AVIAN BORNAVIRUS: RECOMBINANT N PROTEIN VACCINE AND PATHOGENESIS

A Dissertation

by

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ABSTRACT

Proventricular dilatation disease (PDD) is a fatal viral disease that affects mainly psittacine birds, although some non-psittacine species can be affected. Gastrointestinal and/or neurological signs can be seen in PDD-affected birds. These signs are likely a result of non-suppurative inflammation of the central, peripheral and autonomic nervous systems. Parrot bornavirus (PaBV) was discovered as a causative agent of PDD in 2008. The prognosis of PDD is very poor once clinical signs have developed and no specific treatments or commercial vaccines are available to date.

In order to investigate methods of protection, we vaccinated cockatiels using inactivated whole PaBV and/or recombinant PaBV N protein vaccines followed by challenge with virulent PaBV-2. In the first experiment, inactivated PaBV-2-infected cells were administrated via intramuscular (IM) inoculation, three times, followed by one IM injection of recombinant N protein vaccine. In a second experiment, the recombinant N protein vaccine was administered alone, two times, via the IM route. Over 28 weeks post challenge, cloacal swabs were collected and body weights and any clinical signs were noted. Using tissues collected at necropsy, histopathologic examination was used to detect the presence of microscopic lesions characteristic of PDD. Quantitative Real-Time PCR (qRT-PCR) and immunohistochemistry (IHC) were used to detect PaBV-RNA and antigen respectively. Using western blots assays, we were not able to detect a

humoral response after vaccination with the inactivated vaccine. Conversely, the recombinant N protein vaccine stimulated production of anti-N antibodies.

The results of both vaccine studies indicated that the recombinant N protein vaccine was able to protect subsequently challenged birds from lesions associated with PDD. Further, the vaccine protected birds from PDD-related morbidity and mortality. However, the vaccine did not protect "efficiently" against infection, as PaBV- RNA and antigen were detected in organs and cloacal swabs of vaccinated and unvaccinated birds. Moreover, there was no evidence that vaccination, either before or after challenge, increased the severity of the infection. We hypothesized that this protective response was a result of a switch from a type 1 cell-mediated immune response to a type 2 humoral immune response. This hypothesis was supported by a third study which revealed that treatment of cockatiels with cyclosporine A, an immunosuppressant, at the time of challenge with virulent PaBV-2, also conferred complete protection against PDD at the expense of increasing the viral load. This experiment showed that PDD might be an immunologically mediated disease, similar to Borna disease in mammals.

DEDICATION

This dissertation is dedicated to my family: to my parents Sadeq Ettaimesh & Suad Al Jawhar, my brother Ali, my sisters Rasha & Saba, my wife Ghusn Al Khateeb & my children Mohammed Ali, Zain Alabideen, Fatimah.

For My uncles & my cousins especially Hayder Ettaimesh, my brother-in-law Kamal Al Obaidi, my father-and mother-in-law Sabah Al Khateeb & Amal Al Alawi.

In the memory of my great-great-grandfather Sadeq Ettaimesh & my grandmother Salman Al Jawhar.

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TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Proventricular Dilatation Disease (PDD)

PDD is a serious and devastating viral disease of captive parrots worldwide. It is typically characterized by the presence of lymphoplasmacytic infiltrates in central, peripheral and automatic nervous tissues. PDD was first recognized in the late 1970s and early 1980s in the Unites States and Germany during outbreaks which affected macaws (*Ara spp*.). The disease was initially named macaw wasting disease [1] and other names for the disease include macaw fading disease, infiltrative splanchnic neuropathy and myenteric ganglioneuritis [2-4]. PDD has been reported in more than 50 psittacine species, but some non- psittacine species can also be affected [5, 6]. After the disease was first described, research was begun to identify and isolate the responsible etiological agent(s). It was widely accepted that the disease agent was a viral agent and several viruses were considered as candidates. These included a paramyxovirus [7], togavirus, picornavirus [8] and a coronavirus [9]. However, further studies were unable to link these viruses with PDD and the fulfillment of Henle-Koch's postulates was not achieved [9-11]. In 2008, using pyrosequencing, a pan viral microarray approach and conventional PCR, two independent research groups isolated and identified a novel bornavirus from PDD- positive parrots and called it avian bornavirus. This new virus had <70% shared sequence at the nucleotide sequence level and <80% at the overall amino acid level with BoVD-1, a mammalian bornavirus. [12, 13].

PDD causes severe non- suppurative inflammation of the autonomic nervous system which innervates the myenteric ganglia of the gastrointestinal tract (GIT). Esophagus, crop, proventriculus and ventriculus are the most affected parts of the GIT. The neurological function of the myenteric ganglia is impaired and as a result, and the peristaltic movement of the GIT is impaired or lost. Consequently, food accumulates in the proventriculus, leading eventually to crop stasis and enlargement and dilatation of the proventriculus and intestine. Several clinical signs may occur as a result. These include anorexia, lethargy, passage of undigested food with fecal material, regurgitation, diarrhea, dysphagia, emaciation leading to cachexia, and vomiting. Death is often due to starvation and sometimes is caused by sepsis as a result of microbial overgrowth in the enlarged non-motile proventriculus [14-16]. Non-purulent inflammation of the CNS [1, 17], can cause neurological signs such as depression, ataxia, tremor, incoordination, blindness and seizures [1, 18]. These signs may occur alone or in parallel with clinical signs of a dysfunctional digestive tract [1, 19-21]. Mortality may reach 100% [1].

In general, clinical signs and symptoms of PDD, as in Borna disease in horses and sheep, are not specific (pathognomonic) for this disease. Definitive diagnosis is based on postmortem histopathological examination to reveal infiltration of lymphocytes and plasma cells in the myenteric ganglia of the proventriculus and ventriculus [6, 22]. Most cases also show non-purulent encephalomyelitis and ganglioradiculoneuritis of the spinal nerves. Inflammation of peripheral nerves and gliosis may also occur [10, 11, 14, 15, 18, 23]. Cardiac problems due to conduction system deficiency of some PDD- affected birds might be a cause of sudden death [17].

Crop biopsies can be used as a useful confirmative intra-vitam diagnostic tool (especially for expensive or highly endangered birds) to reveal the presence of a lymphoplasmacytic infiltration in the myenteric ganglia. However, the segmental distribution of lesions decreases the accuracy of this method. Radiography, such as X-ray and contrast imaging using barium can also be used to detect the presence of an enlarged proventriculus [24, 25]. Several serological techniques such as western blot (WB), indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA) can be used to detect the presence of specific antibodies against the causative agent, PaBV. However, negative results are not a helpful diagnostic indicator as many symptomatic birds lack detectable antibodies to PaBV [20, 26]. Molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) assays can be used to detect viral sequences in tissues, feces, cloacal and/or crop swabs as well as feather calami [27]. A positive PCR result in the feces is an indication of current infection; however, a negative result is inconclusive as it is well documented that virus shedding is intermittent [25]. Repeated testing of multiple samples can be helpful, as can using a combination of serology and PCR which together are considered to be the most dependable diagnostic approaches. Unfortunately, neither of these diagnostic tools (alone) is highly reliable in detecting infection. In addition, neither is helpful in predicting outcome, as many infected birds remain healthy for many years and die of other causes. There is no single noninvasive test that is entirely suitable for the diagnosis of PaBV infection [27-29].

For postmortem diagnosis, typical histopathologic lesions can be found primarily in GIT and CNS. Viral RNA can be detected in the GIT and nervous system and often in other tissues. Viral antigen can be detected by immunohistochemistry (IHC) using antibodies to PaBV nucleoprotein (N) or phosphoprotein (P). Duck embryo fibroblast (DEF) or quail cell lines CEC-32 and QM7 can be used to isolate the virus [28, 30, 31]

Parrot Bornavirus (PaBV)

PaBV (previously known as avian bornavirus, ABV) was first discovered in 2008 when independent research groups from the United States detected a novel borna virus among several PDD- positive birds by using pyrosequencing [13] and micro array approaches [12]. The virus was named avian borna virus because it is shared several features characteristic of the *Bornaviridae* family (Order *Mononegavirales*) but less than 70% nucleotide sequence identity with mammalian Borna virus BoDV-1 [30, 32]. To date, eight bornavirus genotypes have been identified from psittacines (PaBV 1-8) and seven from nonpsittacine birds. These include strains isolated from Canada geese, trumpeter swans, canaries and estrilid finishes [12, 13, 16, 33-39]. The family *Bornaviridae* currently contains one genus, *Bornavirus*, which includes five species: *Mammalian 1 bornavirus* which includes BoDV-1 and 2; *Psittaciform 1 bornavirus* which includes PaBV1,2,3,4 and 7; *Passeriform1 bornavirus* which includes the strains isolated from canary birds and Bengalese finches; *Passeriform 2 bornavirus* which includes an isolate from an estrilid finch and *Waterbird 1 bornavirus* which includes isolates from Canada geese, mute swans and a variety of ducks. PaBV 1-4 and 7 have all been isolated from

parrots and identified as causative agents of PDD [32, 40]. PaBV- 5, 6 and 8 are not yet officially classified but have been isolated from birds diagnosed with PDD [41]. Another bornavirus has recently been isolated from Loveridge's garter snake. This virus belongs to a novel species (*Elapid 1 bornavirus*) and is named Loveridge's garter snake 1virus (LGSV-1). Because of insufficient characterization, this virus has been placed in the family *Bornaviridae* but not in the genus *Bornavirus* [32]. In 2015 a novel bornavirus was linked to three cases of human fatal encephalitis in three squirrel breeders in Germany [42].Genetic analysis revealed that this virus is less than 77% homology with mammalian 1 Bornavirus cluster [42].

