antigen source and serum from the same birds to determine the presence of the 38 kDa antigen (Villanueva et al., 2008). A third group from Columbia University was the first to publish the DNA primer sequences to detect a newly discovered BV in parrots infected with PDD. This group subsequently published their findings in which this newly described BV was detected in 3 PDD positive birds and none in 4 PDD negative birds, all confirmed by histopathology. Honkavuori and colleagues also determined that antibodies against mammalian BV N and P proteins were not reactive with antigen from PDD infected bird tissue homogenates (Honkavuori et al., 2008). The lack of reactivity between mammalian BV proteins and avian antibodies from birds infected with PDD was observed at the Schubot Center at Texas A&M in a collaborative effort with Honkavuori and colleagues at Columbia University. These findings reiterated the lack of antigenic cross reactivity between avian and mammalian Borna viruses (Villanueva et al., unpublished observations). Researchers also determined that ABV was capable of infecting both neuronal and non-neuronal tissue, was able to grow in chicken and quail cell lines, and antibody against mammalian P protein was cross reactive with P protein from a genotype 2 virus (Rinder et al., 2009). That a new pathogen had been discovered was not debatable. However, if ABV was the etiological agent of PDD was not proven until a year later. Cockatiels that were determined to be PDD negative by PCR detection of ABV in fecal samples were inoculated orally with brain

homogenate from a PDD infected bird, or from a PDD negative bird. Cockatiels infected with PDD homogenate succumbed to disease that clinically and histopathologically resembled PDD. Though the brain homogenates contained 2 other viruses, it was not shown that these viruses caused infection; control birds remained normal (Gancz et al., 2009). ABV infection studies with similar results were carried out at the Schubot Center. Patagonian conures succumbed to PDD following inoculation with ABV infected cell culture while a conure inoculated with ABV negative cell culture remained healthy (Gray, 2009). ABV tropism in the central nervous system was further elucidated by histopathology and immunohistochemistry performed in PDD infected birds and PDD negative birds. These studies showed that ABV infected primarily neurons, and glial cells. ABV had a detrimental effect on the Purkinje cell layer of the cerebellum. Cell atrophy in the Purkinje cell layer was curious in that it was thought to be caused by cell death of surrounding subpopulations of neighboring cells (Ouyang et al., 2009).

The following chapters describe the development of a serodiagnostic test for PDD and the implementation of such a test to determine its diagnostic value as a *bona fide* test for PDD. The main antigen of this test is then purified, revealing some interesting characteristics about the AVB nucleoprotein as an immunogen.

CHAPTER II

DETECTION OF AN ANTIGEN SPECIFIC FOR PROVENTRICULAR DILATATION DISEASE IN PSITTACINE BIRDS

1. Introduction

Proventricular dilatation disease (PDD) is an often fatal disease of psittacine birds and perhaps wild birds (Daoust et al., 1991; Gregory, 1997b; Perpinan et al., 2007; Weissenbock et al., 2009b). PDD has been documented in South American, African, and Australian parrots, suggesting that this disease may have world-wide distribution (Clark, 1984; Doneley et al., 2007). The cases documented have mainly been on parrots in the pet trade, but it is entirely plausible that wild parrots may harbor the disease as well. The classical presenting sign is paralysis and dilatation of the proventriculus secondary to nerve damage in the affected organs. Birds afflicted with PDD will classically present undigested seeds in their feces, regurgitation, lethargy, and ataxia and even blindness (Steinmetz et al., 2008). Some birds may present only gastrointestinal symptoms whereas others will present mainly neurological symptoms. Oftentimes, birds with PDD will display both types of clinical symptoms, attesting to the complex nature of the disease and its pathogenesis. Grund and others (2002) have suggested that PDD is caused by a paramyxovirus, whereas Gough and colleagues (2006) have suggested the disease is associated with a coronavirus. Though a virus like particle has been observed in the feces of a bird with PDD, the disease has only reportedly been duplicated after brain homogenate from a PDD infected bird was fed to other parrots (Gough et al., 1996). Unfortunately, evidence of these and other viruses in birds affected with PDD has been erratic and inconsistent.

Diagnosis of the disease is therefore largely based on clinical signs supplemented by histological examination of the crop tissue. Lymphocytic infiltration of the ganglia of the crop occurs in about 60 to 70 per cent of PDD cases (Doolen, 1994b).

One consistent feature of PDD affected birds is the presence of lymphoplasmocytic encephalitis in the form of perivascular cuffing on postmortem examination. Because large numbers of plasma cells in these perivascular cuffs implies a robust antibody response by the host, studies were initiated to determine which antigens were being targeted by these plasma cells. We therefore homogenized brain tissue samples from parrots that were euthanized. Serum, or plasma, was also submitted along with the bird's tissue samples in order to test the bird's serum against its own tissues and with tissues of birds known to be PDD positive or negative by postmortem examination.

2. Materials and Methods

2.1 Sample materials

One scarlet macaw (*Ara macao*), one yellow-collared macaw (*Primolius auricollis*), two blue-and-gold macaws (*Ara ararauna*), and one African grey parrot (*Psittacus erithacus*) were all diagnosed as being affected with PDD by a board certified avian pathologist at the Texas Veterinary Medical Diagnostic Laboratory at Texas A&M University. Tissues obtained during the necropsy were placed in 1.7 ml microcentrifuge tubes on ice for temporary storage and then at -80° C until further use. Blood samples were obtained by clinicians and interns at the Texas A&M College of Veterinary Medicine, Small Animal Hospital, Exotic Zoo Ward in red top tubes and allowed to clot. The blood was then spun down in a microcentrifuge for 6000*g* for 10 minutes at 4°C. Serum was stored at -80°C until further use.

2.2 Antigen preparation

Tissue samples were thawed and a small piece was cut then weighed. The sample was then put into a glass grinder and a 1:10 g/ml suspension was made with PBS (Sigma, St. Louis, MO, USA) pH 7.4. All samples were kept on ice until aliquoted and stored at -80°C. Homogenized tissue samples were centrifuged at 200*g* for 5 minutes at 4°C to remove large debris from solution.

2.3 SDS-PAGE and protein stain

Homogenized tissue samples were loaded onto 10% Tris-HCl polyacrylamide minigels prepared in Tizard/Ni lab according to the lab manual. Tissue samples to be tested were prepared by adding 12 µL of reducing, denaturing sample buffer (BioRad, Hercules, CA, USA), prepared according to manufacturer's instructions, along with 12 µL of homogenized tissue extract in a 1.7 mL microcentrifuge tube. The samples were boiled in water for 5 minutes and 20 µL of each tissue sample was added to each well of the SDS gel. A pre-stained broad range molecular weight ladder (BioRad, Hercules, CA, USA) was used to estimate the molecular weight of reactive proteins. SDS gels were run for 40 minutes at 80 amps, or 40 amps for one gel. SDS gels were stained for protein by incubation with Coomassie stain for 20 minutes on a shaker. Gels were de-stained by incubation in 7% acetic acid, 10% methanol in ultrapure water overnight then stored in ultrapure water. Gels were also silver stained (Pierce, Rockford, IL, USA) to visualize smaller quantities of protein.

2.4 Western blot

Western blots were performed based on methods originally described elsewhere (Towbin et al., 1979). Tissue extracts were electrophoresed on 10% polyacrylamide gels. Proteins were transferred to a polyvinylidene fluoride (PVDF) in transfer buffer at 100 mA for 2 hours.

Membranes were blocked with PBS (Sigma, St. Louis, Missouri USA) pH 7.4 plus 3% non-fat dry milk and 0.05% Tween 20 shaking at room temperature for 2 h. Test sera were diluted 1:100 in PBS plus 1.5% BSA and 0.05% Tween 20 and exposed to the antigen blots at room temperature for 2 h. Membranes were rinsed 3 times for 5 min with PBS plus 0.05% Tween 20. Membranes were then incubated with alkaline phosphatase labeled goat anti-macaw IgY (Bethyl Laboratories, Montgomery, TX) at a dilution of 1:10,000. The membrane was rinsed again and developed for 10 minutes with 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride (BCIP/NBT) from Sigma. Membranes were rinsed briefly with ultrapure water and air dried for about 30 minutes before determining final results.

3. Results

3.1 Identification of 38 kDa antigen

Tissue samples collected from necropsied birds were thawed and homogenized. Nervous tissue was very fatty and therefore hard to homogenize with PBS alone at room temperature. At the other extreme were the proventriculus and ventriculus which consist of tough muscle and were also hard to homogenize. Given the scarcity of the tissue samples obtained, and the different composition of lipid and protein of the different tissue samples, protein concentration assays were not originally performed. On some birds, not all tissues desired were obtained due to elapsed time since death which causes parts of the brain and spinal cord to autolyse. The adrenal glands were not positively identified on some birds because of elapsed time from death to necropsy. Coomassie stains suggested protein samples contained roughly the same amount of protein. SDS-PAGE and Coomassie stains were originally performed with dilutions of tissue homogenates at 1:10, 1:100, and 1:1000. Western blots were performed with a serum dilution of 1:100 and reacted very strongly with numerous proteins at all homogenate dilutions. It was

found that serum diluted 1:5000 was still able to clearly detect PDD antigen in the western blot. When compared to western blots performed with tissue homogenates from birds that were diagnosed as PDD negative post-mortem, the dominant antigen that came to be associated with PDD was a 38 kDa protein. Other antigens that were reactive with serum from a PDD bird were presumably antigens associated with chronic encephalitis, autoantigens, or other viral antigens.

3.2 Standardization of western blot

Based on preliminary results, a standard western blot protocol was established in an attempt to validate the correlation of PDD with the newly discovered 38 kDa antigen. Serum samples of birds to be tested were diluted at 1:100, unless otherwise noted. Positive control western blots were performed with known PDD positive sera at 1:5000. In fact, some sera reacted just as intensely with the 38 kDa antigen at a dilution of 1:5000 as they did at a 1:100 dilution. Antigen source for western blots were brain or heart tissue homogenate from PDD positive and PDD negative birds, diluted 1:50 w/v in PBS. Some western blots were performed with tissue homogenates from multiple organs to determine PDD tropism. Western blots revealed that tissues of all PDD-affected birds contained an antigen of approximately 38 kDa that was recognized by positive bird serum. The antigen was present in the forebrain, midbrain, hindbrain, spinal cord, sciatic nerve, and in small quantities in the adrenal gland of some birds. In the yellow-collared macaw, the PDD antigen was additionally present in the heart and vagus nerve. Surprisingly, the antigen was not detected in the gastrointestinal tract including the crop, proventriculus, ventriculus, small intestine (Fig. 1). The antigen was also not detected in the feces, blood, liver, or kidney of PDD affected birds. A western blot performed with only the secondary antibody showed it was reactive with light and heavy chains of IgY present in the tissue homogenates (Fig. 2). The 38 kDa antigen was absent from the tissues of a scarlet macaw

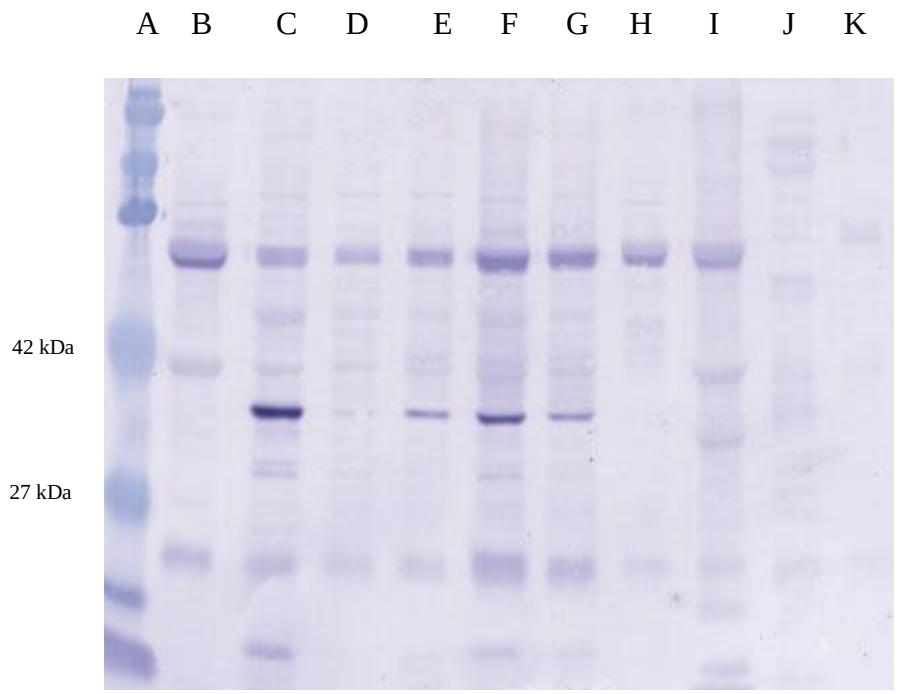


Figure 1. Western blot of infected macaw. Western blot of tissues from a scarlet macaw with proventricular dilatation disease confirmed at postmortem examination. Serum from the same bird was used as the primary antibody. A, molecular weight ladder; B, Adrenal gland; C, Forebrain; D, brainstem; E, Cervical spinal cord; F, Thoracic spinal cord; G, Sacral spinal cord; H, Ventriculus; I, Proventriculus; J, Intestinal contents; K, Cloacal swab. The positive band at approximately 50 kDa reflects the presence of immunoglobulin heavy chains and the band at approximately 25 kDa reflects the presence of light chains.

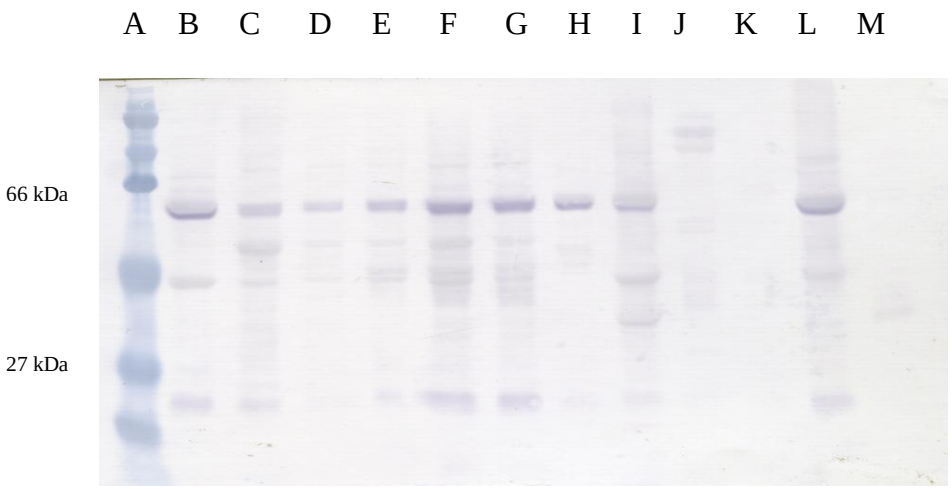


Figure 2. Western blot of infected macaw, secondary antibody control. Western blot of tissues from a scarlet macaw with proventricular dilatation disease confirmed at postmortem examination. Serum from the same bird was used as the primary antibody. A, molecular weight ladder; B, Adrenal gland; C, Forebrain; D, brainstem; E, Cervical spinal cord; F, Sacral spinal cord; G, Thoracic spinal cord; H, Crop; I, Ventriculus; J, Intestinal contents; K, Cloacal swab; L, Heart; M, Proventriculus. The positive band at approximately 50 kDa reflects the presence of immunoglobulin heavy chains and the band at approximately 25 kDa reflects the presence of light chains.

and two blue-and-gold macaws that were euthanized for chronic health problems unrelated to PDD.

3.3 Seroprevalence of antigen in live birds

We next sought to test serum from birds held at the Schubot aviary for the presence of antibodies to the 38 kDa antigen. Sixteen birds of varying species, mostly macaw, were determined to be PDD positive based on crop biopsy and/or a history of clinical symptoms found to be associated with PDD. Of the 16 birds, 12 were found to have antibodies to the 38 kDa protein. A population of 15 birds, deemed to be PDD negative by the same criteria, was tested for the presence of the 38 kDa protein. Two out of the 15 sera gave weak positive reactions.

3.4 Presence of antibodies to other pathogens

In an attempt to determine the nature of the antigen detected by the western blot, a search was undertaken to find antigens of known pathogens that cause a similar disease in their hosts. A leading candidate was the VP1 protein of Theiler's murine encephalitis virus, a picornavirus, with a molecular weight of approximately 33-35 kDa (Lipton and Friedmann, 1980). In collaboration with Dr. Jane Welsh's lab at Texas A&M University, ELISAs were performed by Dr. Andrew Steelman demonstrating that serum from PDD infected birds, as well as some PDD negative birds, contained antibodies against strains of Theiler's murine encephalitis virus (TMEV) BeAn and GDVII strains. However, an ELISA with influenza virus A/Singapore/97 H5N3 hemagglutinin protein presented results similar to the ones obtained with TMEV. Flaviviruses are known to cause encephalitis in susceptible hosts, but have thus far not been associated with PDD. The question was posed as to whether we were detecting an antigen from a previously uncharacterized flavivirus. Western blots were carried out in which serum from West Nile virus positive raptors was exposed to PDD brain homogenate. Results from these

western blots showed that West Nile antibodies were not cross reactive with antigens from the PDD agent. However, it was not ruled out that raptors may also be affected with PDD. Finally, it has been reported that avian paramyxovirus-1 may be associated with PDD. Therefore, a hemagglutination inhibition assay was performed in Dr. Sanjay Reddy's lab by Dr. Pam Farrow to determine if PDD positive serum was cross-reactive with avian paramyxovirus-1 antigen. A hemagglutination inhibition assay was performed according to a protocol established by TVMDL. Results, including PDD negative serum, indicated that none of the birds were making antibodies against avian paramyxovirus-1 HN protein.

4. Discussion

PDD is of serious concern to aviculturalists and bird owners alike. This disease was first described in the 1970's and has been recorded in over 50 species of parrots as well as non-psittacine birds (Clark, 1984; Daoust et al., 1991; Weissenbock et al., 2009b). PDD is mostly described as a non-suppurative encephalomyelitis in conjuction with peripheral ganglioneuritis of the gastrointestinal tract upon post-mortem examination. This disease is fatal and has been studied for about 30 years. Despite decades of research, many important questions remain as to the etiological agent, mode of transmission, diagnosis, and treatment of this disease. Currently, diagnosis of PDD in live birds is done by crop biopsy, which is about 40-60% accurate, and by radiographs of the digestive system (Dennison et al., 2009; Doolen, 1994b). Serological testing as a means of identifying novel pathogens does not have much precedence given the advent of pan-viral microarrays and high throughput pyrosequencing as the preferred methods of pathogen identification. These methods not only require expertise from personnel, but may be expensive if a lab is not equipped to run such experiments. Our approach was to exploit the antigenic specificity of the humoral immune response in order to identify its target antigen. An abundance

of lymphoplasmacytic infiltrates are found in the brains of birds diagnosed with PDD in the form of perivascular cuffing. Therefore, experiments were devised to find an antigen associated with the birds diagnosed with PDD but absent from non-PDD birds.

One such antigen was readily detected by serum from PDD infected birds against proteins found in their own brain homogenates. Most importantly, no antigen or antibodies against the 38 kDa antigen was found in PDD negative birds. While the nature of this antigen remains unclear, it is suspected to be of viral origin or perhaps a neurotoxin. It is also possible that this may be a virally produced toxin. The serum of affected birds does not react significantly with any antigens in normal macaw brains; therefore, it is unlikely to be an autoantigen. The antigen is found at all levels in the spinal cord and in the fore-, mid-, and hindbrain. Its absence from Western blots of affected gastrointestinal tract may simply reflect the relative paucity of nervous tissue within these organs. It's presence in one of five hearts suggests that the heart sample taken may possibly have included some nerve tissue, although heart lesions were present in this bird on histopathological examination and have been associated with PDD (Perpinan et al., 2007; Vice, 1992). The antigen was not detected in any tissue from the three apparently PDD negative birds tested.

The presence of antibodies to this protein in 12 of 16 PDD-confirmed parrots and their absence in apparently normal birds may provide a basis for a serodiagnostic test for this disease. It is important to note that the sera were found to be reactive with the antigen at a dilution of 1:5000. Less diluted samples may result in a higher seroprevalence. There were four birds with negative serology that were crop biopsy positive. In testing 15 birds believed to be negative for PDD, two serum samples gave weak positive reactions. Given the insidious nature of PDD, it is not surprising that otherwise healthy birds may in fact be positive for the disease. Nevertheless,

two possibly false positives out of 15, and four false negatives out of 16 suggest that detection of antibodies to this antigen by Western blotting or by other serological methods may be useful in confirming the diagnosis of PDD.

The presence of antibodies to other viruses was not surprising. The birds tested are all housed in an outdoor aviary and some have been adopted from previous owners. Parrots housed in outdoor aviaries are exposed to the same pathogens that may be carried by rodents and wild birds. Parrots that are housed indoors and therefore have more intimate contact with their owners are also exposed to pathogens carried by their owners, and vice versa. Therefore, it is entirely expected that these birds are exposed to viruses such as influenza, which can be carried by humans and birds as well as many mammals. It was surprising that these birds had antibodies to TMEV since this virus is not thought of as an avian pathogen. It seemed plausible that these birds had come into contact with wild mice that were carriers of TMEV, since TMEV in wild mice has been observed (Moro et al., 2003). However, there was no correlation between anti-TMEV antibodies and PDD status. Serum from birds with high titers of antibodies against WNV, a virus known to cause avian encephalitis, was not found to be reactive with the 38 kDa antigen. Though this does not imply that these birds have not been exposed to WNV, it does show that this antigen is immunologically different from WNV. Antibodies against one type of flavivirus E protein, the major antigenic envelope protein, are broadly cross reactive against other members of the flaviviradae family (Stiasny et al., 2006). Therefore, it is likely that the 38 kDa antigen is not from a flavivirus. Avian Paramyxovirus-1 has been implicated as a possible cause of PDD (Grund, 1999; Grund et al., 2002). We found that none of our birds produced detectable antibodies against AMPV-1 HN protein.

By use of Western blotting, a dominant antigen was detected in the brains of birds affected with PDD by use of the bird's own serum. This serological test could be developed and standardized as perhaps a supplement to crop biopsy for PDD diagnosis. In these studies, assuming that all birds believed to be PDD positive were in fact positive, then the serological test has an accuracy of 75%. This represents an improvement in PDD diagnostic accuracy by 5 to 15%, but a larger sample size will have to be tested in order to verify if there is an improvement. This PDD antigen is most likely not from a flavivirus, or the long-suspected APMV-1. Thus far, the specificity of the test for the PDD antigen appears to be 86.6%. Immediately, this antigen will need to be isolated and characterized in hopes of identifying the pathogen that may be responsible for PDD. More studies will have to be undertaken to determine the value of the Western blot as a diagnostic test for PDD.

CHAPTER III

PURIFICATION AND IDENTIFICATION OF A 38 KDA ANTIGEN ASSOCIATED WITH PDD

1. Introduction

Proventricular dilatation disease (PDD) is a fatal neurologic disease of mainly psittacine birds that has been described since the 1970's (Gerlach, 1991)Birds suspected of having PDD will exhibit neurologic signs that include ataxia, abnormal head movements, and depression. Neurologic signs are often seen in conjunction with gastrointestinal signs such as crop stasis, regurgitation and passage of undigested seeds in the feces (Gregory, 1994, 1997b; Mannl et al., 1987). Historically, only postmortem diagnosis has been considered definitive for PDD. Histopathology of diseased birds reveals an infiltration of T cells, plasma cells and macrophages in the myenteric plexus of the proventriculus and the ventriculus. In addition to gastrointestinal pathology, there is an abundance of nonpurulent inflammatory lesions in the brain, spinal cord and autonomic nerves (Berhane et al., 2001; Lublin, 2006; Mannl et al., 1987). Many viruses including Eastern equine encephalitis virus, coronavirus, and avian paramyxovirus 1 have been proposed as the possible etiologic agent of PDD, but proof has not been established (Gough et al., 2006; Gregory, 1997a; Grund et al., 2002). The only way that PDD was able to be replicated in birds was by exposure to tissue homogenate from birds that were diagnosed with PDD (Gregory, 1998).

Avian Borna Virus (ABV), a newly discovered member of the family of the *bornaviridae,* has recently been implicated as a possible etiologic agent in PDD(Kistler, et al, 2008, Honkavouri et al, 2008). Like it's mammalian counterpart, ABV is a single-stranded

negative sense RNA virus that codes for 6 proteins including L, G, N, P, X, and M. ABV is sequentially divergent from mammalian Borna virus (BV) with less than 70% nucleotide homology to Borna virus, possibly representing a new virus (Honkavuori et al., 2008; Kistler et al., 2008). Under these auspices, it may be assumed that ABV proteins might behave in a different biological and perhaps pathological manner to that of BV viral proteins. Unlike BV in a mammalian host, ABV in an avian host seems to have the added effect of a wasting disease in addition to a neurological component of the disease. Cockatiels naturally infected with ABV genotype 4 were clinically normal until superinfection with ABV genotype 4 induced disease. Though too early to tell, this may imply higher pathogenicity of certain strains over others (Tizard, 2009a). Additionally, ABV is consistently found in extraneuronal tissues of infected birds (Gancz et al., 2009; Honkavuori et al., 2008; Lierz et al., 2009; Rinder et al., 2009; Weissenbock et al., 2009a).

Nucleoprotein (N) and the phosphoprotein (P) are the major immunogenic proteins of Borna virus (Schwemmle et al., 1998). The humoral immune response of the BV infected host is mounted against either the N, P, or both proteins. It is thought that birds infected with ABV may mount a similar humoral immune response (Villanueva, et al, 2009, unpublished observations). The N protein of 7 ABV isolates share anywhere between 70% to 100% sequence homology at the nucleotide level amongst each other (Weissenbock et al., 2009a). The ABV nucleoprotein is also significantly different from the BV nucleoprotein (Honkavuori et al., 2008; Kistler et al., 2008). The N protein exists as two isoforms at 38 kDa and 40 kDa, due to alternative translation initiation sites in the viral genome (Kobayashi et al., 1998). Though both isoforms of N contain an internal nuclear export signal, N40 kDa contains an extra 13 amino acid sequence in the N-terminal region that codes for a nuclear localization sequence (Kobayashi et al., 2001; Kobayashi

et al., 1998). Both avian and mammalian nucleoprotein is located in the nucleus and cytoplasm of the infected cell (Ouyang et al., 2009). Mammalian Borna virus N protein has been purified in part by salting out with ammonium chloride, so this method was employed first (Bause-Niedrig et al., 1992). Several attempts at purifying ABV N in this manner were unsuccessful and so a modified or alternative method had to be established.

Throughout the years, many methods have been used to purify proteins. Researchers have taken advantage of proteins' specific biological and physicochemical properties in order to devise purification techniques. Chromatography describes a method used to separate complex mixtures with a stationary phase and a mobile phase that flows through the stationary phase. For the purification of ABV nucleoprotein, the protein purification method used was hydrophobic interaction chromatography (HIC) due to the unusually hydrophobic nature the protein seemed to exhibit. HIC has been widely used as a powerful tool for protein separation and purification in industry and research (Lu et al., 2009). This method of chromatography separates proteins based on their hydrophobic properties with varying affinity through electrostatic and van der Waals forces. A hydrophobicity column was ideal for the separation of the N protein not only due to its hydrophobicity, but also because the protein was only present in small amounts of homogenized tissue preparations according to Coomassie and silver stains. Thus, the HIC column would serve to concentrate the N protein. SDS-PAGE and immunoaffinity were used in conjunction with HIC in order to effectively purify the ABV nucleoprotein. Identification of the purified protein was undertaken with MS/MS mass spectroscopy after in gel digestion of the protein.

2. Materials and Methods

2.1 Antigen sources

A female eclectus parrot (*Eclectus roratus)* was euthanized for humane reasons as a result of PDD. The bird tested seropositive for ABV (case number 09-096) based on western blotting with duck embryo fibroblasts as the antigen source. Aqueous and vitreous fluids were collected together out of the left eye with a 21 gauge syringe during necropsy. The fluids were dispensed into a microcentrifuge tube and kept in ice until the necropsy was finished. The sample was then kept at 4ºC for 48 hours until a western blot could be performed to determine if ABV was present in the eye. Previous attempts to purify the ABV nucleoprotein used different antigen sources. Homogenized heart tissue from a PDD positive yellow collared macaw (*Primolius auricollis*), confirmed by histopathology, was used at a 1:50 w/v tissue in PBS (pH 7.4) as described before (Villanueva, et al 2008). Another source for purification of ABV nucleoprotein was obtained from duck embryo fibroblasts (DEF) infected with ABV according to the method of Dr Patricia Gray and Dr Paulette Suchodolski. The infected DEFs were shown to contain ABV nucleoprotein by western blot.

2.2 2-D gel electrophoresis

Two-dimensional gel electrophoresis was performed by Dr. Larry Dangot at the Protein Chemistry laboratory at Texas A&M University. Briefly, isoelectric focusing of protein sample was performed overnight at room temperature on an Amersham Pharmacia Biotic IPTGPhor electrophoresis module using an immobilized pH gradient on a polyacrylamide strip. The second dimension is run on a vertical slab gel electrophoresis unit. In experiments where a western blot was to be performed, Dr. Dangot's laboratory also performed electrophoretic transfer of the protein gel onto PVDF membranes according to their laboratory's protocol (www.pcltamu.com).

It is worth mentioning that the PCL protocol is similar to our electrophoretic protocol, except that the transfer buffer used by their laboratory uses CAPS (3-[cyclohexylamino]-1-propane-sulfonic acid, Sigma, St. Louis, MO USA) buffer (10mM CAPS plus 10% methanol, pH 11.0) whereas our protocol uses Tris-HCl, Glycine, 10% methanol,

2.3 Purification with magnetic beads

Dynabeads® M-280 Tosylactivated magnetic beads used to purify antigen were used according to manufacturer's instructions (Carlsbad, CA USA). Briefly, beads were sufficiently washed according to manufacturer's instructions, except that no BSA was used in any of the solutions. Seventy microliters of IgY, precipitated by ammonium sulfate, was diluted in 230 µL of PBS from a PDD infected bird (M24, yellow collared macaw) was incubated with 60 µL of magnetic beads overnight at room temperature. Beads were then washed 3 times with PBS plus 0.05% Tween 20 to wash beads of unbound protein from serum. Various elution methods were attempted in order to desorb the antigen from the antibody. One such method was the addition 100 µL of 100 mM glycine, pH 2 followed by immediate addition of 300 µL of 1mM Tris-HCl to unbound antigen. Addition of different detergents to desorb antigen was also attempted.

2.4 Hydrophobic interaction chromatography

A method originally suggested by Dr. Paul Stroud was modified and used to isolate the ABV nucleoprotein. This method may also be described as reversed-phased chromatography. Briefly, 50 µl of eye fluid was diluted in 150 µl of PBS. This sample was then pipetted into a 200 µl Phenyl-Sepharose® 6 Fast Flow (Sigma, St. Louis, MO USA) column that had been adequately rinsed with PBS. The column was then rinsed with 400 µl 0.5% Tween 20 followed by a rinse with 400 µl PBS to elute proteins that were not of interest off of the column. The column was then rinsed with 200 methanol (Fisher Scientific, Fair Lawn, NJ USA) followed by

200 µl of PBS as a final rinse. All eluates were collected in microcentrifuge tubes and immediately stored at 4ºC. Samples were loaded onto an SDS gel shortly thereafter on the same day for a western blot and Coomassie stain. The column was then thoroughly rinsed with PBS and then stored in PBS plus 0.01% sodium azide until further use.