Before the discovery of PaBV in 2008, the only known member of the family *Bornaviridae* was the mammalian Borna disease virus, BoDV-1, the causative agent of Borna disease of mammals, especially horses and sheep. Various isolates of this virus show very high sequence conservation, an uncommon feature among RNA viruses [12, 43]. In contrast, PaBV is more divergent and to date, 8 different genotypes of this virus have been recognized [32, 41]. PaBV 2 and 4 are the most predominant worldwide [18, 30]. Co-infection with more than one strain has frequently been noted; it appears that the severity of disease and the lesions in a co-infection do not significantly differ from those in a single infection [6, 34, 44].

Genome Organization

Bornaviruses are enveloped, 80-100 nm in diameter, spherical viruses containing a single- stranded, non-segmented negative sense (NNS) RNA genome of about 8.9 kb. The bornavirus genome is considered the smallest among NNS viruses [31, 45]. Unlike other families of the order *Mononegavirales* (*Paramyxoviridae, Rhabdoviridae* and *Filoviridae*), bornavirus transcription and replication take place in the nucleus of host cells. Therefore, the splicing machinery of the host cell allows for alternatively spliced transcripts for protein expression [46, 47]. Short heterogenous untranslated regions (UTR) are located at the 3'- and 5'- ends of the bornaviral genome. These UTRs contain promoter elements for the transcription and replication of the genome. These UTRs differ from those of other NNS viruses in which UTR sequences (up to 20 nucleotides) are complementary to each other [47-50]. Moreover, the bornavirus genome, in contrast to other NNS RNA viruses, lacks specific, repetitive intergenic regions; instead, it has overlapping open reading frames (ORFs) [51, 52]. Bornaviral genomes contain three transcription units (III), defined by three different transcription initiation sites (S1-S3). Four polyadenylation signals/termination sites (T1-T4) (Fig1) have been identified and some transcripts are formed by read-through of these termination sites. In fact, the *pol* transcript is produced only by read-through of the T3 site [53]. Mammalian and parrot Borna viruses share the same general genome organization in terms of gene order, number of genes and organization of sites for initiation and termination of transcription [12, 13]. However, there are minor differences in the region between the N and X genes. BoDV-1 contains a regulatory element for synthesis of the X protein that is lacking in

most of the avian bornaviruses[12, 34, 54]. In fact, among the avian bornaviruses the N/X intergenic region is the most variable region of the genome. Neither the importance of these differences, nor mechanisms for regulation of X protein synthesis have been carefully examined for PaBVs. The Bornaviral ribonucleoprotein (RNP) complex is composed of N and P in association with genomic RNA [55, 56]. Both arms of immune system, humoral and cellular, recognize the nucleoprotein and to lesser extent the phosphoprotein as major targets [57].

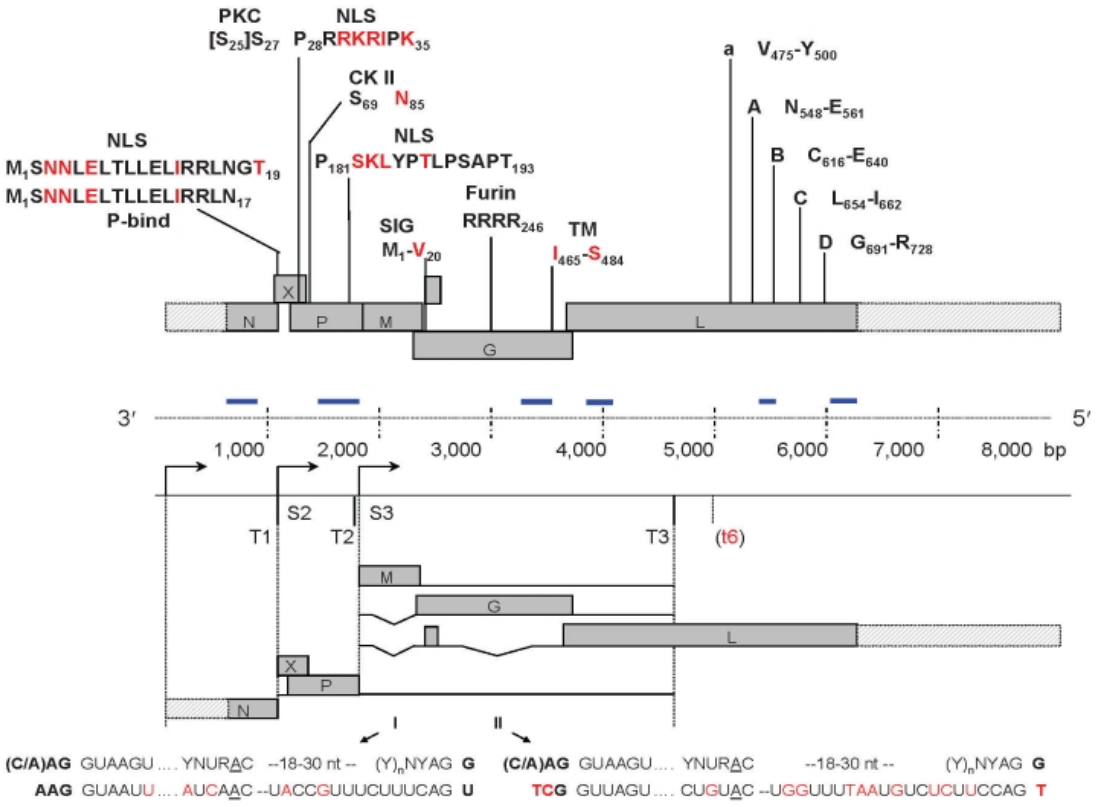


Figure 1. Borna disease virus genome organization. Conservation of genome organization, regulatory sequences, and protein domains of Borna disease virus (BDV) in novel strains from parrots 1034, 1322, and 1367. N, nucleoprotein; P, phosphoprotein; X, X protein; M, matrix protein; G, glycoprotein; L, L-polymerase protein. Genome regions not yet sequenced in the novel strains are shaded. P-bind, binding site for P on X; NLS, nuclear localization signals of X and P; PKC, protein kinase C epsilon phosphorylation sites in P; CK II, casein kinase phosphorylation sites in P; SIG, signal peptide; Furin, furin cleavage site; TM, transmembrane anchor of G; A – D, conserved RNA-dependent RNA polymerase motifs. Conserved sites/residues with respect to BDV strain V are shown in black; divergent sites/residues are indicated in red; K32 in P NLS-1 is divergent only in 1034/1322, K35 in NLS-1 and K183 in NLS-2 are divergent only in 1367. S2 and S3, start sites of transcription units 2 and 3, respectively, showing the conserved GAA initiation triplet; T1, T2, and T3, transcription termination sites showing the conserved TA6 consensus sequence; (t6) indicates a nonconserved TA6 sequence found in some BDV isolates. Blue bars indicate the 6 clusters represented by contigs obtained through pyrosequencing. Consensus splice site sequences corresponding to established introns I and II in genes for M and G of BDV strain V are aligned to corresponding sequences of the novel strains.
This figure was adapted from reference [13]

Bornavirus Proteins

The bornavirus genome encodes for at least six proteins. Starting at the genomic 3′ end, the gene order is: nucleoprotein (N), X-protein (X), phosphoprotein (P), matrix protein (M), membrane glycoprotein (G), and the RNA polymerase (L) [58, 59].

Glycoprotein (G): G encodes a 56-kDa polypeptide, however, a 94 kDa protein (gp 94) results from posttranslational modification through N-glycosylation with high- mannose oligosaccharide complexes [60-64]. This protein may play a crucial rule in persistence of bornaviral infection by protecting the antigenic epitopes through mimicking host N-glycans [65]. G is cleaved into N-(GP-N, 51 kDa) and C-(GP-C, 43kDa) terminal subunits by cellular furin- like proteases [60, 62]. Only cleaved G has been detected in virus particles [65]. GP-N is sufficient for virus entry into cells via a receptor recognition mechanism. GP-C, on the other hand, might be needed for fusion, which occurs after internalization of the virus through endocytosis [66, 67].

Matrix protein (M): M is a 14.5-kDa protein (142 amino acids). M is the smallest matrix protein among NNS viruses. M forms noncovalently linked tetramers to serve as the bornavirus matrix protein [68, 69]. This protein plays a major role in particle formation. M can bind to P and thus is part of the viral RNP complex [70].

Unlike other matrix proteins of NNS RNA viruses, bornaviruses M does not appear to interfere with polymerase activity [70, 71].

Nucleoprotein (N): N is the major component of the RNP complex which in turn acts as a template for the L [51, 72]. In the case of BoDV-1, 371 amino acid N, is found in two forms, 40-kDa (p40) and 38kDa (p38) [51, 52, 73]. In contrast to BoDV-1 N, the smaller (p38 kDa) form of N has not been identified for the avian bornaviruses., BoDV-1 p40 contains a nuclear localization signal (NLS; P3KRRLVDDA11) and a nuclear export signal (NES; 128LTELEISSIFSHCC141) [29, 74, 75]. Both are important for movement of the ribonucleoprotein (RNP) complex into and out of the nucleus [51, 52, 56]. These roles are compatible with the distribution of N isoforms in infected cells, with p40 primarily found in the nucleus and p38 primarily found in the cytoplasm [51, 52]. Although p38 can accumulate in the nucleus at levels similar to p40, it cannot alone support the transcription/replication process of the bornaviral genome. This suggests that the amino terminal sequence of p40 may have functions in addition to RNP translocation [76]. Co-localization and purification experiments indicate that p40 and p38, as well as P and M, associate with genomic RNA to form the RNP complex [70, 71]. [55, 72, 77]. Encapsidation of genomic RNA by multimeric N does not protect genomic RNA against enzymatic attack [78, 79].