2.5 SDS-PAGE

Protein samples were loaded onto 10% Tris-HCl polyacrylamide minigels prepared in Tizard/Ni lab according to standard lab protocols. Protein samples for electrophoresis were prepared by adding 12 µL of reducing, denaturing sample buffer (BioRad, Hercules, CA, USA), prepared according to manufacturer's instructions, along with 12 µL of protein sample in a 1.7 mL microcentrifuge tube. The samples were boiled in water for 5 minutes and 20 µL of each tissue sample was added to each well of the SDS gel. A pre-stained broad range molecular weight ladder (BioRad, Hercules, CA, USA) was used to estimate the molecular weight of reactive proteins. SDS gels were run for 40 minutes at 80 amps, or 40 amps for one gel. SDS gels were stained for protein by incubation with Coomassie stain for 20 minutes on a shaker. Gels were de-stained by incubation in 7% acetic acid, 10% methanol in ultrapure water overnight then stored in ultrapure water. Gels were also silver stained (Pierce, Rockford, IL, USA) to visualize smaller quantities of protein.

2.6 Western blot

Western blots were performed based on methods originally described by Towbin, et al. (Towbin et al., 1979). Proteins were transferred to a polyvinylidene fluoride (PVDF) in transfer buffer at 100 mA for 2 hours. Membranes were blocked with PBS (Sigma, St. Louis, Missouri USA) pH 7.4 plus 3% non-fat dry milk and 0.05% Tween 20 shaking at room temperature for 2h. Test sera were diluted 1:100 in PBS plus 1.5% BSA and 0.05% Tween 20 and exposed to the

antigen blots at room temperature for 2 h. Membranes were rinsed 3 times for 5 min with PBS plus 0.05% Tween 20. Membranes were then incubated with alkaline phosphatase labeled goat anti-macaw IgY (Bethyl Laboratories, Montgomery, TX) at a dilution of 1:10,000. The membrane was rinsed again and developed for 10 minutes with 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride (BCIP/NBT) from Sigma. Membranes were air dried for about 30 minutes before reading. Positive control serum was obtained from M24, a yellow-collared macaw with clinically, necropsy- and histologically confirmed PDD. For control purposes, serum from M21, a blue-and-gold macaw (*Ara ararauna*) histopathologically confirmed to be free of PDD lesions, was used as a negative control.

2.7 MS/MS analysis

Protein samples identified on SDS gel with Coomassie stain were stored in ultrapure water at 4°C until they could be submitted for identification. Protein samples were submitted to Dr William Russell for MS and MS/MS analysis at the Laboratory for Biological Mass Spectrometry at Texas A&M University. Protein sequences obtained were searched against protein databases including NCBI, Swiss Prot and the chicken genome for possible identification.

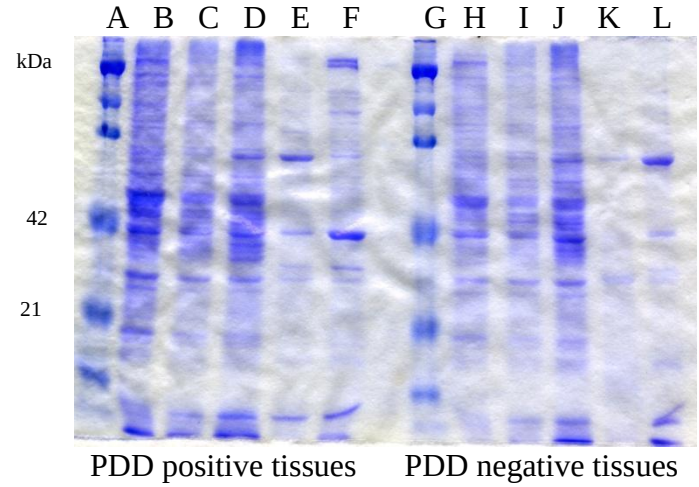
3. Results

3.1 Antigen sources and initial descriptions

Four different antigen sources were used in attempts to purify the 38 kDa antigen that appeared on western blots. Homogenate from the hind brain of a yellow-collared macaw was first used because it was in abundant supply. Previous results had shown the antigen to be present in the hind brain of PDD affected birds. Brain homogenate is very abundant in lipids and therefore difficult to work with because of the formation of precipitates. Protein solutions are

constantly maintained on ice in order to avoid excessive protein degradation. When brain homogenate was maintained in cold temperatures, large precipitates formed. Because of this, it was difficult to concentrate the brain homogenate and maintain uniform solutions for testing. Another consequence was that such lipid heavy solutions were not conducive to downstream applications of protein purification. It was soon discovered from a Western blot that the same yellow-collared macaw, from which the brain homogenate was generated, also contained the 38 kDa antigen in heart homogenate. Heart homogenate contained much less lipid and therefore greatly alleviated the problems associated with purification of protein from lipid rich solutions. However, previous results along with newly acquired results suggested conflicting conclusions. The western blot stained brightly, yet no protein was ever detected on the corresponding Coomassie, or silver stained gel (Fig. 3). This was also the case for the PDD-infected tissue culture homogenate from which antigen purification was also attempted. At a concentration of 300 µL of infected tissue culture, there was still no visible protein band that corresponded to the bright band of the Western blot. It was concluded that the antigen was present in very small amounts in the homogenates, but readily detected by an overwhelming antibody response. Additionaly, antigen from tissue culture was subjected to n-glycanase treatment at which it was determined that the antigen contained an n-linked glycosylation site (Fig. 4A). In order to disprove the theory that the antigen may perhaps be a prion, M24 infected tissue culture was submitted to proteinase K treatment for 1 hr. at 37°C (Fig. 4B). Failure of a western blot to detect the antigen after proteinase K treatment showed that it was most likely not a prion protein.

A.



B.

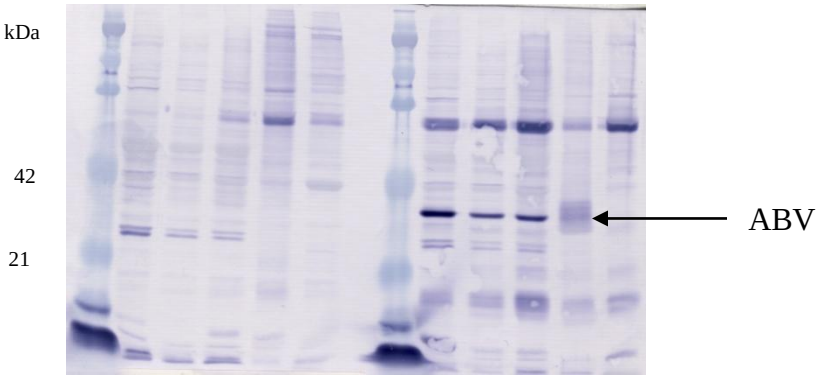


Figure 3. Western blot and Coomassie stain of infected macaw. Coomassie (top, A) and Western blot (bottom, B) of tissues from histopathology confirmed PDD negative and PDD positive scarlet macaws (*Ara macao)*. Tissues were homogenized and diluted in PBS at 1:50 g/ml then exposed to serum diluted at 1:5000. (A) Broad range ladder, (B) Brain, (C) Brain stem, (D) Sacral cord, (E) Proventriculus, (F) Ventriculus, (G) Broad range ladder, (H) Brain, (I) Brain stem, (J) Sacral cord, (K) Proventriculus, (L) Ventriculus. The Western blot is a replica of the Coomassie stain. The ABV nucleoprotein is present in PDD affected tissues and is detected by PDD positive serum.

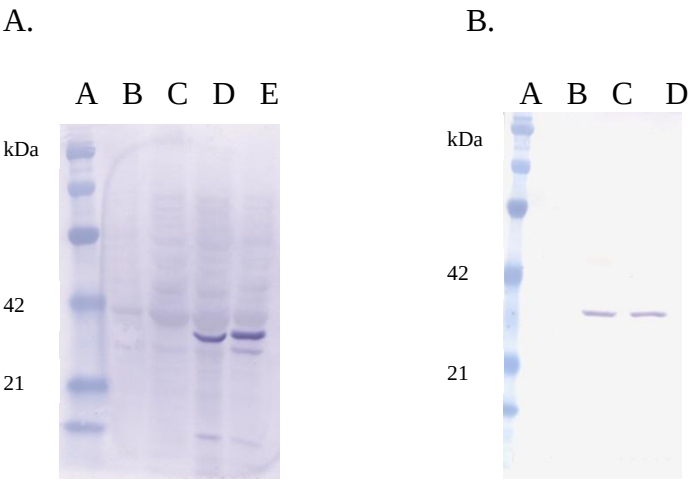


Figure 4. Western blots with enzymatic treatment of antigen. Western blots of the ABV nucleoprotein subjected to different types of enzymatic treatment. A. Brain homogenates subjected to treatment with n-glycanase. (A) Broad range ladder, brain homogenate of PDD negative bird without (B) and with (C) n-glycanase treatment, brain homogenate of PDD positive bird without (D) and with (E) n-glycanase treatment. B. Brain homogenate subjected to protease K treatment. (A) Broad range ladder, (B) brain homogenate treated with protease K at 37°C for 1 hr., (C) brain homogenate incubated at 37°C for 1 hr., (D) untreated brain homogenate. Western blots were developed with PDD positive serum diluted at 1:5000. ABV nucleoprotein is indicated by the positive reactions between the 42 and 21 kDa markers on both blots.

3.2 2-D gel electrophoresis as a method of antigen purification

Conventional two-dimensional gel electrophoresis was performed in order to separate the antigen from the rest of the proteins. PDD negative and PDD positive heart homogenates, as well as normal and PDD-infected DEF tissue culture cells were given to the Protein Chemistry Laboratory at Texas A&M for 2-D gel electrophoresis. Western blots were negative for the presence of the 38 kDa antigen and the corresponding SDS gel also contained considerably less amount of protein than demonstrated by single-dimension SDS-PAGE. A technique termed detergent swapping was attempted in order to better dissolve the proteins and better separate them in the isoelectric focusing step. Briefly, the protein solution was first subjected to SDS treatment in order to completely separate the proteins from each other. The SDS was then "swapped out" for a non-ionic detergent so that isoelectric focusing could take place. Even after such attempts, it appeared that the antigen was not undergoing proper isoelectric focusing and was perhaps lost on the polyacrylamide strip. An older method, in which isoelectric focusing was performed in a gel tubes as opposed to immobilized pH gradient strips, was also used in an attempt to purifiy the protein. This technique also produced negative results and the whereabouts of the protein could not be accounted for. These results indicated that the antigen was unable to go into solution for isoelectric focusing, perhaps due to high lipid content.

3.3 Antigen purification using magnetic beads

Another approach undertaken to purify the 38 kDa antigen from tissue homogenate was to utilize antibody conjugated magnetic beads to bind the antigen and separate it from other unwanted proteins. Antibodies in the form of whole serum were conjugated to the magnetic beads according to the manufacturer's instructions overnight at room temperature. Protein dilutions were made so as to saturate the magnetic beads with protein. To determine success of

antibody binding, a small amount of the magnetic beads were boiled in denaturing sample buffer and a western blot using only secondary anti-macaw antibody conjugated to alkaline phosphatase was performed to verify the presence of the heavy and light chains of macaw IgY. Brain homogenate as the antigen source was first used, but quickly abandoned because of its heavy lipid content. This did not allow the magnetic beads to properly incubate with the antigen solution at room temperature. Tissue culture homogenate was next used as the antigen source. In order to further eliminate unwanted proteins, tissue culture homogenate was subjected to MEM-PER and NE-PER protein purification kits in order to determine if the antigen was present in the cellular membrane or the nucleus of infected cells, respectively. Western blot results indicated that the antigen was present in both the nucleus and cytoplasm. This finding was in accordance with results found in separate ongoing investigations by Gray, et al, and Ouyang, et al, 2009. Therefore, whole cell tissue culture homogenate was used for subsequent experiments. Using the Western blot to track the presence of the protein, results indicated that the antibody was binding to the antibody, or perhaps the magnetic beads, and was not eluted by addition of glycine at pH 2, or by addition of 0.5 % Tween 20. Addition of denaturing SDS sample buffer was able to elute the antigen and produce western blot positive for the 38 kDa antigen. Only through this drastic method was the antigen liberated, yet the amount was still not visible on a Coomassie or silver stain. Furthermore, the antigen was not stable in SDS sample buffer and downstream applications for protein purification were not possible. Not only were the antibodies highly specific for the antigen, but they also had a very high affinity for the antigen.

3.4 Antigen isolated with hydrophobic interaction chromatography

A fourth source of antigen was discovered from a bird in a form that was ideal for protein purification. An eclectus parrot, which was seropositive for PDD antigen antibodies, was

euthanized for humane reasons. In addition to tissues routinely obtained for histopathology to confirm PDD diagnosis, aqueous and vitreous fluid were both obtained and stored at -80°C according to protocol. This mixture of eye fluids was found to be rich in PDD antigen and relatively free of other proteins based on protein staining and western blots. A new method for purification, hydrophobic interaction chromatography, was attempted. Eye fluid was run through the column and the antigen was eluted with PBS after undiluted ACS grade methanol was run through the column. Upon detection of the antigen with western blot, an increase of about 6-8 kDa of molecular weight occurred (Fig. 5A-B) The protein on the Coomassie-stained SDS gel that corresponded to the positive signal on the western blot was cut out of the gel and analyzed by double mass spectroscopy.

3.5 Mass spectroscopy

In-gel protein sample was submitted to Dr William Russell for MS and MS/MS analysis at the Laboratory for Biological Mass Spectrometry at Texas A&M University. The sequence of an ABV nucleoprotein isolated from a previous PDD case (M14, scarlet macaw) was kindly provided by Dr. Thomas Briese at the Center for Infection and Immunity, Mailman School of Public Health, Columbia University. This sequence was used for comparison to amino acids obtained by mass spectroscopy analysis after in-gel trypsin digestion. Four amino acid sequences from the antigen isolated from the ecclectus readily matched the M14 nucleoprotein amino acid sequence (Fig. 6). The isolated antigen was identified as the nucleoprotein of ABV. Furthermore, the eye fluid from a PDD affected eclectus parrot was frozen at -80°C at necropsy. This fluid was found to be reactive with antibodies from PDD affected birds and was also the source of antigen used to identify the antigen as the ABV nucleoprotein. The fluid was then

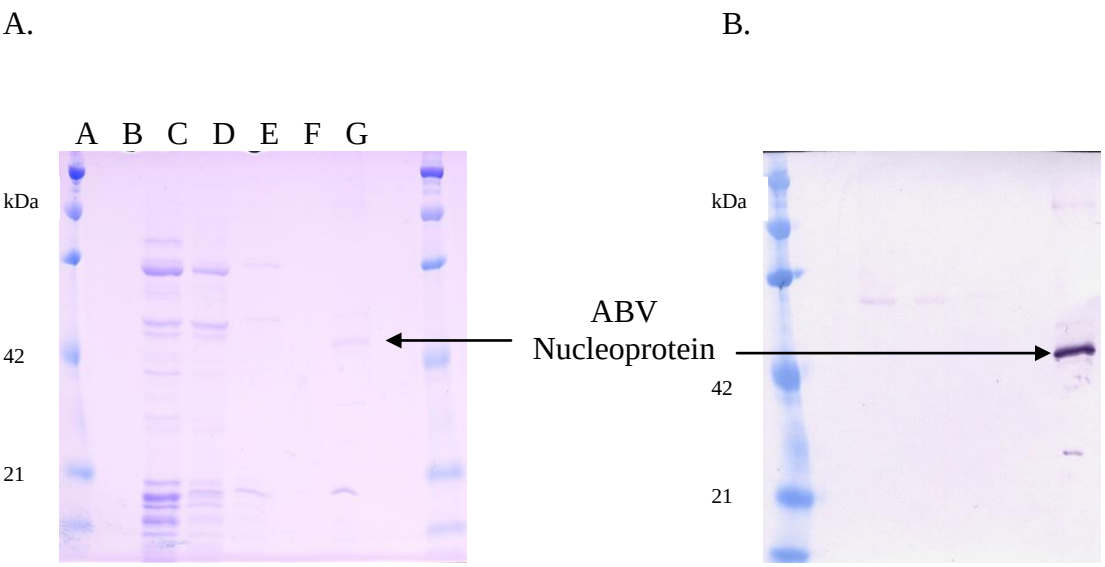


Figure 5. Western blot and Coomassie stain of purified antigen. Coomassie (left, A), and corresponding Western blot (right, B) of samples acquired from the ABV nucleoprotein purification process using hydrophobic interaction chromatography. (A) Broad range marker, (B) sample collected after eye fluid was added to column, (C) sample collected after column is washed with PBS, (D) sample collected after column is washed with 0.5% Tween 20 diluted in PBS, (E) sample collected after column is washed a second time with PBS, (F) sample collected after undiluted ACS grade methanol is added to column, (G) sample collected after column is washed for the last time with PBS.

MPPKRQRSPNDQDEEMDSGEPAASRSHFPSLTGAFLQYTQGGVDPHPGIGNEKDIHKNA

VALLDQSRRELYHSVTPSLVFLCLLIPGLHSALLFAGVQRESYLTTPVKQGERLITKTANF

FGEKTMDQELTELQISSIFNHCCSLLIGVVIGSSAKIKAGAEQIKKRFKTLMASINRPGHG

ETANLLSVFNPHEAIDWINAQPWVGSFVLALLTTDFESPGKEFMDQIKLVAGFAQMTTY

TTIKEYLNECMDATLTIPAVALEIKEFLDTTAKLKAEHGDMFKYLGAIRHSDAIKLAPRN

FPNLASAAFYWSKKENPTMAGYRASTIQPGSIVKEAQLARFRRREITRGDDGTTMPPEIA

EVMKLIGVTGFAN

Figure 6. Amino acid fragments of isolated nucleoprotein. Peptide sequences identified from MS and MS/MS analysis. Peptide sequence is from the nucleoprotein of ABV isolated from a scarlet macaw that was histopathologically confirmed as PDD positive and was seropositive for ABV nucleoprotein. Amino acids highlighted in red are peptide fragments that were successfully matched with the protein isolated by hydrophobic interaction chromatography and SDS-PAGE. The amino acids highlighted in green represent the predicted cytotoxic T cell epitope as identified for BV by Schamel, et al. (2001).

taken to Dr. Ross Payne who then took various pictures of the eye fluid using electron microscopy. Results suggest a presence of virus-like particles in the eye fluid (Fig.7).

4. Discussion

Birds are a valued part of our ecosystem and are an important part of the pet trade. These birds are highly valued not only for their plumage, but also for their longevity. Proventricular dilatation disease has been a problem for bird owners and aviculturalists alike since it was first described in the 1970's (Clark, 1984). Researchers have speculated on a variety of viruses that could be the etiological agent of PDD with limited success. A breakthrough came in 2008 and 2009 with the identification of a newly characterized Borna virus, termed avian Borna virus, and its ability to illicit PDD in birds infected with ABV (Gancz et al., 2009; Gray, 2009; Honkavuori et al., 2008; Kistler et al., 2008; Tizard, 2009a). An antigen associated with birds affected with PDD, but absent from PDD-negative birds, was identified but not fully characterized at the time (Villanueva et al., 2008). The antigen, present in cell culture and brain homogenate, was subjected to different protein purification methods as the extent of the antigen's hydrophobicity became increasingly apparent. Different methods were used conjunction to develop a final protocol to successfully identify the previously unidentified antigen as the ABV nucleoprotein.

Two-dimensional gel electrophoresis was unsuccessful in isolation of the AVB nucleoprotein, despite its abundant presence in both the nucleus and cytoplasm of ABV infected cells(Ouyang et al., 2009). A possible explanation for this may be that hydrophobic proteins are difficult to detect by such methods (Zuobi-Hasona et al., 2005). Hydrophobic proteins tend to be insoluble during isoelectric focusing and tend to aggregate, particularly membrane associated proteins. It is likely that optimization of ABV nucleoprotein solubility for successful 2-D gel electrophoresis will involve experimentation with different detergents such as n-dodecyl-β-

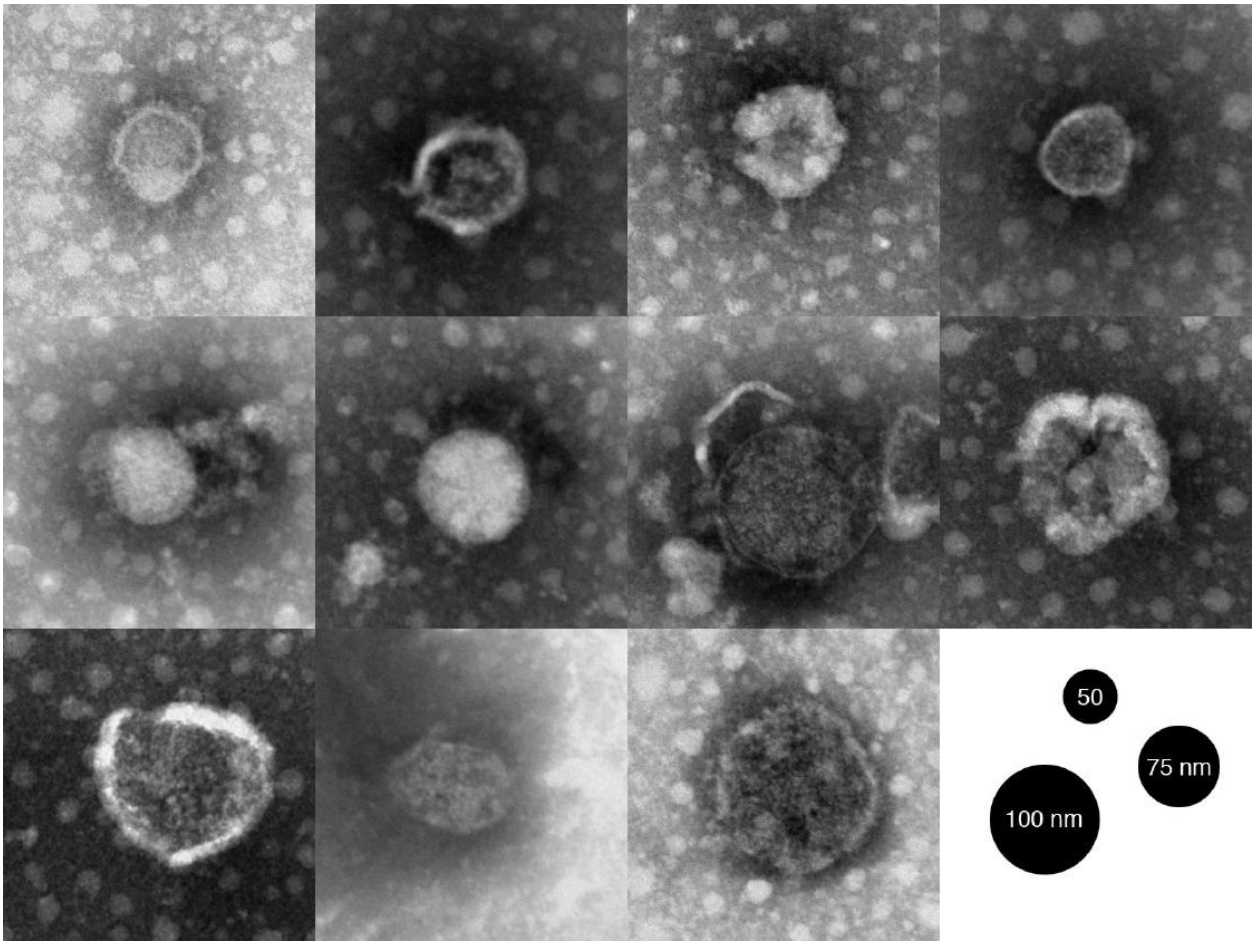


Figure 7. Electron microscopy of possible Avian Borna Virus. Electron microscopy pictures of possible ABV virions from eye fluid from PDD confirmed eclectus parrot. Courtesy of Dr. Ross Payne.

maltoside (DDM), or amido sulphobetaine-14 (ASB-14) (Devraj et al., 2009). It has been determined that serum from a PDD affected bird is able to detect the 38 kDa antigen. Therefore, it was expected that the antigen would be detected on a western blot from a 2-D gel using serum from a PDD affected bird, even though the protein was not identified after Coomassie staining. The western blots from 2-D gels were negative, except for the first time they were performed and gave a faint positive signal in the 38 kDa region. Because of the sensitivity of the assay, it was determined that the antigen was not present in the gel. Another possibility was that the chemicals involved in isoelectric focusing interfered with the integrity of the epitope(s) on the 38 kDa antigen. However, treatment of the antigen with different detergents, treatment with 2M urea, and the stability of the antigen in brain homogenate stored at 4°C after 2 months without protease inhibitor made this explanation unlikely. Attempts to isolate the antigen using antibody conjugated beads were only successful after the beads were boiled in SDS sample buffer for 5 minutes. From this, it was concluded that the antiserum has a very high avidity for the 38 kDa antigen.

In order to further characterize the 38 kDa antigen, enzymatic treatment of the antigen was performed. Treatment of the antigen from brain homogenate with n-glycanase resulted in a subset of antigen detected at about 36 kDa. N-glycanase enzymatically cleaves off carbohydrate residues that are linked to the amino acid asparagine. These carbohydrate residues are necessary for protein folding, conformation and targeting to subcellular regions (Gupta et al., 2009). This is a significant difference since the nucleoprotein of BV is not glycosylated. This difference raises questions as to the difference in function and host range between the ABV and BV and their respective nucleoproteins, since the nucleoprotein of BV is not glycosylated (Kobayashi et al., 1998). Previous attempts to isolate ABV nucleoprotein according to methods described by

Bause-Niedrig et al. (1992) by ammonium sulfate precipitation were unsuccessful. ABV nucleoprotein shares about 72% homology on the amino acid level to BV nucleoprotein and, given the different diseases that these viruses cause to their respective hosts, adds to the speculation about the role the nucleoprotein plays in the pathogenesis of ABV (Kistler et al., 2008). It is thought that BV spreads along axonal networks, or perhaps through some other form of cell to cell spread because of the lack of whole BV virions present in infected cell culture (Carbone et al., 1987; Gosztonyi et al., 1993). Because of the way the RNA of BV is thought to be tightly associated with abundant nucleoprotein, and because there is such a robust humoral response in birds infected with ABV, the differences in properties between the nucleoproteins may account for the differences in disease manifestation (Rudolph et al., 2003). Second, antigen present in brain homogenate was subjected to protease K treatment commonly employed by researchers involved in prion research (Biasini et al., 2009). Earlier accounts of the pathology caused by Borna virus disease described some of the cases as resembling spongiform encephalitis (Ludwig et al., 1973). The antigen proved to be susceptible to protease K treatment based on western blot analysis. The fact that PDD is caused by ABV is indisputable. That ABV is the only agent that can cause PDD like symptoms is debatable. There are cases in which histopathologically confirmed PDD birds were found to be ABV negative (Kistler et al., 2008; Weissenbock et al., 2009a). Prion disease research is a rapidly growing field and it may be that an uncharacterized prion disease circulates in psittacine birds, as it has already be described in numerous hosts with potential for trans-species infection (Seuberlich and Zurbriggen, 2010).

Hydrophobic interaction chromatography was able to effectively isolate the 38 kDa antigen, but there was a significant molecular weight change in the protein which could not be explained at the time. The weight change was an increase of about 5 to 6 kDa from the 38 kDa

weight of the originally identified antigen. This increase in weight may be due to interaction of the antigen with some of the reagents used during purification, or perhaps co-purification with an unidentified protein. The antigen was then identified by in-gel digestion followed by MS and MS/MS analysis with protein fragments matching several amino acid residues of a sequenced ABV. One of the fragments identified is close to the sequence TELQISSI, which is similar to the CD8 T cell epitope characterized for BV (Schamel et al., 2001).

ABV nucleoprotein was isolated and identified from a PDD confirmed bird using the bird's own serum. This validates the detection of the ABV nucleoprotein by western blotting as a serologic test for the presence of ABV. Since there are cases of antibodies present in the serum of birds that are clinically normal and biopsy negative for PDD, the presence of ABV nucleoprotein cannot provide a definitive diagnosis of PDD. Likewise, a fecal sample from a bird that is positive for ABV RNA is not a definitive diagnosis for PDD, raising the issue of a carrier state for ABV (Kistler et al., 2009). The ABV nucleoprotein appears to have hydrophobic properties and carbohydrate moieties that make it significantly different from BV nucleoprotein. Further analysis of the ABV nucleoprotein will shed light on the pathogenesis and the ensuing immune response to this virus.

CHAPTER IV

THE DIAGNOSIS OF PROVENTRICULAR DILATATION DISEASE: USE OF A WESTERN BLOT ASSAY TO DETECT ANTIBODIES AGAINST AVIAN BORNA VIRUS

1. Introduction

Proventricular dilatation disease (PDD) has caused significant losses in captive birds since the early 1980s when it was first recognized in Europe and North America. PDD affects at least 50 species of psittacine birds as well as other bird species (Clark, 1984; Gregory, 1994). The clinical signs of PDD vary between individuals and species, but in general they exhibit two general types of disease. Some develop neurologic signs such as depression, seizures, ataxia, blindness, tremors and incoordination (Steinmetz et al., 2008). A peripheral neuritis has also been reported in some birds involving the sciatic, brachial and vagal nerves (Berhane et al., 2001). Alternatively, birds may develop gastrointestinal problems such as crop stasis, regurgitation, inappetance, and undigested food in feces secondary to damage to the enteric nervous system. This damage leads eventually to starvation and death. Death due to circulatory collapse or food aspiration is also common. Birds may show neurologic signs or gastrointestinal signs or both. It is also suspected that some affected birds may show minor or no clinical signs. Definitive diagnosis of PDD is based on detection of a lymphoplasmacytic infiltration in the ganglia and nerve plexus, especially the myenteric plexus of the gastrointestinal tract (Schmidt, 2003). This diagnosis is however made difficult by the variable distribution of lesions in birds. For example, in one series of 14 birds (Berhane et al., 2001) lesions were seen in the crop in 43% of cases, proventriculus 36%, ventriculus 93%, duodenum 21%, heart 79%, adrenal gland 50%, spinal cord 69%, brain 46%, sciatic nerve 58%, brachial nerve 46% and vagus nerve 46%. In

another series (Shivaprasad, 1995), 72% of 61 PDD cases had lesions in the crop. While crop biopsy has been considered the most definitive diagnostic test, it clearly fails to detect a significant portion of the affected population. PDD has long been considered to have an infectious etiology. Recently, the use of high-throughput viral screens has enabled investigators to identify the presence of a new virus, avian Borna virus (ABV), from several cases of biopsy-confirmed PDD (Honkavouri et al., 2008; Kistler et al., 2008). Subsequent studies using PCR and immunohistochemistry on tissues of affected birds have confirmed the association between ABV infection and PDD (Rinder et al., 2009; Weissenbock et al., 2009a). Ouyang et al. (2009) have demonstrated the presence of ABV by immunohistochemistry in the central nervous system of multiple clinical cases of PDD. PDD has been induced experimentally in cockatiels (*Nymphicus hollandicus*) by Gancz et al. (2009) using infected bird brain and in Patagonian conures (*Cyanoliseus patagonus*) by Gray et al. (2009) using ABV4 passed for six passages in duck embryo fibroblasts. We have also reported on the presence of ABV antigen in the tissues of birds with PDD using a Western blot assay (Villanueva et al., 2008). Borna viruses are nonsegmented, negative strand RNA viruses belonging to the family Bornaviridae. Unique characteristics of Borna viruses (nuclear localization of transcription, alternative splicing, and a differential use of initiation and termination signals) justified their classification into a separate family in the order Mononegavirales (Briese et al., 1992). Until recently, only one member of the Bornaviridae was known, Borna disease virus (BDV), the cause of a meningoencephalitis in horses and sheep largely restricted to central Europe (Ludwig and Bode, 2000). However pyrosequencing of cDNA from the brains of parrots with PDD identified two strains of a novel Borna virus (Honkavuori et al., 2008). Using real time PCR, the study confirmed the presence of this virus in brain, proventriculus and adrenal gland in three birds with PDD but not in four

unaffected birds. Kistler et al. (2008) used a microarray approach to identify a Borna virus hybridization signature in five of eight PDD cases and none of eight controls. Using high-throughput pyrosequencing in combination with conventional PCR cloning and sequencing, these investigators were able to recover the complete viral genome sequence of one strain and named this virus avian Borna virus. Subsequent investigations have identified seven genotypes of this virus (Weissenbock et al., 2009a). Our ability to culture ABV from cases of PDD provides a convenient source of antigen for serologic assays. We have therefore developed a Western blot assay that can detect antibodies to ABV in the serum of psittacids. We report here on the use of this test as an aid to the diagnosis of PDD and provide further evidence for the linkage between PDD and ABV infection.