Phosphoprotein (P): P is a 24 kDa, 201 amino acid protein that plays an important role in the replication cycle of bornaviruses. P acts as a transcriptional activator by binding and interacting with other viral proteins (X, N, M, and L) and with itself [64, 77, 80, 81]. P plays a pivotal role as a mediator for regulation and assembly of the polymerase complex [77]. P has two NLS and one NES [82, 83]. Infection of neurons by

bornaviruses results in interference with the recycling process of synaptic vesicles by blocking protein kinase C (PKC) -phosphorylation of key substrates. Evidence indicates that P plays a role in bornavirus pathogenesis by acting as a decoy substrate for phosphorylation by PKC within infected neurons [84, 85].

L protein: L, the RNA dependent RNA polymerase, is by far the largest protein of bornaviruses (190 kDa, 1711 amino acids). This protein is translated from a spliced mRNA [63, 86]. Nuclear localization of this protein in infected cells is achieved by an NLS motif (R844VVKLRIAP852) positioned in the center of this protein, or by interaction with P [87]. Bornavirus L contains conserved domains and motifs found in other NNS viral L proteins [48, 88].

X protein: X is a nonstructural protein whose ORF overlaps the P ORF. X is the smallest bornaviral protein (10 kDa, 87 amino acids residues). Although X is not found in infectious particles, it plays an important role in the bornavirus replication cycle. Bornavirus genomes with nonfunctional X ORFs are not viable [89, 90]. Assembly of an active polymerase complex depends on the relative abundance of X and P in infected cells [91, 92]. X can be identified in both nuclear and cytoplasmic compartments. Nucleocytoplasmic shuttling of X protein can be achieved via its interaction with P [70, 71, 93].The P interaction site has been mapped to amino acids 3-16 (SDLRLTLLELVRRL), at the N-terminus of X. This sequence partially overlaps an

importin-binding sequence mapped to amino acids 6-19 (RLTLLELVRRNGN) of X and important for nuclear shuttling [94-96].

Bornavirus Replication Cycle

To date, our understanding of the replication cycle and of PaBV is largely based on results of intensive research on BoDV-1 [54, 97, 98]. BoDV-1 attachment and entry seem to be comparable to rhabdoviruses and filoviruses, with pH-dependent entry through intracellular vesicles [99-101]. The BoDV-1 surface glycoprotein complex (GP-N & GP-C) plays an important role for receptor recognition and cell entry. BoDV- 1 is believed to enter the cells by means of receptor-mediated endocytosis with the help of unknown surface receptors on infected cells [66, 102]. Interactions between bornavirus G and the cellular receptor triggers the internalization of the virus via an energy-dependent mechanism using a clathrin-mediated (caveola-independent) pathway which requires the Ras-related protein Rab5 and microtubules [66, 103]. GP-N is the receptor binding protein while the hydrophobic amino-terminus of GP-C is required for pH-dependent membrane fusion in the endosome [61, 65, 103]. Fusion results in release of the viral RNP into the cytoplasm of infected cells [66]. However, no data are available concerning intracellular trafficking of the released RNP after membrane fusion [31]. Genome replication and transcription occur within the nucleus of infected cells. Thus, after translation, several viral proteins (N, P, X and L) move back into the nucleus via NLS. These proteins also have NES that may facilitate movement of newly assembled RNPs from the nucleus [56, 83]. Bornavirus N, P and L are required for viral genome

transcription and replication. Genomic RNA encapsidated by N protein acts as a template for the associated polymerase complex which consists of L and P. Even though X is not part of the RNP complex, it plays a major role in the replication cycle by modifying the formation and activity of the polymerase complex via interactions with P. Interactions of X and P results in changing the ratio of N to P, thus modifying the enzymatic activity of L [71, 76, 78, 93, 104]. Bornaviruses use mainly polycistronic mRNAs are transcribed from three transcriptional initiation sites [47, 53]. The only monocistronic product of the genome is a 1.2-kb transcript which is translated to generate the p40 and p38 isoforms of N [51, 52]. BoDV-1 packaging and release from infected cells are still not fully understood. Virions have been observed to bud from the plasma membrane of persistently infected cells *in vitro* [105, 106]. However, there is evidence of cell to cell spreading of the RNP complex [45]. Therefore, it seems that replication of bornaviruses in the central nervous system of susceptible hosts includes primary infection in which receptor-mediated endocytosis is followed by cell to cell propagation [107]. BoDV-1 can be detected, late in infection, in many tissues and organs as a result of its centrifugal spread via the axoplasm of peripheral neurons [108].

Pathogenesis and Pathogenicity

The pathogenesis of PDD is still not fully understood even though it is well accepted that PaBV is the sole causative agent of this disease [12, 13, 23]. Many questions about PDD pathogenesis remain to be answered. Many infected birds remain asymptomatic (clinically healthy) for long periods of time, sometimes for many years. The mechanisms and/or circumstances that trigger development of clinical disease are still not understood [109, 110]. PaBV is less restricted to neuronal tissues than BoDV-1, which is almost exclusively neurotropic and is seldom detected in other tissues [111, 112]. In PaBV-infected, but healthy birds, the virus is most often detected in the brain while in clinically sick birds the virus is more widespread, present in many organs [30, 110]. It is not known if vigorous immune responses are the cause or the effect of more widespread tissue tropism. It is possible that the immune responses might be more vigorous in clinically diseased birds. Alternatively, differences in tissue distribution and pathogenesis might be a result of genotypic differences among PaBVs. [38, 54]. Another difference between BoDV-1 and PaBV is the route of inoculation. It has not been possible to infect rats with BoDV-1 using the intravenous route [113]. On the other hand, this method of inoculation was successful in cockatiels [114]. Thus, it is fair to suppose that PaBV and BoDV-1 share some parallel features of pathogenesis but that differences also might be present.

The pathogenesis of BoDV is attributed to immunopathological events in the CNS. In the absence of cytotoxic T lymphocytes (CTL), BoDV-1 can cause persistent non-cytopathic infection of the CNS and is present both in neurons and astrocytes [98, 115, 116]. Transfer of T cells from clinically diseased rats to infected but clinically healthy rats initiates the development of Borna disease [23, 117, 118]. Thus, cellular damage occurs due to CD8 T lymphocytes, not directly by BoDV-1 [119]. In general, Borna disease is associated with infiltration of large numbers of immune cells into the CNS [120]. The severity of BoDV disease and the viral load in the CNS depends on the host age and species [120]. Persistent infection with high virus loads in the CNS has been found in neonatal rats. In contrast, adult immunocompetent rats develop meningoencephalitis [120]. CD8+ T cells play an important role in this scenario as the decline of BoDV 1– specific inflammation occurs when CD8+ T cell responses are reduced, despite continued virus replication [121]. CD8+ T cell play a key role for both virus- triggered clinical disease and antiviral defense activity. Virus- specific CD8+ T cell can provide protection against infection as a result of immunization. However, once a large number of neurons in the CNS become infected, the antiviral activities of CD8+ T cells become harmful [117, 118, 122, 123]. In addition to CD8+ T cells, CD4+ T cells also play a role in the pathogenesis of Borna disease. Establishing BoDV- specific T cell lines by using lymphocytes from N (p40)-immunized rats enabled researchers to select T helper lymphocytes bearing MHC class II and CD4. Passive transfer of these cells into BoDV infected, immunosuppressed healthy rats resulted in serious acute disease and death within 5 days post transfusion. However, transferring the same cells into

uninfected rats caused no disease. These results strongly indicate that this disease might be a result of delayed type hypersensitivity immune reaction (Type IV hypersensitivity) [118, 124-126].

Innate responses to viral infections are multifaceted and complex. The complete picture of interactions of BoDV-1 with antiviral defenses is not clear. However, there is evidence that BoDV-1 evades innate responses by trimming the 5’ termini of genomic and antigenomic RNA. As a result of genome trimming, the RNA strands carry a terminal monophosphate, instead of a triphosphate. Thus (retinoic acid-inducible gene 1) (RIG-I) cannot sense the presence of virus in the infected cells; as a consequence, the production of type I interferon (IFN) is blocked [127, 128]. A study using PaBV infected quail cells lines showed that viral infection of cell cultures was reduced when type I IFN was added [127], moreover, viral load in the cell cultures was reduced. In contrast, cell cultures with high viral load did not produce detectable levels of type I IFN. Another study showed that both PaBV and BoDV-1 X demonstrate similar type I IFN-inhibiting capacity, and the reduction in IFN is dose dependent upon X [129]. Kinetic analyses were used to compare the genomic termini during acute and persistent bornavirus infections and revealed that an accumulation of terminal truncations of one to three nucleotides at 3' terminus of the bornavirus genome resulted in attenuation of replication and transcription, probably contributing to lifelong, persistent infection [130]. Negative strand RNA virus genomes have ITRs that initiate synthesis of genomic and antigenomic RNA. Trimming of the bornaviral genome generates incomplete ITRs that are highly

attenuated as promoters for genome and antigenome synthesis [131]. In contrast, protein levels are not affected by incomplete ITRs. Therefore, complete ITRs appear to be important for genome replication but not for mRNA synthesis. This is compatible with bornavirus noncytolytic, persist infection where low levels of infectious virus particles, in combination with high antigen levels are observed [131]. It has been also shown that PaBV uses a similar mechanism (trimming the 5' terminus) to escape innate responses [127]. It has been suggested that bornaviruses might be used as novel RNA virus vectors for stable foreign gene expression in the CNS [132]. However, it is not known whether viral or host functions are responsible for genome trimming [31].