2. Materials and Methods

2.1. Sample material

Schubot Center birds: The Schubot Center at Texas A&M University had available a collection of 23 psittacine birds of seven species donated in the belief that they suffered from or were in close contact with cases of PDD. All birds underwent crop biopsy and blood was taken for serology. Birds that died were necropsied and the cause of death was determined.

Submitted samples: A total of 94 serum samples from 33 species of normal psittacines and suspected cases of PDD were submitted by veterinarians who were members of the American Association of Avian veterinarians. A confirmed diagnosis of PDD was based on a positive necropsy or crop biopsy showing the characteristic lymphoplasmacytic infiltration of the neuronal ganglia. A negative diagnosis was based on a histopathologically negative necropsy or biopsy, apparent good health, or clear evidence from necropsy that the bird was suffering from a

disease other than PDD. A diagnosis of PDD could not therefore be positively excluded in these birds.

Cockatiels: Fifteen apparently healthy cockatiels (*Nymphicus hollandicus*) were purchased from a single breeder.

Wild birds: Blood samples were obtained from seven wild scarlet macaws (Ara macao) and one mealy Amazon parrot (*Amazona farinosa*). The samples were taken at the Tambopata research center, Peru (_13.1382908,_69.6079608) from birds trapped for radiotelemetry studies, sampled, and released immediately afterwards.

2.2. Antigen preparation

The antigen preparation used for immunoblotting was a lysate from duck embryo fibroblast (DEF) cultures infected with ABV strain M24 originally isolated from a yellow collared macaw (*Primolius auricollis*) and passaged six times in the duck embryo fibroblasts. M24 belongs to ABV genotype 4. Cultures were harvested at 5 days post-infection when the ABV-infected or normal DEF cells were lysed by freezing and thawing. Recombinant ABV N protein, also used as a source of antigen, was generated as follows: RNA was isolated from DEF inoculated with the brain homogenates of a PDD-positive bird (M14) using the Qiagen RNeasy Mini kit (Qiagen, Hilden, Germany). First strand cDNA was generated using the High Capacity cDNA Reverse Transcription Kit and random primers (Applied Biosytems, Foster City, CA, USA). ABV N sequence was amplified using primers 50 GCG GTA ACA ACC AAC CAG CAA 30 and 50 GTT CAT TAG TTT GCR AAT CCR GTT A 30. The amplified region was initially cloned into pTOPO vector (Invitrogen, Carlsbad, CA, USA) and sequenced. The N open reading frame was then reamplified using forward primer Borna N attB1 (50-AAA AAG CAG GCT TCA CCA TGG AAATGC CAC CCA ARA GAC AAA-30) and reverse primer N1-V5 attB2 (50-AGA

AAG CTG GGT GTT TGC RAA TCC GST-30), to generate a V5-tagged N product, or Borna N1 attB2 (50-AGA AAG CTG GGT TCA TTA GTT TGC RAA TCC G-30). The amplified products were moved into pcDNA3.2/V5 DEST (Invitrogen, Carlsbad, CA, USA) by recombination-mediated cloning. N expression vectors were transfected into CHO-K1 cells using Lipofectamine 2000 (Invitrogen) and after 36–48 h the cells were collected and lysed with RIPA buffer (150mM NaCl2, 0.5% DOC, 0.1% SDS, 50mM Tris–HCl, 1% NP-40) by incubation for 5 min on ice. Expression of V5-tagged N-protein was verified by Western blot with anti-V5 antibody. In addition, cloned histidine-tagged ABV N (strain 1367; 49 kDa, including tag and vector sequence) purified on Ni-NTA-Agarose (Qiagen) was used to confirm the identity of the dominant antigen.

2.3. Western blot assay

Western blot analysis was used to test the serum or plasma of birds for the presence of ABV-specific antibodies. Western blots were performed based on methods originally described by Towbin et al. (1979). Infected DEF lysates were standardized to contain 30 µg of protein/lane and electrophoresed on 10% polyacrylamide gels. Proteins were transferred to a polyvinylidene fluoride (PVDF) in transfer buffer at 100mA for 2 h. Membranes were blocked with PBS pH 7.4 (Sigma, St. Louis, MO, USA) plus 3% non-fat dry milk and 0.05% Tween 20 at room temperature for 2 h. Test sera were diluted 1:100 in PBS plus 1.5% BSA and 0.05% Tween 20 and exposed to the antigen blots at room temperature for 2 h. Membranes were rinsed three times for 5 min with PBS plus 0.05% Tween 20. Membranes were then incubated with alkaline phosphatase labeled goat anti-macaw IgY (Bethyl Laboratories, Montgomery, TX) at a dilution of 1:10,000. The membrane was rinsed again and developed for 10 min with 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride (BCIP/NBT) from Sigma. Membranes were

air dried for about 30 min before reading. Positive control serum was obtained from M24, a yellow-collared macaw with clinically, necropsy and histologically confirmed PDD. For control purposes, an antibody-negative serum from M21, a blue-and-gold macaw (*Ara ararauna*) histopathologically confirmed to be free of PDD lesions, was used.

2.4. Fecal PCR assay

Fresh fecal samples were suspended in 500–750ml of sterile saline on ice. The samples were mixed and the solids were pelleted by brief centrifugation at 5000 rpm. RNA was purified from the supernatants using either the Ambion MagMAXTM Viral RNA Isolation Kit or the QIAamp Viral RNA Mini kit, according to manufacturer's recommendations. Approximately 1mg of RNA was used for the synthesis of cDNA (Applied Biosystems1 High Capacity cDNA Reverse Transcription Kit) using random primers. ABV sequences were amplified using primer sets: forward (50-CAG ACA GCA CGT CGA GTG AGA-30) and reverse (50- GGC TCT TGG TCT GAG ATC ATG GAA-30). The PCR conditions were as follows: initial denaturation, 94 8Cfor 3 min, followed by 35 cycles of 94 8C, 30 s, 54 8C, 30 s and 72 8C, 20 s followed by a final extension of 5 min at 72 8C. Samples were analyzed by agarose gel electrophoresis.

3. Results

3.1. Sera from PDD-affected birds recognize ABV N-protein

Western blot assays using antigen from infected DEF and serum from PDD confirmed birds showed that most birds reacted strongly with a single 38–40 kDa protein (Fig. 8). This antigen was also found in infected tissues, especially the central nervous system of PDD cases (Villanueva et al., 2008). It is expressed in ABV-infected DEF where its quantity increases over time. Its molecular weight is compatible with the predicted size of ABV N protein. Antisera reacting with this band reacted with cloned ABV N-protein derived by two different procedures

(Fig. 9). The specificity of the reaction to N-protein in tissue cultures was verified by preadsorption; antibodies to the N-protein were adsorbed out using an infected DEF lysate but not an uninfected DEF lysate (not shown).

3.2. Western blot assay

Of the 23 birds from the Schubot Center aviary at Texas A&M University that were crop biopsied or necropsied, 11 had characteristic lymphoplasmacytic infiltrates in their ganglia and thus were considered to have confirmed PDD. Of these 11 biopsy/necropsy-positive birds, nine (82%) were also seropositive (Table 1). Three of these positive birds were African grey parrots in apparent good health. Twelve birds in the aviary were healthy and had no detectable crop lesions so their disease status was unclear. Of these 12 birds, four (33%) were also seropositive. Since it has been determined that crop biopsy is positive in only 40–70% of PDD cases (Berhane et al., 2001; Shivaprasad et al., 1995), it is entirely possible that these four seropositive, biopsy-negative birds were subclinical PDD cases. Ninety-four serum samples were submitted by veterinarians together with clinical and histopathological documentation of their PDD status. Of 19 birds with necropsy or biopsy-confirmed PDD, 18 (94%) were seropositive. Of 75 ‘‘healthy’’ birds that did not appear to have PDD, 65 (85%) were seronegative, while 10 were seropositive. Based on veterinarian's reports, at least nine of these 10 had been in “contact” with known PDD cases. Serum samples obtained from eight wild psittacids captured at the Tambopata Research Center, Peru were negative for antibodies to ABV.

3.3. Detection of ABV by virus culture or PCR

PCR was performed on fresh droppings and/or cloacal swabs from the 15 healthy cockatiels. On initial testing, fecal PCR was positive in seven of these birds. Two birds were seropositive, one fecal PCR-negative and one positive. Subsequent weekly testing of the eight remaining negative

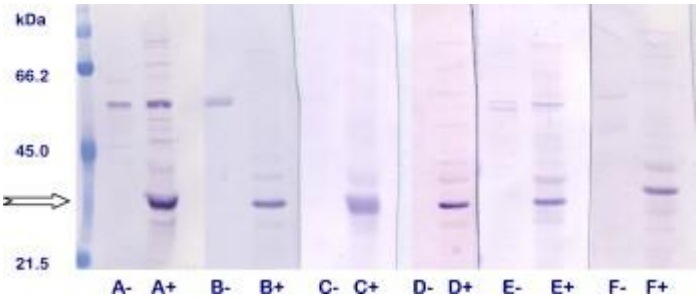


Figure 8. Western blot patterns of serum from infected birds. The predominant Western blot patterns observed using a standard tissue culture-derived antigen preparation from ABV-infected (+) or non-infected (−) cultures and exposed to six positive avian sera. Each serum was diluted 1:100. (A) Nanday conure, (B) Cockatiel, (C) African grey, (D) White cockatoo, (E) DuCorp's Cockatoo, and (F) Golden Conure. The arrow denotes the location of ABV N-protein.

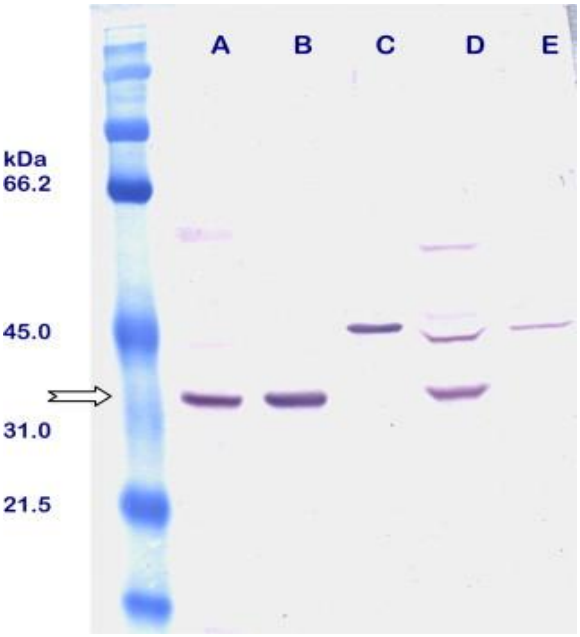


Figure 9. Western blot of ABV serum with cloned ABV nucleoprotein. Comparison of reactivity between lane A, crude brain-derived antigen from a confirmed case of PDD; lane B, cell lysate from ABV-infected tissue culture-derived; and two cloned ABV N-protein preparations, lanes C and D using a single positive macaw serum (M24). The cloned N-protein in lane C carries a 7 kDa histidine-tag. The cloned N-protein in lane D is unpurified and grown in Chinese hamster ovary (CHO) cells. Lane E consists of a lysate of normal CHO cells. The positive serum (M24) used in this immunoblot was adsorbed once with a CHO cell lysate in order to reduce the background in lanes D and E. The arrow denotes the location of the ABV N-protein.

Table 1. Summary of Western Blot Results

Summary of Western blot results in serum samples from birds housed in the Schubot Center aviary and samples submitted by participating veterinarians. Confirmed PDD-positive birds had the characteristic histopathological lesions as determine by biopsy or necropsy.

	Schubot Center confirmed positive	**Schubot Center confirmed negative**	**Submitted confirmed positive**	**Submitted believed "healthy"**	**Total**
Seropositive	9	4	18	10	41
Seronegative	2	8	1	65	76
Total	11	12	19	75	117

birds by fecal PCR over the next 4 weeks eventually identified an additional six PCR-positive birds for a total of 13/15 positive cockatiels. None of these birds changed their serologic status during the month-long observation period. In addition, fecal samples from 13 birds housed at the Schubot Center aviary were also tested and five were found to be PCR-positive. Thus of 28 fecal samples tested, 18 (64%) were positive for fecal ABV (Fig. 10). Six of these 18 birds with positive PCR were also seropositive; three of the 10 PCR-negative birds were also seropositive (Table 2).

3.4. Sensitivity and specificity of the serological assay

When the results of testing serum from both Schubot Center and submitted samples are combined, 27 of 30 confirmed PDD cases were seropositive giving a sensitivity of 90%. Of 87 apparently healthy or non-PDD cases, 14 were seropositive, a specificity of 82% (Table 1). In contrast however, in those birds where fecal PCRs were performed (Table 2), only six of 18 PCR-positive birds were seropositive while three of 10 PCR-negative birds were seropositive giving the Western blot procedure an apparent sensitivity of 33% and a specificity of 30%.

4. Discussion

Serology has been widely employed in the diagnosis of mammalian Borna virus infection although its interpretation has been controversial (Ludwig and Bode, 2000). The most common tests employed have been immunofluorescence, ELISA and Western blot assays using persistently infected cells, lysates of such cells, or cloned recombinant proteins (Briese et al., 1995; Hsu et al., 1994; Rott et al., 1991; Thiedemann et al., 1992; Wagner et al., 1968). Much of the controversy regarding the significance of serologic assays has centered on the proposed association between human neuropsychiatric illness and the presence of antibodies to Borna disease virus. This association has generally been disproven, serologic results obtained have not

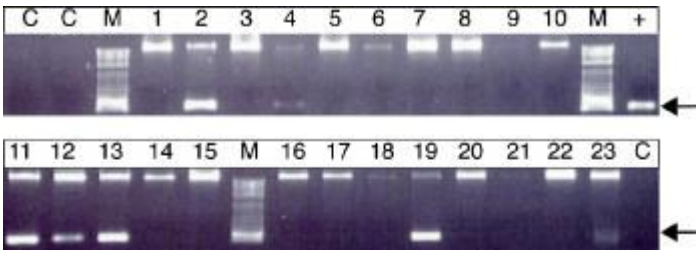


Figure 10. PCR detection of ABV from bird feces. Use of PCR for the detection of ABV sequences in bird feces. Lanes 1–10 and 16–23: PCR products from fecal samples from healthy cockatiels. Lanes 11–15: PCR products from fecal samples from healthy African gray parrots. Lanes 2, 4, 11, 12, 13, 19 and 23 show the presence of a band corresponding to the expected 131 bp fragment. Lanes labeled M contain 100 bp ladder. Lanes labeled C are negative reagent controls. The positive control (+) lane is PCR product from the brain of a PDD case. The high molecular weight band seen in most fecal samples is a nonspecific product.