Genetically, BoDV is very homogeneous, with the exception of a strain named No/98, which differs by more than 15% at the nucleotide sequence level when compared to reference BoDV-1 strains [133]. However, variants that replicate better in mouse and rat models have been isolated. The consequences of divergent viral genotypes have not been of particular interest in studies of BoDV pathogenesis. In contrast, PaBV is much more genetically diverse.

PaBV-4 and PaBV-2 genotypes are most commonly recovered from parrots with PDD [26, 133-135]. PaBV-2 appears to be more pathogenic for cockatiels than PaBV-4. Moreover, cockatiels experimentally infected with PaBV-2 develop mainly gastrointestinal signs whereas neurological signs were observed more frequently in PaBV-4 infected cockatiels [135].

Even though seroconversion in PaBV-2 infected cockatiels appears to occur earlier than in PaBV-4 infected cockatiels, the antibody titers are lower. Moreover, viral RNA shedding was detected later in PaBV-2 infected cockatiels than during PaBV-4 infection. In this experimental model, earlier immune responses may have limited PaBV-2 replication but exacerbated disease [135].

In spite of less obvious clinical signs, viral loads in organs of PaBV-4 infected birds were significantly higher than in PaBV-2 infected birds. These results were independent of the route of inoculation (intracerebral or intravenous) and were also independent of the time point of death post infection. On the other hand, birds infected with PaBV-2 via the intracerebral route had higher viral loads compared with birds infected via the intravenous route. Therefore, these birds showed a clear depended in term of viral load in different organs with the route of inoculation as well as time point of death (late death birds had higher viral load in comparison with early death). Additionally, in PaBV-4 infected birds, viral antigen was more frequently detected in the CNS in comparison with PaBV-2 infected birds, in which the levels of viral antigen were increased in the GIT [23, 135, 136]. Moreover, re-isolation of PaBV-4 was easier from almost all tissues compared to PaBV-2 infected birds. Re-isolation of virus from birds that died later required only one passage in cell culture, while virus re-isolation from birds that died earlier post infection required several passages. Finally, virus shedding was detected later in PaBV-2 compared to PaBV-4infected birds. These results demonstrate that neither the severity nor progress of the disease is correlated with the replication of the

virus. [136] Instead, disease is triggered through mechanisms independent of viral load. In fact, one explanation for these results is that early immune system activation reduces viral load at the expense of disease severity.

It appears that the PaBV-2 infection may be similar to BoDV-1 infection in that even minimal virus replication triggers clinical disease, and disease progression is a result of T-cell responses [98, 135, 136]. These differences between infection patterns of PaBV-2 and PaBV-4 might be the result of variations in genome sequence. It is also possible that there are virulent variants of PaBV-4 and benign varients of PaBV-2 [119, 136]. More detailed study of PaBV replication, perhaps focusing on differences in G sequences and/or differences in X, are necessary to define the virulence determinants of these viruses.

The immunopathogenic mechanisms of PaBV infection remain to be determined. In the case of BoDV, clinical disease depends largely on the development of immune responses [98, 135, 137]. Before the identification of PaBVs as the etiological agent of PDD in parrots, it was proposed by some that PDD might be an auto-immune disease similar to Guillain Barré syndrome in humans [138]. Rossi et al. (2008) claimed that the non-purulent inflammation typical of PDD was a result of antiganglioside antibodies, and that these antibodies were significantly higher PDD- birds than in healthy birds [138]. Later, these authors concluded that these antibodies can be triggered as a result of PaBV infection, or any other viral infection. However, studies in our laboratory have revealed

no connections between clinical disease and the presence of antiganglioside antibodies [139]. We have attempted to induce PDD-like disease by inducing production of antiganglioside antibodies in chickens. (Leal *et al* Unpublished) reported that antiganglioside antibodies are not associated with the development of PDD.

Tizard *et al* (2016) noted three potential events that could lead to proventricular dilatation in PDD. First: Excessive relaxation (or contraction failure) of smooth muscle fibers in the proventriculus as a result of prolonged *stimulation* of non-adrenergic non-cholinergic (NANC) neurons in the myenteric plexus. Taking into consideration the destructive nature of the lesions of myenteric plexus as a result of bornavirus infection makes this scenario unlikely. Second: Obstruction of the so- called intermediate zone, located between proventriculus and the ventriculus, as a result of damage to pacemaker cells of the isthmus resulting from accumulation of ingesta in proventriculus. Third: Impairment of satiation signals from the proventriculus to the brain as a result of failure of vagus nerve afferent signals, so the affected birds might be continuing to eat. This is unlikely however to be the primary cause, as many PDD affected birds observed stopped eating early in the course of the disease [140].

Transmission of PaBV

The natural mechanisms of PaBV transmission are unknown. This virus is frequently detected in crop and cloacal swabs, so the fecal- oral route of transmission has been proposed [18, 30]. It is well documented that PDD can develop in a flock after new PaBV- positive birds are mixed with resident birds [14], clearly indicating horizontal transmission. However, some investigators note that not all PaBV-negative birds become positive even when in direct contact with infected birds [38, 39]. Some researchers have demonstrated that mixing PaBV- experimentally infected cockatiels with non-infected cockatiels, results in deposition of PaBV on the skin and feathers of the non-infected birds but that their internal organs remain virus-negative [23]. The extent of fecal- oral PaBV transmission among birds remains an enigma, especially when negative birds remain negative after years of contact with positive PaBV- positive birds [110].

Vertical transmission of PaBV also has been investigated. In one study, several embryos tested positive for PaBV-RNA and these embryos were of psittacine parents positive for PaBV [141]. However, it is not possible to conclude that the embryos were effectively infected, as the assay method was for PaBV RNA, not infectious virus. PaBV- negative offspring can also be obtained from positive parents by hand rearing [142]. In order to prove vertical transmission, viable virus needs to be isolated from PaBV- positive eggs or from offspring hatched in incubators [141, 143].

In general, there are difficulties explaining PaBV transmission between birds whether horizontally or vertically. Part of these difficulties come from the nature of the virus itself since PaBV establishes persistent infections of cells, and these cells release very low numbers of infectious particles [30, 54]. It may be that just a few cell types, for example kidneys, are capable of releasing enough infectious particles for effective transmission [144].

PaBV transmission is an area that needs to be extensively studied in well-controlled experimental systems. In a recent study, different routes of infection were assessed, as wells as different doses of infection, in order to determine the effectiveness of PaBV-2 infection in cockatiels [145]. Mucosal routes of infection failed to establish persistent infection with PaBV-2 in cockatiels, in contrast to parenteral routes in which all the inoculated birds were infected [145].

Seroconversion among Naturally and Experimentally Infected Birds

PaBV- specific antibodies have been detected in both PDD- affected and asymptomatic birds [27, 146]. However, some infected birds never produce detectable antibodies. On the other hand, sudden seroconversion sometimes occurs just before the development of clinical disease [26]. Cockatiels can develop antibodies to PaBV as early as 6 days after experimental infection [147]. Most PaBV- antibodies are directed against N (P40), and have been identified in birds with recent or past infection. Anti-P and anti M were also detected but with greater variability, thus, these antibodies are less useful as markers for PaBV infection [27]. Currently, no studies that have looked for, or identified the presence of neutralizing antibodies in bornavirus-infected birds. In the case of BoDV-1, anti-G and anti M neutralizing antibodies have been reported [148, 149]. Additionally, monoclonal antibodies directed toward the major glycoprotein (gp94) of BoDV-1 were capable of preventing Borna disease when given early in rats; a significant finding as regards vaccine development [150]. It seems that neutralizing antibodies might play a role in restriction of the infection into neural tissues in rats [149]. It has also been shown that anti-N and anti-P antibodies are non-neutralizing in the acute phase of Borna disease.

In the case of PaBV infected psittacine birds, anti-G antibodies (C-terminal) as well as the antibodies against the L protein have never been detected, even in long-term persistently infected birds. This might be a result of the inability of recombinant proteins to interact with those antibodies, or the failure of the host to produce antibodies against

those proteins [27, 142]. These results are in partial agreement with BoDV-1 infection in mammals, where non-neutralizing antibodies can be abundantly detected as early as two weeks after experimental infection in rats whilst the neutralizing antibodies, if they develop at all, can only be detected late, during chronic, infection [150, 151].

There are several examples whereby specific virus-neutralizing antibodies (generated early after infection) play a major role against cytopathic viruses. These examples include poliovirus, rabies virus, influenza virus and vesicular stomatitis virus [152-155]. On the other hand, cellular immunity plays the dominant role in controlling the infection of noncytopathic viruses like BoDV-1 in rats, lymphocytic choriomeningitis virus in mice, human immunodeficiency virus, and hepatitis B and C viruses in humans [156-159]. Thus, it is appears that in general, antibodies (humoral response) against PaBV in clinically infected birds are not protective, and in fact might be positively correlated with an increased severity of disease [18, 160].

A strict correlation is not always observed between seroconversion, positive fecal PaBV-RNA and virus load in tissues for the same bird [38, 109, 161]. A one year survey of naturally PaBV-infected psittacines from three bird collections revealed that PDD is more likely to develop in birds demonstrating high anti-PaBV antibodies in combination with a high PaBV RNA load in crop and cloacal swabs [161]. Additionally, some PDD-positive birds die in spite of high titer of antibodies against PaBV indicating that humoral immunity is not protective [161]. A similar result, mortality due to Borna

disease in the presence of high anti-BoDV antibodies, was obtained from studies of immunocompetent rats [121].

Variable levels of anti-PaBV antibodies have been detected in egg yolk and serum of late-stage embryos hatched from PaBV-2 infected parents. Egg yolk immunoglobulin (Ig) Y composition reflected the IgG composition of serum antibodies from female parents. However, these antibodies were absent from hatchlings 2-5 weeks later which is similar to chickens [142, 162].

Additionally, there is little data on whether or not there is any cross-protection between different species of PaBV. In one experiment, healthy cockatiels naturally infected with PaBV-4 produced unusual and severe PDD- lesions after inoculation with a different strain of PaBV-4 [36].

Potential Therapy

To date, the treatment of PDD is nonspecific and it is based on symptomatic treatment with supportive regimens; some treated birds have survived for months to years [163-165]. Just a few studies have been published about the potential therapeutic effect of certain drugs [18, 25, 166]. On the other hand, antiviral therapy has never been reported as a successful treatment against PaBV infection [54, 119].

Since non-purulent inflammation is the pathognomonic histological lesion for PDD, several studies examined the therapeutic effects of anti- inflammatory drugs. Meloxicam, celecoxib and tepoxalin are non-steroidal anti-inflammatory drugs (NSAIDs) that have been recommended to reduce inflammation in PDD birds. Celecoxib (cyclooxygenase 2 inhibition) was reported as a candidate to treat PDD-affected birds (with improvement of the clinical signs), but this trial was done without using a control group [167, 168]. In one study, experimentally infected cockatiels were treated with meloxicam. In that study, however, the treated cockatiels developed more severe lesions in comparison with control group in which cockatiels were PaBV positive but not treated [166].

Amantadine hydrochloride as an antiviral drug has been reported to reduce the clinical signs in PDD-affected birds [25, 168]. On the other hand, some researchers reported that amantadine hydrochloride has no effect on viral shedding of the infected birds [18]. Therefore, it's unclear how this drug reduces the symptoms. Further studies need to be done to identify the mode of action [119]. Ribavirin was reported to reduce the infection of PaBV- infected cells in cell culture but had no measurable effect on viral shedding in infected birds [26].

As no antiviral drugs have been identified that can directly reduce infection by PaBV, it might be that the interruption of pathogenesis is a good therapeutic approach. Therefore, prevention of clinical disease by using immunosuppressive drugs such as cyclosporine A

was studied in mammals. Cyclosporine A (a T- cells – specific immunosuppressive drug) increased survival time, with no reported encephalitis or clinical symptoms, in experimentally infected rats [169]. Thus, similar treatment, of PDD in birds could be beneficial. In one case report, an African grey parrot suffering from PDD clinically recovered after administration of cyclosporine A [170].

An immunomodulating therapeutic approach was suggested by some authors by using robenacoxib as an anti-COX-2 nonsteroidal anti-inflammatory drug in combination with *Mycobacterium bovis* extracts in order to re-direct activated T cells into local inflammatory sites. However, these studies were done with no proper case-controls [147, 171], thus the results should be considered inconclusive.

Many palliative therapeutic protocols have been used for the management of PDD-affected birds and responses could be evaluated by doing serial crop biopsies, detecting viral shedding in feces and/or radiographic imaging of the proventriculus [25, 168].

Development of a Vaccine for PDD

Natural protective immunity to PaBV has not been demonstrated and there is no correlation between antibody levels and recovery. In fact, high antibody levels sometimes precede disease in persistently infected birds [170]. Studies of BoDV-1 pathogenesis predict that some immune responses are directly linked to disease development [117, 122]. Therefore, it remains to be seen if vaccination is protective or

detrimental in experimental PaBV infection. Recently, one study described a vaccination strategy that reduced viral load in tissues, as well as bornavirus RNA shedding, in experimentally infected cockatiels and common canaries. These promising results were achieved by employing two viral vectors. Recombinant Newcastle disease virus and modified vaccinia virus Ankara were designed to express the nucleoprotein and phosphoprotein genes of PaBV-4 and canary bornavirus 2 (CnBV-2). These viral vectors demonstrated self-limiting infection in a prime/booster vaccination protocol and PaBV specific antibodies were detected in vaccinated birds [172]. These results showed for the first time that the bornavirus infection can be markedly reduced by vaccination, a very important step towards protection against PDD in psittacine birds [172]. A subsequent study from same authors indicated that using these vaccines via the same prime- boost vaccination regime in cockatiels protected against low dose PaBV-2 challenge and development of PDD [145].

We have developed and tested inactivated whole virus and recombinant N protein vaccines in cockatiels as a part of our efforts to protect against PDD in parrots. Even though vaccination with inactivated vaccine failed to stimulate humoral responses, one or two doses of recombinant N protein vaccine triggered anti N antibodies and protected vaccinated birds against PDD. QRT-PCR and IHC revealed that the recombinant N protein vaccine did not "efficiently" protect against infection. Based on these results, we hypothesized that protection against PDD in vaccinated birds occurred as a result of stimulation of Th2 responses and suppression Th1 responses. To test this hypothesis, we

used cyclosporine A as an immune suppressive drug. Indeed, the cyclosporine treated birds were protected against PDD. However, qRT-PCR also showed that the viral load was higher in the cyclosporine treated group.

CHAPTER II

MATERIALS AND METHODS

Vaccines

Inactivated whole virus and recombinant N- protein vaccines, as well as virus stocks for challenge were prepared in the Schubot Exotic Bird Health Center- in a BSL-2 laboratory.

Virus Culture and Quantitation

PaBV-free primary duck embryo fibroblasts (DEF) were infected with a cockatiel (*Nymphicus hollandicus*) derived PaBV-2 isolate and cell cultures were passaged 7 times. Focus forming unit (FFU) assays were used to determine the virus titer because bornaviruses are non- cytopathic both *in vivo* and *in vitro* [173-176]. FFU assays were performed, as described previously [177], with PaBV anti-N antibodies (1:500) as a primary antibody and alkaline phosphatase (AP)-conjugated goat anti-macaw IgY serum (Bethyl Laboratories TX, USA) as a secondary antibody.

Inactivated Whole Virus Vaccine

PaBV free primary DEFs were infected with a PaBV-2 isolate from cockatiels (*Nymphicus hollandicus*). Cells from passage 7, containing 6.5 x 10^5 FFU/ml of PaBV-2 were suspended in phosphate-buffered saline (PBS). After brief sonication, the cell

suspension was inactivated by using formalin (36.5-38% in H_2O), final concentration 0.025%. Formalin treatment was done for 7 days.

Recombinant N-Protein Vaccine

Recombinant N protein was prepared as described previously [177]. Briefly, frozen brain tissue from a PaBV4- infected golden-collared macaw (*Primolius auricollis*) was used as source of viral RNA. RNA was extracted, using a Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In this protocol, the selective binding characteristics of a silica -based membrane were used to obtain up to 100 µg of RNA per sample. RNA was used to generate first-strand cDNA by reverse transcription (High Capacity Reverse Transcription Kit from Applied Biosystems Foster City, CA 94404 USA,) and random hexamers. PCR was performed to amplify the nucleoprotein gene of PaBV-4 (Accession number JN014948.1) using the following primers with BamHI and XhoI restriction enzyme sites added for cloning at 5' and 3' end respectively.

Forward primer for N cloning:

5'-CATG CAT ATG CCA CCC AAG AGA CAA AGA AGC (SEQ ID NO: 1)

Reverse primer for N cloning:

5'- GTAC CTC GAG GTT TGG GAA TCC GGT TAC ACC (SEQ ID NO: 2).

The resulting PCR product was cloned into the TOPO™ cloning vector (Invitrogen™ Carlsbad, CA 92008 USA). *E. coli* cells were transformed and plasmids were prepared.

Plasmids were sequenced (Gene Technology Lab of Texas A&M University) using Sanger sequencing to confirm the correct sequence of the inserted PCR products. The N gene was moved into the pET 21a vector (Novagen™ Hornsby Westfield, NSW 1635, Australia) to generate a histidine-tagged fusion protein. Bacterial cells containing the recombinant expression plasmid were grown and protein expression was induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested by centrifugation, resuspended in PBS buffer and sonicated. Sonicated material was centrifuged to remove insoluble product and the supernatant was applied to a Ni-NTA Agarose column (Qiagen, Hilden, Germany). The column was washed and eluted as recommended by the Qiagen manual. Material was then further purified by Sepharose CL-4B column chromatography. Purified protein preparations were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and WB. The purity of this protein was estimated to be greater than >95% pure by SDS-PAGE. Protein concentration was determined by using the BCA protein assay kit (Pierce, Rockford, IL).

To prepare the vaccine, the protein concentration was adjusted to 25 µg per 75 µl of sterile saline. Each 0.1ml vaccine dose was contained 75µl of recombinant N protein in saline and 25µl alum (Invitrogen^{TM}). Vaccine was administered by intramuscular injection into the right pectoral muscle.