Table 2. Summary of Western Blot Results Compared to PCR Results

Summary of Western blot results for birds from the Schubot Aviary tested by fecal RT-PCR for the presence of ABV.

	Fecal PCR-positive	**Fecal PCR-negative**	**Total**
Seropositive	6	3	9
Seronegative	12	7	19
Total	18	10	28

been reproducible and are probably low-titred false positive reactions or a result of laboratory contamination (Durrwald et al., 2007; Staeheli et al., 2000). It is perhaps appropriate to consider the significance of all bornaviral serology with some caution based on this human experience.

Nevertheless, sera from mammals with confirmed BDV infection and tested by Western blotting usually recognize the viral N, and P antigens. Triggered by the susceptibility of chickens to experimental infection (Ludwig, 1985), Borna virus infection in birds has been investigated previously (Berg et al., 2001; Malkinson et al., 1993). For example, Borna disease virus has been detected in the feces of wild birds, notably mallards (*Anas platyrhyncos*) and jackdaws (*Corvus monedula*) by PCR (Berg et al., 2001). However, the analyzed viral sequences were a match for those of mammalian BDV and different from those of the recently characterized ABV.

In this study, sera from PDD-affected birds reacted strongly with cloned N-protein indicating that the immunodominant antigen detected in Western blot assays is the 38 kDa N-protein of ABV. A comparison of Western blotting results with fecal PCR indicates that the Western blot assay does not detect all birds infected with ABV. The survey of cockatiels showed that almost all the birds tested had positive fecal PCRs yet were seronegative. The birds appeared to be carriers of ABV that showed no clinical symptoms. Repeated testing of these birds also indicated that seroconversion does not necessarily occur rapidly after the appearance of ABV sequence in feces samples and may not even develop months after ABV is detectable by PCR (Unpublished observations).

Given the absence of a satisfactory antemortem diagnostic test for PDD, it is very difficult, if not impossible, to certify any bird free of this disease. Crop biopsy is not only a highly insensitive procedure but also a significant surgical procedure not undertaken lightly. Furthermore crop biopsy is only positive in 40–70% of PDD cases. It is difficult to justify this

procedure in clinically healthy, normal birds just to confirm the absence of PDD. Thus a "healthy" bird cannot be guaranteed to be free of PDD. As a result, it is very difficult to determine the true sensitivity of the Western blot assay. Seropositivity in "healthy" birds is also difficult to interpret. As in most other infectious diseases, a population of seropositive healthy birds can be expected to exist that may include infected animals about to develop disease or recovered animals that are immune to the disease. The relatively few seropositive healthy birds observed in this study (16% or less) is compatible with two hypotheses that reflect what we know about PDD. First, few birds may survive an infection to become seropositive. Second, seropositivity may develop only immediately prior to disease onset–a situation analogous to mammalian Borna disease where clinical disease is a result of immunopathologic mechanisms (Ludwig and Bode, 2000). Although seropositivity may therefore serve as a useful non-invasive diagnostic marker in general, in some cases a more complex scenario may need to be considered. Three of our African Grey parrots were crop biopsy positive, seropositive and fecal PCR-positive but remained clinically healthy for at least 1 year. However our finding that all eight healthy, wild-caught birds from Tambopata, Peru were seronegative provides some assurance that ABV is not present in that specific region of the Amazon basin.

Western blotting represents a significantly improved diagnostic test for PDD when compared to the currently available crop biopsy. Given the finding that seronegative birds that appear to be shedding the virus as detected by PCR, it is however not feasible to eliminate infection from an aviary by the use of Western blotting alone. Western blotting requires only a small blood sample and offers in combination with fecal PCR a practical tool for *intra-vitam* diagnosis and surveillance.

CHAPTER V

AVIAN BORNA VIRUS DISCUSSION

PDD is a disease that is just beginning to be understood. Since its initial description in the early 1970's, it has been the scourge of bird owners because questions of cause, containment and prevention remained unanswered. Recent advances in molecular diagnostics have revealed the agent that causes PDD, and continue to be a major factor in pathogen discovery and surveillance (Honkavuori et al., 2008; Kistler et al., 2008; Lipkin, 2008). Concordantly, a second group employed more traditional methods of pathogen identification, based on the host's immune response (Villanueva et al., 2008). Similar methods had been used to help identify the coronavirus responsible for the 2003 Severe Acute Respiratory Syndrome (SARS) outbreak (Peiris et al., 2003). Both molecular and serological methods provide an optimal strategy in the diagnosis of ABV. Shedding of ABV by infected birds has not been adequately studied. Conversely, serological tests have not been able to detect all ABV PCR positive animals. This may reflect a difference in antigenic cross-reactivity since strain ABV4 nucleoprotein was used for the serological studies. To date, there have been seven strains identified with varying genetic homology (Weissenbock et al., 2009a). Thus, both tests have their limitations and ideally should be performed together for a more definitive diagnosis.

When a susceptible host is infected by a pathogen, the host mounts an antibody response directed against it. An exception to this process is when the pathogen causes an acute, fatal infection as in the case of Ebola virus. Antibodies serve to neutralize virus infectivity, target virus infected cells for antibody-mediated endocytosis resulting in antigen cross-presentation by antigen presenting cells, and targeting cells for complement activation and the membrane attack

complex resulting in cell lysis, among other functions (Tizard, 2009b). In the case of ABV infection in birds, the presence of antibodies against the ABV nucleoprotein implies viral infection. However, the function of the antibodies in the course of the disease is unclear. Two different cases of PDD illustrate the complexity of this question. First, a green winged macaw (*Ara chloroptera*) was housed with other birds that were confirmed PDD positive by crop biopsy, serology, and fecal shedding of ABV by PCR analysis. The macaw was crop biopsy negative, seronegative on January 10, 2009, but ABV positive by PCR testing of the feces. The bird was clinically normal otherwise. Approximately one month later, the macaw displayed classic PDD symptoms including constant regurgitation, ataxia, lethargy, and crop statsis confirmed by Dr. Belcher based on barium fluoroscopy examination. His serum tested positive for the presence of antibodies against ABV nucleoprotein at the time the symptoms were first noticed by a student worker who observed the birds daily. One week after the onset of clinical symptoms, the macaw was euthanized due to poor condition and prognosis. Serum was collected upon euthanasia and corresponded to a more robust response against the ABV nucleoprotein. Histopathological examination confirmed the macaw was affected with PDD. At the time of this writing, there were three African grey parrots that were seropositive for ABV, shed ABV in their feces, and were also crop biopsy positive. Despite this, the 3 birds remained healthy with little to no clinical symptoms for over a year after their original diagnosis. Given these different scenarios, the production of antibodies against ABV may be either protective, or immunopathologic. A carrier state for ABV has been recognized and only adds to the anxiety aviculturalists and clinicians share about PDD (Kistler et al., 2009; Lierz et al., 2009; Villanueva et al., 2009). Several factors need to be considered in the different scenarios of ABV infection including species difference, ABV strain difference, the effects of a possible autoimmune

response, the role of T cells in ABV pathology, and perhaps differences in antibody allotype and function. For example, the presence of certain carbohydrate moieties on the Fc region of IgG antibodies has been shown to affect the antibody's affinity to different Fcγ receptors, potentially targeting the antigen for endocytosis and subsequent antigen presentation (Radaev and Sun, 2002). Monitoring the avian IgM response during the course of PDD may address some of these questions.

The role of autoimmunity in PDD pathogenesis is unknown. A recent paper has suggested that BV, and presumably ABV, is able to insert itself into the genome of the host. It was also demonstrated that the BV nucleoprotein has significant homology to host proteins (Horie et al., 2010). This finding presents BV pathogenesis in a new perspective. In the case of ABV, the virus may simply become latent, or undergo very low levels of replication. The nucleoprotein may be recognized as self by regulatory T cells and the ensuing immune response may simply reflect a breakdown in immunotolerance. Epitope spreading and molecular mimicry may also play a significant role in ABV pathogenesis and eventual progression into PDD. Analogous situations occur in the case of Theiler's murine encephalitis virus infection in the mouse as a model for pathogen-borne multiple sclerosis, or infection of rats with Kilham rat virus resulting in autoimmune diabetes (Zipris et al., 2007). Another factor may be a change in the intestinal flora of the infected bird, caused by impaired gastrointestinal motility, resulting in an autoimmune response by the host (Chervonsky, 2010). Autoantibodies have been characterized in birds afflicted with PDD. Anti-ganglioside antibodies were documented in birds diagnosed with PDD, leading researchers to believe that PDD may be primarily an autoimmune disease (Pesaro, 2009). Additionally, anti-myelin antibodies were observed in a sun conure (*Aratinga solstitialis*) who remained clinically normal after one year of being diagnosed as

seropositive antibodies against ABV (Tizard, 2009a). Whether these antibodies are a consequence of PDD, or they represent a separate underlying condition remains to be determined. It has already been demonstrated that some of these birds have been exposed to a number of pathogens, including Theiler's murine encephalitis virus, or an agent antigenically similar. The role of autoimmunity in PDD remains unclear, but it is likely that many factors aside from the infection of the host with ABV are involved.

Borna virus is able to infect chickens, ostriches, and has been speculated to be present in wild birds including mallards and jackdaws, just as PDD has been recorded in Canada geese and ABV has been cultured in chicken embryo fibroblasts (Berg et al., 2001; Daoust et al., 1991; Ludwig et al., 1973; Rinder et al., 2009; Verwoerd, 2000). These two viruses are capable of infecting similar hosts and yet cause different diseases. Given that the BV cell receptor has not been definitively identified, it seems entirely plausible that a host may be susceptible to both ABV and BV. Infection of a mammalian host with ABV, or infection of a psittacine host, such as cockatiels, with BV may be experiments of interest. Rat C6, Madin-Darbin Canine Kidney (MDCK), and Vero cells, traditionally permissive to BV, are not permissive to ABV infection (Rinder et al., 2009). Hosts that may succumb to infection with both viruses may be bats. Afterall, 74 different species of bats were shown to carry more than 65 viruses, of which only 3 viruses were found to be DNA viruses (Calisher et al., 2006). The propensity for bats to carry RNA viruses and their ability to tolerate these viruses remains speculative.

A protein identified as the ABV nucleoprotein was isolated using a combination of protein purification techniques and a serodiagnostic test was developed and implemented with results faring better than the standard crop biopsy (Villanueva et al., 2009). ABV has been shown to cause PDD by both natural and experimental infection of previously naïve birds (Gancz

et al., 2009; Gray, 2009; Kistler et al., 2009). Though Koch's postulates have been fulfilled, important differences between BV and ABV pathogenesis have been observed. Unlike mammalian BV, ABV RNA and nucleoprotein have been found in tissues outside of the nervous system (Lierz et al., 2009; Rinder et al., 2009; Villanueva et al., 2008). In mammalian BV, it is speculated that antibodies from the host serve to restrict the virus to nervous tissue (Stitz et al., 1998a). Host antibodies against ABV do not seem to inhibit viremia (De Kloet and Dorrestein, 2009; Villanueva et al., 2009). In our personal observations and according to others, birds that suffered clinically from a PDD-like disease, and in some cases were histologically confirmed PDD cases, were not found to harbor ABV (Kistler et al., 2008). More mysteriously, birds have been shown to co-habit with other birds that have died of PDD, and yet remain ABV negative by PCR and serology (De Kloet and Dorrestein, 2009; Kistler et al., 2009; Villanueva et al., 2009). Indeed, a pair of pionuses (*Pionus menstruus*), one cagemate was found to be seropositive and PCR positive for ABV while the other cagemate remained negative in both tests after over a year of testing.

There are other conditions that may cause proventricular impaction, including lead poisoning and mycobacteriosis (Gray, personal communication). However, that some other agent may be able to cause a disease similar to PDD should not be ignored. A wasting disease caused by a prion has not been described in birds. The avian prion protein has been characterized and is found to have about 55% homology to mammalian prion protein. Avian prion protein appears to have copper binding properties and function in cell cycle regulation, but has not been associated with an avian wasting disease. Avian prion protein's true function has yet to be defined (Atoji and Ishiguro, 2009). Additionally, emerging molecular tools in disease surveillance may uncover other, previously uncharacterized agents present in diseased birds.

CHAPTER VI

INFLUENZA VIRUS

1. Introduction

Influenza is responsible for 36,000 deaths in the United States annually (Draghi et al., 2007). In the event of a human influenza pandemic, an estimated 20% of the world's population could become ill resulting in 30 million hospitalizations and a possible 6.5 million deaths (Fouchier et al., 2005; Stohr, 2004). Influenza epidemics are nothing new to humans, but the capricious nature of the hemagglutanin antigen allows the virus to re-infect the same populations year after year. Since 1918, there have been 4 pandemics with three different antigenic subtypes: the "Spanish" flu of 1918 (H1N1), the "Asian" flu of 1957 (H2N2), the "Hong Kong" flu of 1968 (H3N2), and the reintroduction of H1N1 in the "Russian" flu of 1977. Though H2N2 seems to have disappeared, descendents of the H1N1 and H3N2 pandemic viruses seem to have established a permanent presence in the human population. According to the WHO, since 2003, 282 people have died out of 471 confirmed cases of H5N1 infection as of January 28, 2010. Since 1997, the focus has been mainly on the ability of H5N1-infected chickens to directly infect humans, but it is also worth noting that H7 and H9 avian influenza subtypes have also infected humans. An influenza pandemic appears to be a substantial and credible threat at this moment. For an influenza pandemic to occur, the H5N1 virus must first successfully transmit from birds to humans. Second, the virus must cause illness and transmit easily between humans. Transmission of avian influenza to humans has been a relatively rare phenomenon considering the number of people that may have been exposed to sick poultry (Fielding et al., 2007; Vong et

al., 2006). Transmission of H5N1 virus among humans has been even more rare (Ungchusak et al., 2005; Vong et al., 2006).