Serological and Molecular Tests

Western blot assays

WB assays were used to assess anti-N antibody levels in plasma or serum collected at the following times: Before starting pilot, vaccine and vaccine safety experiments; three weeks after each vaccine dose (pilot and vaccine experiments) and at the end of each experiments (pilot, vaccine and cyclosporine A trials). For the vaccine safety trial, the second serum sample was collected 17 days after the booster dose of this vaccine. WB assays were performed as previously described [177]. Briefly, recombinant N- protein was adjusted to 1µg/ lane and electrophoresed on 10% polyacrylamide gels (size-fractionation). Proteins were then transferred to polyvinylidene fluoride PVDF (in transfer buffer, consisted of Trizma base (Sigma), glycine, H2O and methanol, at 90 V for 1.5 hr.) and the membrane blocked with 5% nonfat dry milk in phosphate buffered saline (PBS) plus 0.05% Tween 20 for two hrs. at room temperature. Membranes were incubated with diluted bird sera (1:200) for two hrs. at room temperature followed by: Washing for three times, ten minutes each, with wash buffer (0.02 M sodium phosphate (Na2HPO4·7H2O), 0.15 M sodium chloride (NaCl), pH 7.2 and 0.05% (v/v) Tween-20. Membranes were then incubated for one hr. at room temperature with alkaline phosphatase labeled goat anti-macaw IgY (Bethyl Laboratories, Montgomery, TX). diluted 1:5000 in blocking buffer. Membranes were then rinsed three times for 10 min each with washing buffer and developed using 5-bromo-4-chloro-3-indolyl phosphate/p-nitroblue tetrazolium chloride (BCIP/NBT) from Sigma (St. Louis, Missouri, USA) and

air dried for 30 min before reading. Sera from histopathological-PDD positive parrots, diluted 1:5000 in PBST blocking buffer, was used as a positive control.

Quantitative real-time PCR (qRT-PCR)

This test was performed to detect the presence and relative quantity of viral RNA in tissues from test birds. Cloacal swabs were taken at various times to assess levels of virus shedding. Tissues were collected at necropsy and frozen at-80°C. RNA was extracted from tissue samples and cloacal swabs using RNeasy Mini Kits and QIAapm viral RNA mini kits respectively (Qiagen, Hilden, Germany). Extracted RNA from cockatiel samples were analyzed using a qRT-PCR assay targeting the P gene of PaBV. qScript XLT One-Step RT-qPCR ToughMix ROX (Eurofins company, Val Fleuri, Luxembourg) was used to carry out First-strand cDNA and PCR amplification in a single tube. Each reaction contained TaqMan probe and primers:

ABV P9F primer-5_-AAGAAGAA[Y]CC[Y]TCCATGATCTC-3_ (36 μM).

ABV P10R primer-5_-AA[Y]TGCCGAAT[B]A[R]GTCATC-3_ (36 μM).

ABV P TaqMan

Probe-5_FAM-TCGATAACTG [Y]TCCCTTCCGGTC-3_-BHQ (10 μM).

Avian β actin was used as housekeeping gene for the purpose of quantitating the viral load for qPCR. Actin primers were also used to check the success of RNA extraction using. Actin mRNA was amplified using the following primers and probe (Integrated DNA Technologies, Coralville, IA, USA):

Actin F (5'- CATGAA GATCCTGAC AGA-3')

Actin R (5'-TCTCCTGCTCYA AYTCCA-3').

Actin probe (5'-/56FAM/CACCACCACAGCCGAGAGAGAAAT/3BHQ-1/-3').

QPCR was carried out using the ABI (Foster City, CA 94404 USA) 7900HT Fast Real-Time PCR system with SDS 2.4 software. The amplification profile was:

1 cycle: 5 min 50°C (reverse transcription), 1 cycle: 20 sec 95°C (RT inactivation/initial denaturation), 45 cycles: 3 sec 95°C (PCR denaturation), 30 sec 56°C (PCR annealing). Threshold cycle values (Ct) >35.0 were considered negative [13] . Relative change in gene expression (mRNA of P gene) was determined using the $2^{-\Delta Ct}$ method, with beta actin used as the endogenous control [178, 179].

Immunohistochemistry (IHC)

IHC was performed to determine the distribution of PaBV antigen in tissues. This test was done according to method of Wunschmann et al. [180]. An automated staining process was used to perform IHC using polyclonal antiserum against PaBV- N protein (strain 1367; GenBank accession no. FJ169440). This antiserum was used as primary antibody. Briefly: Sections of four-micron thickness were placed on glass slides and then were deparaffinized (clearing agent) and rehydrated (alcohol 100%, 95%, 70%, and 50% distilled H_2O) then incubated with 3% H_2O_2 for 15 min to block the activity of endogenous peroxidase. Tris buffered saline with 0.05% Tween 20 was used to rinse the sections. These sections then were incubated with normal goat serum diluted 1:10 in TBST for blocking unspecific binding sites. Sections were then incubated with anti-PaBV antiserum (diluted 1:1,250) for 45 min at room temperature. Sections were washed with TBST and were then incubated with secondary antibody (polymer-labeled goat anti-rabbit immunoglobulin G in 2% normal chicken serum) for 45 min at room temperature. Positive antigen–antibody reactions were developed with 3-amino-9-ethylcarbazole (AEC) for 10 min. Slides were briefly counterstained with Mayer hematoxylin. Sections were analyzed and reactions were graded as mild, moderate and severe according to the number of PaBV antigen–positive cells. IHC examination was done blindly by an expert pathologist in the California Animal Health & Food Safety Laboratory System (H. Shivaprasad, Pers Comm)

Virus for Challenge and Experimental Infection

PaBV-2 isolate from cockatiels was grown in primary DEF monolayers as described previously [74, 177]. DEF were passaged 5 times. The virus titer was determined by counting FFU as described previously [177]. Virus preparations were adjusted to a concentration of 4×10^4 FFU of PaBV-2 per 0.1 ml saline [177]. Each bird was inoculated intramuscularly (0.1 ml) with this virus with the exception of the cyclosporine A experiment where each bird was inoculated with 8×10^5 FFU.

Samples of each inoculum (0.5 ml) of infected DEFs were retained for further cultivation to ensure that the birds were given viable virus. All birds were kept in isolation rooms in a facility at the College of Veterinary Medicine and Biomedical Sciences avian complex / Texas A&M University under the Biosafety Level 2 (BSL2). (Although PaBV has never been shown to infect humans, bornaviruses have been reported to cause neuropsychiatric disorders of humans [181] and we have recently demonstrated that PaBV is infectious for cultured human astrocytes). Birds were housed in flight cages, food and water were provided *ad libitum*.

Experimental infections were performed under Animal Use Protocol IACUC 2012-0266 approved by the Texas A&M University Institutional Animal Care and Use Committee. In general, any birds that lost 20% of their body weight were euthanized using CO_2 under isofluorane anesthesia.

Experimental Animals and Study Design

Pilot experiment

For the pilot experiment, twenty-six adult male and female cockatiels (*Nymphicus hollandicus*) were clinically healthy and tested free from herpesvirus, chlamydia and PaBV. Herpesvirus and chlamydia tests were performed by an external diagnostic laboratory. WB and RT-PCR assays were performed in-house on plasma and cloacal swabs to detect antibody and PaBV RNA, respectively. All these birds tested negative for both PaBV RNA and antibodies.

The birds were divided into three groups:

Group 1A consisted of 9 birds. Each bird was vaccinated with a single priming dose, followed by two booster doses (three weeks apart) of inactivated whole virus vaccine administered IM, 0.1 ml/bird. Inocula contained 6.5 x 10^5 FFU of PaBV-2. Based on western blot results, this group was subsequently given a dose of recombinant N- protein vaccine intramuscularly (25 µg/ bird) one month after the second booster dose of inactivated vaccine. Western blots were used to detect the presence of anti-PaBV antibodies in the birds in this group. The WB antigen consisted of recombinant N protein. Serum from a PaBV-free cockatiel was used as a negative control.

All birds in this group were challenged with PaBV (4 x 10^4 FFU/ bird) one month after receiving the recombinant N protein vaccine.

Group 1B consisted of 9 birds. These birds received no vaccine. They were challenged on the same day as birds in Group 1A (0.1 ml/bird containing 4 x 10^4 FFU/ bird of PaBV-2). This group was used to determine the virulence of the challenge virus as a control.

Group 1C consisted of 8 birds. These birds were challenged on the same day as birds in groups 1A and 1B. However, these birds were additionally given one dose of recombinant N protein vaccine 30 DPC.

Birds in all groups were monitored for 200 DPC, at which time all remaining birds were euthanized.

Recombinant N protein vaccine study

For this, study we used twenty male and female cockatiels. All tested negative for PaBV (RNA and antibodies), herpesvirus and chlamydia (as described above) and were divided into two equal groups.

Group 2A birds were vaccinated with one prime and one booster dose (21 days apart) of recombinant N protein vaccine (25 µg/ bird). One month later birds were challenged with PaBV-2 (4 x 10^4 FFU/ bird) administered IM.

Group 2B birds received no vaccine, but were challenged with virus as described above for group 2A. All birds were monitored for 200 days post challenge.

Cyclosporine A experiment

Sixteen adult male and female cockatiels were divided into two equal groups:

Group 3A birds were given a dose (0.2 mg/bird) of cyclosporine A, suspended in sesame oil via the oral route daily for 70 days (study period). These birds were challenged with PaBV-2 given by IM route 24 hours after the cyclosporine A treatment began. The dose of virus used was 8×10^5 FFU/ bird.

Group 3B birds were given sesame oil throughout the 70 days study period as a control for the stress of daily handling Group 3A birds. Group 3B birds were challenged as described above for group 3A. A higher challenge dose of virus was used in this study due to the shortened observation period.