2. Pathogenesis and Molecular Biology

Influenza virus is categorized as either low pathogenic avian influenza (LPAI), or highly pathogenic avian influenza (HPAI). LPAI infection in chickens is characterized as not causing any disease, or asymptomatic disease, or a mild disease. HPAI infection in chickens is typically fatal due to broad viral dissemination causing massive cell death leading to organ failure (Pantin-Jackwood and Swayne, 2009). Influenza virus is a single-stranded, segmented, negative-sense RNA virus that codes for 10 to 11 proteins (Spackman, 2008). The protein that plays an important part in viral tropism is the hemagglutinin (HA) protein. The HA protein primarily attaches to sialic acid in the alpha2, 3 configuration in the avian host. Influenza viruses that infect primarily humans recognize sialic acid in the alpha2,6 configuration (Repik et al., 1994). Upon attachment of the HA to the sialic acid, the virus is endocytosed into the cell and HA unfolds due to a decrease in pH in the acidic compartment, exposing the fusion protein. At this stage, the neuraminidase (NA) protein enzymatically cleaves other sialic acids, thus preventing interference viral attachment by other sialic acid moieties. The drop in pH is facilitated by the ion channel protein M2. M2 has been tested as a potential antigen for vaccine development and is also the target of the antiviral amantadine (Chuang et al., 2009; Pei et al., 2009) The viral RNA is then released into the cellular cytoplasm. Each strand of RNA in the viral genome is stabilized and targeted to the nucleus by multiple copies of nucleoprotein via nuclear targeting sequences (Wu et al., 2007). These structures, termed viral ribonucleoprotein complexes, are also outfitted with one copy of the polymerase complex consisting of proteins PA, PB1 and PB2 (Ruigrok and Baudin, 1995). Upon nuclear localization, replication of viral RNA, as well as

anti-sense RNA, is carried out by the polymerase complex. The exact mechanism required to switch from viral RNA production to positive sense RNA production for viral proteins remains speculative. Viral RNA is then exported out of the nucleus by primarily by the NXF1 nuclear export pathway, at least according to the latest research (Erkmann and Kutay, 2004; Fasken and Corbett, 2005; Read and Digard, 2010). Evidence for nuclear exportation of influenza RNA through the CRM1/exportin 1 pathway, the same pathway thought to be utilized by Borna virus RNA exportation, has been presented as well (Read and Digard, 2010). However, the exact mechanism of nuclear exportation of the viral ribonucleocomplexes into the cytoplasm for translation of viral RNA remains under investigation. Viral complexes assemble in the cytoplasm under the direction of the M1 matrix protein. The assembled virion then buds from the cellular surface enveloped by a lipid bilayer. In the case of highly pathogenic viruses, the HA molecule is already cleaved and ready for subsequent infection into another cell. This is because internal cellular proteases are able to recognize and cleave HA at the multiple basic amino acid sequence at the enzymatic cleavage site (Maines et al., 2008).

3. Avian Immune Response

The immune response to influenza virus infection has proven to be uniform among vertebrates, with most of the information coming from infection studies performed on mice (Doherty et al., 2006). Once a highly pathogenic influenza virus infects a chicken, the mean time for mortality is between 1.5 to 5.5 days (Swayne and Kapczynski, 2008). Influenza infection in chickens is primarily gastrointestinal, whereas in humans the infection is primarily upper respiratory. The exception is highly pathogenic influenza, in which the host may become viremic. Chickens do not have lymph nodes as humans do, but have Peyer's patches and mucosal lymphoid aggregates. The paranasal Harderian gland and the Meckel's diverticulum act as

germinal centers and areas of plasma cell aggregation and the bursa of Fabricius are involved in antigen processing (Doherty et al., 2009; Swayne and Kapczynski, 2008). Influenza viruses first invade epithelial cells and then go on to infect other permissive cells, including dendritic cells, as in the case of highly pathogenic influenza virus (Swayne and Kapczynski, 2008). Activation of the innate immune system occurs upon infection. Some of the cytokines first produced upon infection include IFNα, as well as TNFα and IFNγ produced by natural killer T cells (Ho et al., 2008). Though cytokines are helpful in controlling viral replication and spread, a phenomenon known as a "cytokine storm" occurs in which the host produces cornucopia of cytokines with unchecked production. This results in severe inflammation and is often fatal to the host (Perrone et al., 2008). Although not adequately studied, it is thought that the avian immune response is similar given the apparent homologues and orthologues of mammalian cytokines contained in the avian genome (Kaiser, 2007). Vaccination of birds against influenza is generally considered successful if antibody titer against HA, the major antigenic protein targeted by the humoral response, is ≥ 32 based on hemagglutinin inhibition. This titer is considered a protective response and has been shown to prevent viral shedding upon influenza re-infection in birds (Lee et al., 2004).

Adaptive immunity plays a critical role in viral clearance from the host. Cytotoxic T cells effectively destroy influenza infected cells by the use of granzymes and perforin, with the aid of IFNγ and TNFα (Doherty et al., 2009; Thomas et al., 2006). T helper cells have also been shown to aid in viral clearance, but to a lesser degree. In humans, T helper cells assist in directing B cells to produce different IgG isotypes. In particular, IgG2a has been shown to be an effective antibody for influenza virus clearance(Brown et al., 2004). However, chickens are as of yet only known to have one isotype of IgY (Tizard, 2009b). Chickens have been shown to have dendritic

cells that function in a manner very similar to human dendritic cells, and indeed other lymphocytes that function in a similar manner to that of their mammalian counterparts (Erf, 2004; Wu et al., 2010). Recently, a T cell epitope, recognized by both CD4 and CD8 T cells was described in the avian response to influenza H5 protein. This is the first T cell epitope of influenza viruse recognized by chicken T cells, corresponding to $H5_{246-260}$, a generally conserved amino acid sequence among influenza HA proteins (Haghighi et al., 2009).

4. Animals as "Mixing Vessels"

Another potential source of pandemic influenza virus is the pig. Pigs have been proposed as a "mixing vessel" for genetic recombination of avian and human influenza viruses (Ito et al., 1998; Kida et al., 1994; Van Reeth, 2007). One of the primary reasons for this theory is that the trachea of a pig contains both NeuAcα2,3Gal (α2,3 sialic acid) and NeuAcα2,6Gal (α2,6 sialic acid) receptors recognized by avian influenza and human influenza viruses, respectively (Repik et al., 1994). Human infections with swine influenza are a rare event given the abundance of α2,6 sialic acid receptors in the porcine trachea and epithelial cells from the upper respiratory tract in humans (Komadina et al., 2007; Van Reeth, 2007). It remains to be determined what the biological barriers, other than receptor specificity, are that prevent the influenza virus from transmitting freely between species. Currently, three main influenza subtypes are circulating in pigs: H1N1, H1N2, and H3N2 (Gourreau et al., 1994; Jung et al., 2007).

Many factors may account for interspecies transmission of influenza virus, including virus receptor specificity, immune status, the correct combination of genes in a virion, and perhaps a genetic susceptibility to infection. It may be posited as to why it is that interspecies transmission of influenza virus does not happen more often. It is thought that barriers which

prevent interspecies transmission of influenza virus may be exploited to develop a more efficacious vaccine.

5. Anti-gal Antibodies

In 1984, Galili and colleagues published results which described a new type of human natural antibody specific for the Galα1,3Galβ1,4GlcNAc (alpha-gal) eptiope. Galili and colleagues named this antibody "anti-gal" (Galili et al., 1985; Galili et al., 1984). It was determined that Old World primates and humans produce anti-gal whereas New World primates and nonprimate mammals do not produce the antibody, but express the alpha-gal epitope. It is hypothesized that the α1,3galactosyltransferase gene, which produces the enzyme responsible for alpha-gal production, was inactivated in Old World primates about 20 million years ago. This phenomenon may have given these animals an immunological advantage against a pathogen (Galili et al., 1987; Galili et al., 1988b).

Like other natural antibodies, such as anti-blood group antibodies, anti-gal is thought to be produced in response to normal bacterial flora of the gut (Galili et al., 1988a; Springer and Horton, 1969; Wiener, 1951). Though the alpha-gal epitope is not found in normal human tissues, it is expressed on senescent red blood cells. It is thought the purpose of anti-gal is to target senescent red blood cells for lysis. Based on the structures below, one can see the similarity that the alpha-gal epitope shares with blood group epitopes.

What sets apart anti-gal from the blood group antibodies is that it's found as IgG, IgM, and IgA isotypes as opposed to mainly IgM isotype of blood group antibodies. Another unique feature of this natural antibody is that it is found in high concentrations in normal serum. Anti-gal makes up about 1% of total IgG, or about 40-100 μg/ml in all four classes(Galili, 2006; Galili et al., 1984). Individuals with blood type A or O produce about 85% of their anti-B antibodies as

anti-gal antibodies, so actual anti-gal IgG concentrations may vary significantly. Anti-gal IgM is found in similar titers as IgG, but levels vary greatly with individuals, just as they do with serum anti-gal IgG. In some studies, there is no significant difference between IgG and IgM levels in serum (Diaz et al., 2003; Hamadeh et al., 1995b; Teranishi et al., 2002). IgA anti-gal levels in serum are low, but are found in significant amounts in whole colostrum, milk, and saliva (Hamadeh et al., 1995b).

The reason for the presence of this antibody in such high titers is unknown. Of particular note is the interaction of anti-gal with microbes. It has been found that anti-gal is able to bind the LPS of *Escherichia coli* and *Serratia marcescens* in normal gut flora and inhibits complement activation against these microbes. When the antibody binds the LPS of pathogenic E. coli, anti-gal is able to activate complement and effectively lyse the bacterium (Hamadeh et al., 1992). Anti-gal can also bind to pili of the human pathogen *Neisseria meningitidis*, but will not lyse the bacterium with complement. In this case, anti-gal IgA is thought to play a role in bacterial pathogenesis by inhibiting complement activation (Hamadeh et al., 1995a). The erythrocytic stages of *Plasmodium falciparum* malaria were found to express the alpha-gal epitope. As a result, people with known malaria infections were found to have elevated levels of anti-gal antibodies (Ravindran et al., 1988). It was also reported that patients infected with human papillomavirus were found to express elevated amounts of anti-gal in their cervical mucus (Hernandez et al., 2002). Based on these findings, anti-gal is able to provide immunogenic tolerance to normal flora and aids in the clearance of some pathogens.

Studies have shown that normal human serum is able to inactivate viruses that express the alpha-gal epitope. Therefore, anti-gal is thought to serve as a potential barrier against viral infections. Viruses passaged through cells expressing the alpha-gal epitope were found to be

inactivated by normal human serum. These viruses include pseudorabies virus, eastern equine encephalitis virus, and porcine endogenous retrovirus, among others (Hayashi et al., 2004; Quinn et al., 2004; Repik et al., 1994). It is of interest to determine if this phenomenon occurs with influenza virus. Galili and colleagues found that influenza virus hemagglutinin (HA) glycoprotein is able to accept alpha-gal residues and antibody complexes with alpha-gal-HA were formed. Furthermore, antigen presentation was enhanced if HA contained the alpha-gal epitope (Galili et al., 1996; Henion et al., 1997).

Incorporation of alpha-gal epitopes in vaccines are not a novel concept. Experimental tumor vaccines and HIV vaccines with alpha-gal have produced higher antibody titers than vaccines without the alpha-gal epitope. Both these vaccines were tested in the alpha-gal "knock out" mouse model (Abdel-Motal et al., 2006; Abdel-Motal et al., 2009b; Galili et al., 2007). Recently, alpha-gal was enzymatically conjugated onto the HA of PR8 H1N1 virus and used as a vaccine in the alpha-gal "knock out" mouse. Results confirmed that mice vaccinated with alpha-gal PR8 HA produced a higher amount of antibodies against HA, as compared to mice vaccinated with PR8 HA without alpha-gal (Abdel-Motal et al., 2007). It is of interest to determine the efficacy of an influenza virus vaccine with respect to incorporation of the alpha-gal epitope. Though mice are not natural hosts of influenza virus, birds are known to be naturally infected with influenza virus. Consequently, anti-gal antibodies have been described in chickens (McKenzie et al., 1999). Therefore, chickens may serve as a better model for the enhancement of the immune response to an alpha-gal influenza vaccine through interaction with anti-gal. These results may encourage influenza vaccine production in tissue culture in order circumvent the current shortcomings of vaccine production in hen's eggs.

CHAPTER VII

ENHANCEMENT OF HUMORAL IMMUNE RESPONSE TO ALPHA-GAL INFLUENZA VACCINE

1. Introduction

Influenza is an RNA virus responsible for upper-respiratory infections in humans and subsequently thousands of hospitalizations, including 36,000 deaths annually in the United States alone (Draghi et al., 2007; Fouchier et al., 2005). The capricious nature of the virus, making it hard to predict the nature of the next influenza pandemic, is best exemplified by the current H1N1 "swine flu" pandemic. Previous attention was focused on the ability of highly pathogenic H5N1 avian influenza to be the source of the next human influenza pandemic, that an H1N1 virus of swine origin swiftly became established in the human population. Fortunately, this virus does not appear to have the potential to become highly pathogenic according to current definitions, and previous exposure to H1 antigen seems to confer some degree of immunity despite the significant amino acid divergence between 2009 pandemic H1 and previous H1 antigens (Herfst et al., 2010; Saxena et al., 2009; Vincent et al., 2010). These findings only reiterate the importance of vaccination as a means of infection prevention.

The need for an influenza vaccine that elicits a robust humoral and adaptive immune response is needed to curb the effects of seasonal influenza infection. Though various types of influenza vaccines are available, there are two primary sources of vaccine production: eggs and cell culture. Though influenza virus grown very well and to high numbers in eggs, there are a few disadvantages to vaccine production in eggs. Production is dependent on the health of available chickens and their egg production, the management of the eggs before and after virus

inoculation, and the co-purification of egg products that some people may be allergic to. An alternative is cell culture based influenza vaccine production. Indeed, the European Union has already approved influenza vaccine produced from MDCK cell culture.

Another benefit to producing the MDCK-based influenza, although perhaps unintended, is the presence of the alpha-gal carbohydrate epitope. This carbohydrate epitope is the target of the natural anti-gal antibody that constitutes about 1% of circulating IgG in old world primates, including humans (Galili et al., 1984; Galili et al., 1988b). It is postulated that intestinal flora assist in the development of this natural antibody, similar to blood group antibodies (Galili et al., 1988a; Springer and Horton, 1969). These antibodies have also been described in newborns, suggesting maternal transfer of these antibodies (Teranishi et al., 2002). Anti-gal has proven to pose a great barrier to successful xenotransplantation (Galili, 2006). Because of this, anti-gal has been exploited to enhance the immune response to vaccines that incorporate the alpha-gal epitope. Alpha-gal has been incorporated into endogenous tumor vaccines as a method to break immunological tolerance and activate the immune system to perceive tumor associated antigens as foreign (Abdel-Motal et al., 2009b; Galili et al., 2007). Alpha-gal has also been enzymatically added to influenza virions to enhance the immune response to this virus, compared to virus that does not possess the alpha-gal epitope (Abdel-Motal et al., 2007; Galili et al., 1996; Henion et al., 1997). The reasoning behind this phenomenon is that anti-gal is able to bind antigens that contain the alpha-gal and target them directly to antigen presenting cells via Fc receptors. Internalization of vaccines in such a manner increases the processing and presentation of antigens to activate T cells (Abdel-Motal et al., 2009c).

In this study, chickens were used as the animal model to test the ability of anti-gal to enhance the immune response against influenza vaccines due to their natural production of anti-gal antibodies

(McKenzie et al., 1999). Chickens have dendritic cells that are thought to function in the same manner as human dendritic cells and even bear the same molecular markers (Wu et al., 2010). Subsequently, chickens are also naturally susceptible to influenza infection, unlike the alpha-gal "knock out" mice used in previous studies (Abdel-Motal et al., 2007). Influenza H5N3 vaccines were formulated with and without alpha-gal epitopes to determine what effect alpha-gal would have in enhancement of the immune response against influenza antigen.

2. Materials and Methods

2.1 Affinity chromatography

Serum from human male (blood type AB), or pooled serum from chickens (Sigma) was thawed. A 2 ml melibiose column on Foxy® Jr fraction collector was set up according to operator's manual and rinsed with 200 ml TN150 buffer (0.025M Tris, 0.150M NaCl in dH_20). Serum was then diluted 1:2 with TN 150 buffer and 0.2µ sterile filtered. Diluted serum was run through the column, then rinsed with 200 ml TN500 buffer (0.5M NaCl) followed by another rinse with 200 ml TN150 buffer. Anti-gal antibody was eluted with 200mM melibiose (Sigma) and fractions were collected. Based on the UV readout graph, necessary fractions were collected to run on a 10% SDS gel and the rest saved at -80°C. Fractions were pooled, dialyzed, and the final product was run on 10% SDS gel. NaN_3 was added to the anti-gal antibody solution to a final concentration of 0.02% and stored at -80ºC.