Differential counts of white blood cells (WBC): All the birds in the cyclosporine A study were bled via jugular venipuncture (~0.05 ml) and approximately three blood smears were prepared without anticoagulant. The blood smears were rapidly air dried and then fixed with methanol. Slides were submitted to the clinical pathology laboratory at Texas A&M university, College of Veterinary Medicine for Modified Wright's staining prior to differential leucocyte counts.

Vaccine safety trial

Fifty adult male and female cockatiels tested negative for the presence of anti PaBV antibodies using WB assays before starting this experiment. The majority of these birds had shed PaBV at least once during the last few years, as indicated by results of PCR on cloacal swabs. Each bird was given two doses of recombinant N protein vaccine, 26 days apart, administered by the IM route and tested for anti-PaBV antibodies after the second dose. The birds were monitored daily for six months for any adverse reaction that might have occurred due to the vaccine.

Pathology

The vaccine experiments continued for 200 DPC and the cyclosporine A experiment for 70 DPC. During this time, birds were visually monitored on a daily basis. Initial weights were taken 2-3 DPC and every 7-14 days thereafter. Any bird that lost more than 20% of its original weight in addition to any PDD - related clinical signs was euthanized. Complete necropsies were performed on each bird whether euthanized or died for other reasons. Tissue samples collected included:

Brain, eye, optic nerve, spinal cord, peripheral nerves, lung, heart, liver, spleen, pancreas, adrenal gland, kidney, gonad, crop, proventriculus, ventriculus, intestine, cloaca, skin and a portion of the pectoral muscle. Tissues samples were placed in 10% buffered formalin and embedded in paraffin for histopathologic examination. Hematoxylin and eosin stains were used to stain 4 µm tissues sections. Bright field microscopy was used to examine the stained slides.

Histopathology examination was done blindly by an expert pathologist in the California Animal Health & Food Safety Laboratory System (H. Shivaprasad) or at the Texas A&M Veterinary Medical Diagnostic Laboratory.

No cockatiels in the safety trial died or were euthanized for any reason, through the observation period.

Statistical Analysis

One-way analysis of variance (ANOVA) with the subsequent Tukey's HSD (honest significant difference) in addition to Unpaired and Paired *t* tests were performed using GraphPad Prism software version 7.02 to analyze differences between groups in respect to mean body weight and for quantification of viral load in organs and cloacal swabs as well as relative semi-quantification analysis of ImageJ software results of WB assays. $p<0.05$ was considered significant differences.

CHAPTER III

RESULTS

Pilot Experiment

This pilot experiment was conducted to answer the following questions:

- Is it possible to demonstrate an immune response in birds vaccinated with a cell-associated, inactivated whole virus vaccine?
- Is this response protective against virus challenge?
- Does the response exacerbate disease in birds in which protection is incomplete?
- Does the response exacerbate disease in previously naïve birds?
- Does the response exacerbate disease in previously infected (healthy) birds?

Three groups of PaBV-negative cockatiels (seronegative and negative for PaBV shedding) were used. Group 1A consisted of 9 birds vaccinated with 3 doses (one prime and two boosters) of formalin inactivated PaBV-infected duck embryo fibroblasts (DEF).

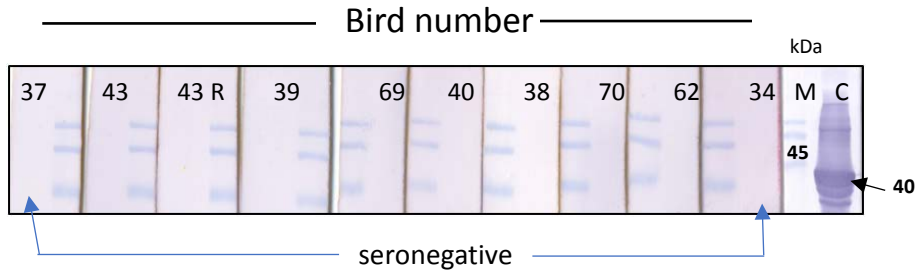
Group 1A (vaccinated group) birds were tested for antibodies against PaBV N protein 3 weeks after each booster dose of inactivated vaccine and all were negative by western blot assay (Fig. 2).

As we were unable to detect an immunologic response to the inactivated vaccine, Group 1A birds were subsequently inoculated with a single dose of recombinant N protein vaccine.

The rationale for changing the pilot experimental design was that we did not wish to challenge birds in which we were unable to measure an immune response. In response to the single dose of recombinant N protein vaccine, 7 of 9 birds in Group 1A seroconverted (were western blot-positive for anti PaBV N antibodies) by 3 weeks (Fig. 2), thus the experiment was continued and the questions were revised:

- Is it possible to demonstrate an immune response in birds vaccinated with a cell-associated, inactivated whole virus vaccine followed by a dose of recombinant N protein vaccine?
- Is this response protective against virus challenge?
- Does the response exacerbate disease in birds in which protection is incomplete?
- Does the response exacerbate disease in previously naïve birds?
- Does a dose of recombinant N protein vaccine exacerbate or protect previously infected birds?

A



B

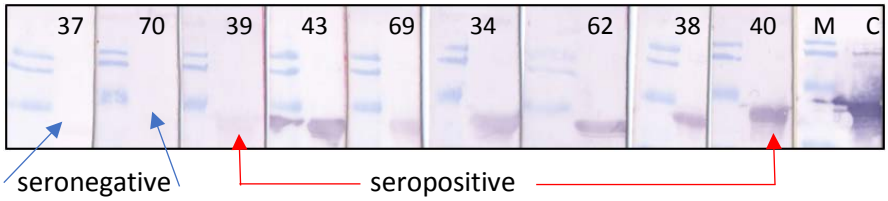


Figure 2. Western blot assay for the pilot study vaccine group. (A) Vaccine group after the second booster dose of inactivated whole virus vaccine. (B) Vaccine group after one dose of recombinant N protein vaccine. M= size marker. C = positive control serum. The black arrow denotes the location of PaBV- N protein. R= test repeated.

Experimental infection, clinical observations and pathology

To test for vaccine efficacy, Groups 1A, 1B and 1C were challenged, on the same day with 4 x 10^4 FFU of PaBV-2 by the intramuscular route. All birds were observed daily and all were weighed at regular intervals. The birds in Group 1A survived, and were apparently healthy through 200 days post challenge (DPC) (Fig. 3). In contrast, many of the birds in Group 1B and 1C, lost weight and sickened, and died. In Group 1B, 7 of 9 birds died or were euthanized before 200 DPC due to PDD- related poor health (Table 1). In Group 1C (vaccinated- post challenge group), 5 of 8 birds also sickened and died or were euthanized before 200 DPC due to PDD- related poor health.

Body weights of all birds were recorded 2-3 DPC and then every 10-14 days through 200 DPC. Average group body weights at each time point are shown in Fig. 4. There was variability within each group, however the overall trend was that birds in Groups 1B and 1C initially lost weight, but that the long-term survivors in these two groups had weights comparable to those of the vaccinated birds by the end of the observation period. The average weights at all time points were then used to determine if there was an overall difference between vaccinated and the control groups. This was accomplished using Tukey's HSD (Fig. 5). The difference in overall average weight over the whole experiment, between vaccinated (1A) and the control group (1B) was significant at $p<0.05$ (Fig. 5). There was no significant difference between Group 1C and the other two groups.

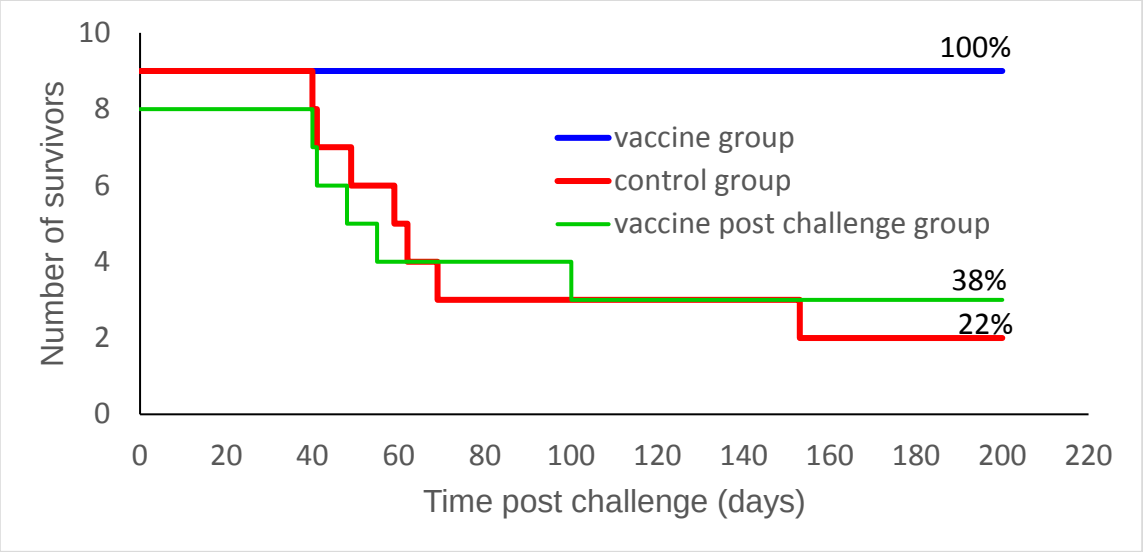


Figure 3. Survival curves for pilot study. Percent survival was calculated for each group.