2.2 SDS-PAGE

SDS-PAGE gels were run based on methods originally described by Laemmli (Laemmli, 1970). Briefly, 10% polyacrylamide 0.75mm thick minigels were made in the lab using the protocol, glass plates, and gel assembly kit from BioRad Biotechnologies, Hercules, CA USA. Protein samples were mixed 1:1 with Laemmli reducing sample buffer (BioRad, Hercules, CA USA) and

boiled in water for 5 minutes. Twenty microliters of sample were loaded into each well. A broad range pre-stained ladder (BioRad, Hercules, CA USA) served as the molecular weight standard. Gels were run in denaturing running buffer at 40 milliAmps for 45 minutes, or until the sample buffer reached the bottom of the gel.

2.3 Western blot

Western blots were performed based on methods originally described by Towbin et al., 1979. Briefly, SDS gels were transferred onto a polyvinylidene fluoride (PVDF) membrane in transfer buffer at 100 milliAmps for 2 hours. The PVDF membranes containing the protein samples were then blocked with PBS (Sigma, St. Louis, Missouri USA) pH 7.4 plus 3% non-fat dry milk and 0.05% Tween 20 shaking at room temperature for 2 hours. Next, serum, or purified antibody were diluted in PBS plus 1.5% BSA and 0.05% Tween 20 at 1:100. Membranes were washed 3 times for 5 minutes with PBS plus 0.05% Tween 20. Next, the membranes were incubated with alkaline phosphatase labeled rabbit-anti-chicken (Bethyl Laboratories) diluted in the same solution as the primary antibody at 1:10,000. Membranes were washed again 3 times and developed for 10 minutes with 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride (BCIP/NBT) from Sigma. Membranes were air dried for about 30 minutes to determine final results.

2.4 Rabbit erythrocyte hemagglutination

SPF New Zealand White rabbit erythrocytes are obtained from LARR courtesy of Dr. Vincent Gresham or Andrea Taylor. Erythrocytes were washed twice with PBS and centrifuged at 930 X *g* (2000RPM) at 4ºC using the Allegra® X-15R centrifuge. Cells were then stored at 4ºC until further use. Cells were kept for 1 week and then discarded if not used. Antibody stock, or whole serum is diluted 1:10 in PBS prior to performing assay. On a 96-well V-bottom microtiter

plate, 50µl of PBS were added to serially dilute the stock antibody solution 2-fold in duplicate. Two wells served as controls (PBS + RBC) for each sample. Once serial dilutions were made, 50 µl of rabbit red blood cells were added to each well. Hemagglutination titer was determined once the cells in the control wells settled at the bottom. For hemagglutination inhibition, antibody was incubated with melibiose for 30 minutes at room temperature before an equal amount of erythrocytes were added.

2.5 Vaccination of chickens with inactivated virus

Forty chickens at 2 weeks age were obtained from the A&M Poultry Science Center. The influenza virus A/Singapore/97 H5N3 was used for the vaccines. Chickens were divided into 4 groups based on the type of vaccine they received: alpha-gal influenza, alpha-galactosidase treated MDCK-grown influenza, PBS with alpha-galactosidase, and PBS. Each chicken received approximately 0.09 mg of HA, according to previous recommendation (Maas et al., 2009). Virus grown in MDCK cells were subjected to alpha-galactosidase (Sigma, St. Louis, MO USA) with 3µl of 5% enzyme stock solution in PBS. This is a modified method of previously described methods (Luo et al., 1999). All viral vaccines were inactivated with formalin based on protocols established by DelSite Biotechnologies, Inc and were administered IM in the left breast muscle at 3 weeks age. Chickens were bled from the jugular vein to check serum anti-gal titers.

3. Results

3.1 Purification of anti-gal antibodies

Anti-gal antibodies from adult chicken serum (Sigma, St. Louis, MO USA) were purified using a melibiose (gal-alpha1,6gal) column with the Foxy® Jr fraction collector at room temperature. Based on UV output, fractions were collected that contained the largest concentrations of protein. Samples of the fractions were run on SDS-PAGE and western blots were performed

with gal-alpha1,3gal-conjugated bovine serum albumin proteins as a positive control. Western blot results were positive for anti-gal-alpha1,3gal antibodies with SDS-PAGE indicating a relatively clean protein preparation of primarily IgY antibodies. These antibodies were used as a positive control for further experiments.

3.2 Alpha-gal epitopes on MDCK passaged virus

Influenza virus A/Singapore/97 H5N3 was grown in Madin-Darby Canine Kidney (MDCK) cells in the presence of trypsin, according to protocols established by DelSite Biotechnologies, Inc. Briefly, MDCK cells were grown in roller bottles with serum free media at 37°C. Cells were then checked for approximately 90% confluency, the old media was discarded and new media was added with freshly thawed seed stock virus and trypsin. Cells were incubated at 33°C and monitored for cytopathic effect, then harvested at approximately 48 hours. Virus was then purified by Dr. Guo Jianhua according to protocols established by DelSite Biotechnologies, Inc. Influenza virus was then inactivated in formalin at 4°C for over 48 hours. Alpha-gal epitopes were determined to be present on the virus by western blot. Furthermore, alpha-gal epitopes were removed by alpha-galactosidase from green coffee bean according to previously described methods (Luo et al., 1999). Anti-gal antibodies previously reactive with MDCK-passaged influenza virus were no longer reactive upon addition of alpha-galactosidase (Fig. 11). As a control, anti-gal antibodies were not reactive with the alpha-galactosidase enzyme. A successful method of determining the effect of anti-gal on enhancing the humoral immune response had been developed. The only difference between these two vaccines was the absence of alpha-gal epitope.

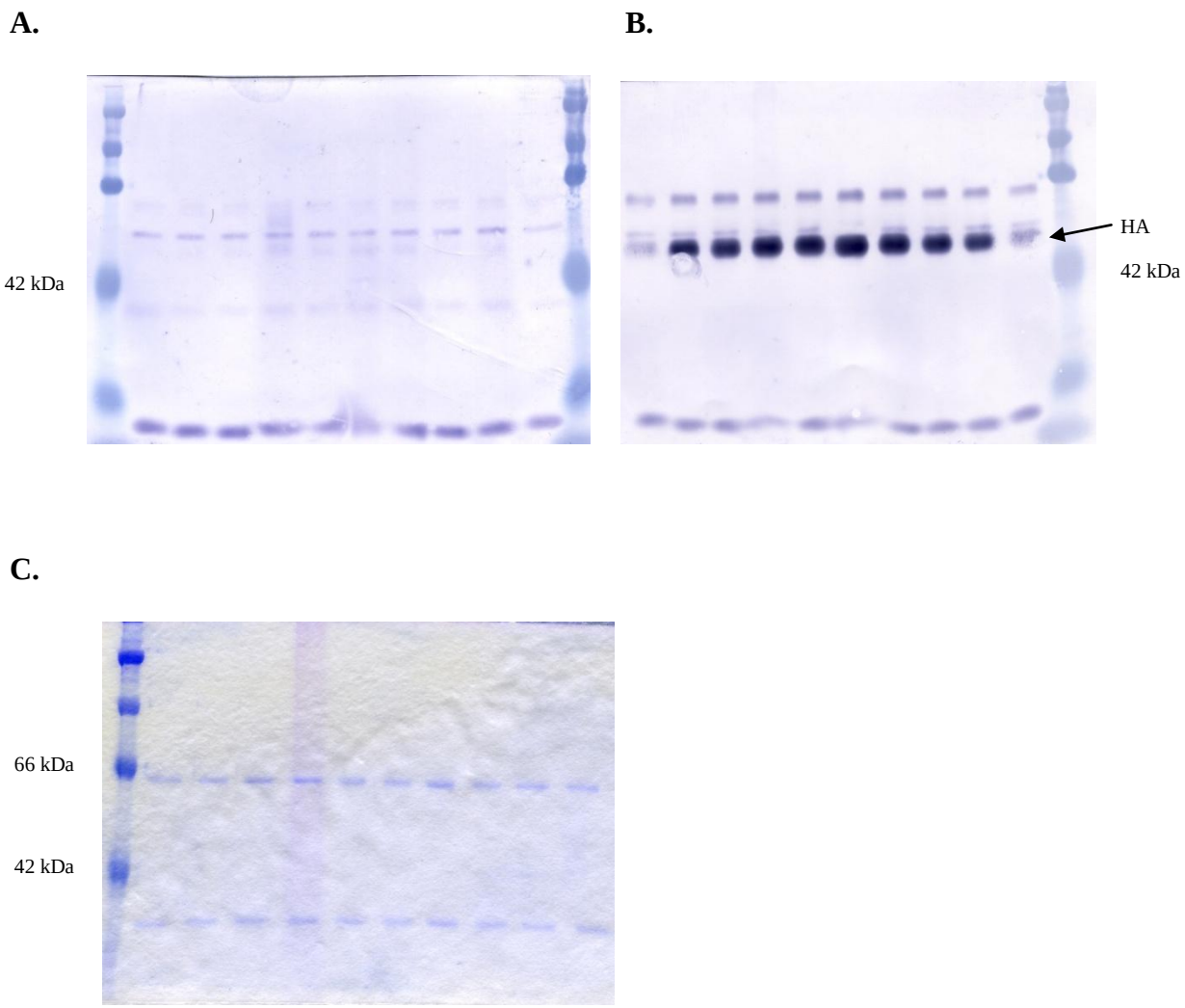


Figure 11. Western blot of influenza vaccines administered to chickens. Western blots of vaccines administered to White Leghorn chicks at 3 weeks of age. Virus for vaccine was grown in MDCK cells. A, western blot of vaccines treated with alpha-galactosidase enzyme and probed with chicken anti-gal antibodies to confirm removal of alpha-gal moieties from HA protein. B, vaccines with alpha-gal moieties reactive with chicken anti-gal on HA glycoprotein (arrow). C, coomassie stain of vaccines, without enzyme, administered to chickens.

3.3 Vaccination of chickens

Day-old white leghorn chicks were collected from the Poultry Center at Texas A&M University and placed in BSL-2 isolation buildings. Chicks were fed starter feed and given water ad-libitum. Chicks were housed in hanging cages (10 per group) which were cleaned and monitored daily. Chicks were bleed at 3 weeks and subsequently vaccinated with either alpha-gal H5N3, H5N3, alpha-galactosidase enzyme, or PBS. Chicks were subsequently bled at 5 weeks, at 9 weeks and again at 11 weeks. A second booster vaccine was given at 9 weeks of age to determine boosting effect.

3.4 Determination of anti-gal titers in chickens

Serum collected from chicks at 3 weeks of age contained no traces of anti-gal antibodies based on rabbit erythrocyte agglutination. Since there were extra chicks, 3 were terminally bled and their serum was pooled. The pooled serum was subjected to affinity chromatography and fractionation. All fractions collected were negative for anti-gal based on SDS-PAGE and western blot. Interestingly, some sera that were not heat inactivated were found to agglutinate rabbit erythrocytes at 2 HA units. At week 5, most chicks were found to have anti-gal at titers ranging from 8 to 64 HA units. One chick still contained no anti-gal in its serum while 3 chicks contained 64 HA units. Anti-gal titers from 32 to 64 HA units among all chicks were established by week 9.

3.5 Virus neutralization assay

Crucially, virus neutralization assay was unable to be performed because of time and resource constraints. Aside from the fact that anti-gal was not present in chicks at the time of vaccination, live H5N3 virus was unable to be worked with because it would require 5 days of no interaction with avian species.

4. Discussion

Though chicken vaccination programs have been largely successful because of the broad cross-reactivity of anti-HA antibodies in chickens, the challenge for a more robust influenza vaccine in humans remains (Doherty et al., 2006; Swayne and Kapczynski, 2008). Alpha-gal epitopes have proven to be beneficial in various vaccine strategies where a more vigorous immune response is warranted. This has proven particularly true for endogenous tumor vaccines containing alpha-gal epitopes in which tumor associated antigens successfully incorporate the carbohydrate epitope and trigger an immune response against the previously tolerated tumor (Abdel-Motal et al., 2009b; Galili et al., 2007). This strategy has been further extended into development of more effective HIV and influenza vaccines (Abdel-Motal et al., 2006; Abdel-Motal et al., 2007; Abdel-Motal et al., 2009a). However, the shortcoming of these experiments is that the alpha-gal "knock-out" mouse is not a natural host to either HIV or influenza. Although anti-gal titers comparable to human anti-gal titers amounting to 1% of total IgG are achieved, mice do not have the same repertoire of IgG isotypes that humans have. The chicken presents a better animal model for the effects alpha-gal epitope would have on enhancement of the immune response. The chicken is not only a natural host of influenza virus, but it has an immune system that is found to function in a similar manner to the human immune system and naturally develops anti-gal antibodies (Erf, 2004; Kaiser, 2007; McKenzie et al., 1999; Wu et al., 2010).

Anti-gal antibodies were not self-reactive (results not shown), but a vaccine with just the enzyme was administered to in order to dismiss the question of a potential adjuvant effect of the enzyme. Anti-gal titers were unable to develop by 3 weeks at the time of vaccination. This may be because the chickens were kept in an environment that did not allow their intestinal flora to develop properly. Indeed, development of a proper intestinal flora depends not only on

environment, but in this case, the development of anti-gal antibodies in chickens depends also on diet (Cotter and Van Eerden, 2006). In this regard, the experiment should be repeated with chickens that are housed in an environment that is more natural to their upbringing. This would, of course present more variables into the experiment. A second option would be to vaccinate chickens at 5 weeks to determine the effects of alpha-gal vaccines. However, by this time most chickens are already consumed, and so the applications may only be beneficial to humans. This experiment must be repeated with special attention to anti-gal development in chickens. As demonstrated by three week old chicks that were terminally bled and found lacking anti-gal antibodies, it does not appear that these antibodies are transferred maternally. That serum which is not heat inactivated is able to agglutinate rabbit erythrocytes suggests the presence of lectins able to bind alpha-gal or perhaps another carbohydrate moiety on these red blood cells.

CHAPTER VIII

SUMMARY

Avian Borna Virus and Influenza are both considered important pathogens of birds. PDD has been a problem for aviculturalists and pet owners alike since the 1970s. Until recently, an etiological agent had not been identified and therefore, a diagnostic test could not be established. In these experiments, an antigen common to these diseased birds was found, isolated, identified, and a resulting serodiagnostic test was developed. This serodiagnostic test may be used in conjunction with PCR tests to more accurately determine the presence of ABV in birds.

Secondly, the effect of a carbohydrate epitope incorporated into influenza vaccine was tested. Chickens produce natural antibodies against the anti-gal epitope. It was thought that this would result in an enhanced humoral immune response by directly targeting these antigens to antigen presenting cells. However, it was not anticipated that these chickens would not be producing anti-gal at the time of vaccination. Although influenza vaccines that differed only in alpha-gal epitope were produced, the ability for this epitope to enhance the humoral immune response in chickens could not be determined.

Birds are an important part of many ecological processes including seed dispersal and insect control. Not to mention, they are also an important source of food. Measures must be taken to insure that these animals are protected.

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VITA

Itamar Davi Schabert Villanueva received his Bachelor of Science in microbiology with a minor in sociology, from Texas A&M University in May of 2003. He entered the doctorate program at Texas A&M University College of Veterinary Medicine and Biomedical Sciences with the Department of Veterinary Pathobiology in September of 2005. While persuing his graduate studies, he was a research assistant in Dr. Yawei Ni's lab with DelSite Biotechnologies, Inc. Dr. Villanueva's research interests include diagnostics and surveillance of infectious diseases and vaccine development.

Itamar Villanueva

c/o Dr. Ian Tizard

Department of Veterinary Pathobiology

Texas A&M University

College Station, TX 77843-4467

E-mail: agita02@hotmail.com.