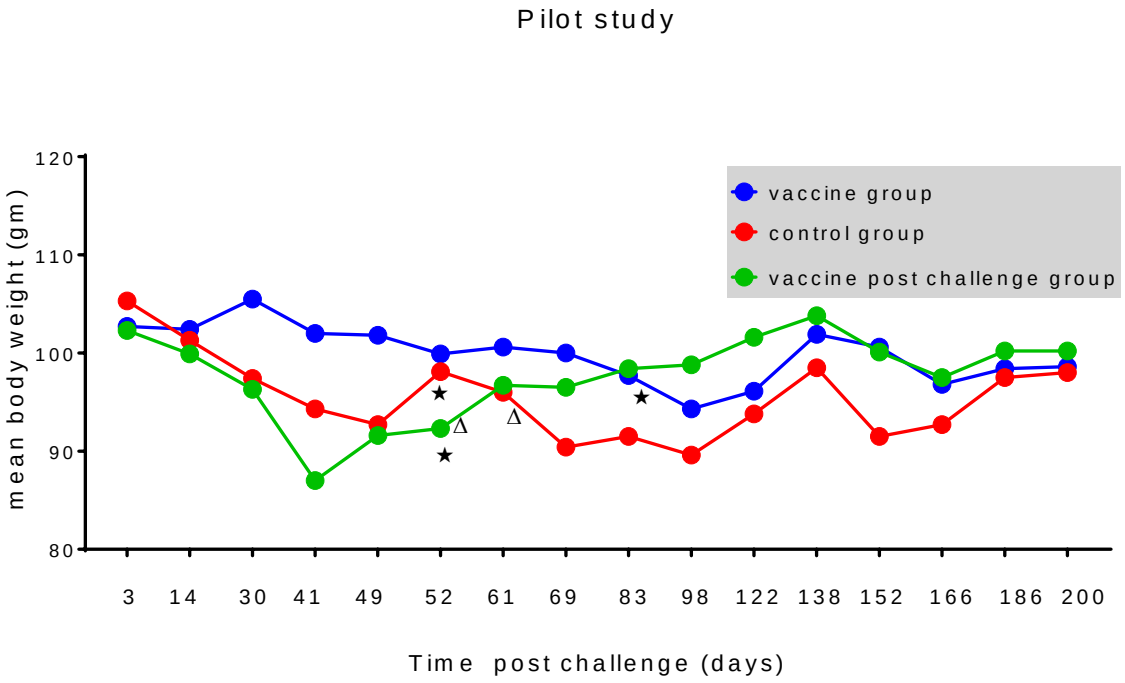


Figure 4. Mean body weight of pilot study birds. ★ marks viral shedding in ≥ 50 % of the birds. Δ marks mortality in ≥ 50% of the birds.

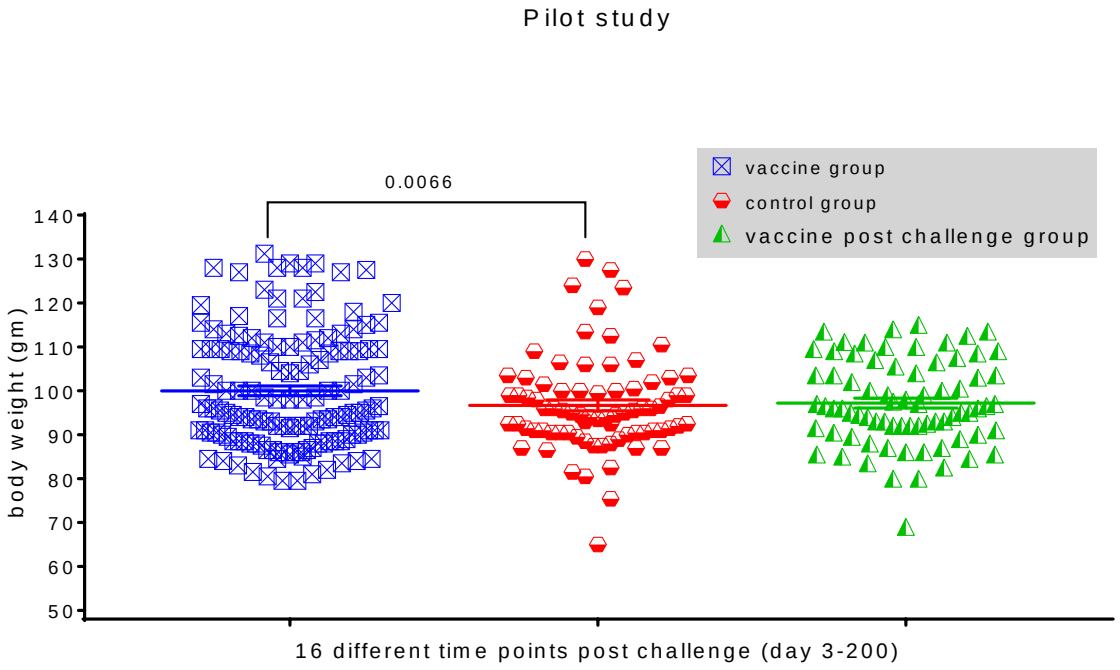


Figure 5. Scatterplot of body weights for pilot study. Mean ± SE. $P = 0.0066$

Table 1. Macroscopic & Microscopic PDD-related lesions from pilot study.

Group	Bird #	Macroscopic	Microscopic	Time point of death/euthanasia (DPC)
1A	62	Neg.	Neg.	200
	70	Neg.	Neg.	200
	38	Pos.	Pos.	200
	40	Neg.	Neg.	200
	39	Neg.	Neg.	200
	69	Neg.	Neg.	200
	34	Neg.	Neg.	200
	43	Neg.	Neg.	200
	37	Neg.	Neg.	200
1B	48	Pos.	Pos.	40
	60	Neg.	Pos.	41
	57	Pos.	Pos.	49
	50	Pos.	Pos.	59
	45	Pos.	Pos.	62
	64	Pos.	Pos.	69
	68	Pos.	Pos.	153
	65	Pos.	Pos.	200
	61	Neg.	Pos.	200
1C	44	Pos.	Pos.	40
	54	Neg.	Pos.	41
	63	Pos.	Pos.	48
	59	Pos.	Pos.	55
	51	Pos.	Pos.	100
	58	Neg.	Neg.	200
	36	Pos.	Pos.	200
	49	Neg.	Neg.	200

Group 1A: vaccinated, Group 1B: unvaccinated control, Group 1C: vaccinated post challenge. Macroscopic lesion= Included not limited to enlarged dilated proventriculus. Microscopic lesion= marked lymphoplasmacytic ganglioneuritis and encephalitis or encephalomyelitis. Pos. = positive; Neg. = negative. DPC= days post challenge.

During the study period, all the vaccinated birds (Group 1A) were clinically healthy; none displayed any PDD- related clinical signs. Moreover, most of these birds showed no gross lesions characteristic of PDD upon necropsy (Table 1) with the exception of bird #38 that had a mildly dilated proventriculus. In contrast to the vaccinated group, birds in Group 1B (unvaccinated control) and 1C (vaccinated post-challenge) showed gastrointestinal clinical signs of PDD, in particular, apathy, emaciation and undigested seeds in the feces. Necropsy of these birds revealed the presence of typical lesions of PDD which included a dilated proventriculus. The exceptions were two birds from Group 1B (# 60 and 61) and three birds from Group 1C (#54,58 and 49).

In Group 1B, 7of 9 birds died or were euthanized due to PDD prior to 200 DPC. One of the two surviving birds also had signs of infection and developing PDD upon necropsy. In the vaccinated post-challenge group, 5of 8 cockatiels died or euthanized due to PDD. Of the 5 birds in groups 1B and 1C that survived the challenge, two had a dilated proventriculus and three others had flaccid hearts, enlarged crops and/or severe enteritis when necropsied at 200 DPC.

Histopathological examination of birds from Group 1A (vaccinated group) revealed only 1 of 9 with lesions (lymphoplasmacytic ganglioneuritis and encephalitis) suggestive of PDD (Table 1). Bird #38 had mild to moderate perivascular cuffing and increased glial cells in brain, with a few scattered lymphoid nodules in and around the serosa and isolated ganglia of the proventriculus, gizzard, and intestine.

The heart had focal infiltration of lymphocytes around isolated epicardial ganglia. In contrast, all birds from the control group (1B) and 6 birds from Group 1C (vaccinated post challenge) had lesions typical of PDD, ranging from mild to severe multifocal perivascular cuffing and gliosis randomly scattered throughout brain. In addition, infiltration of lymphocytes mixed with a few plasma cells in the subserosal layers of the myenteric plexus in the proventriculus and gizzard was noted along with formation of lymphoid nodules.

As summarized in Table 1, all birds in Group 1B displayed either macroscopic or microscopic lesions, thus morbidity was 100%. Mortality by 200 DPC was 78% in this group. In Group 1C, morbidity was 75% and mortality by 200 DPC was 62.5%. This is in contrast to the vaccinated birds (Group 1A) in which morbidity was 11% and mortality was 0%.

Detection of PaBV-RNA in cloacal swabs and organs

qRT-PCR was used to detect and quantify PaBV-RNA (P gene mRNA) in cloacal swabs (Table 2) and several organs (Table 3). Most birds shed viral RNA at least once during the study period. Exceptions included one bird (# 45) from Group 1B and two birds (#44 and #59) from Group 1C. Those birds died or were euthanized relatively early, before 63 DPC. Within these two groups there was no correlation between shedding and survival as non-shedders died at 62, 40 and 55 DPC while, all clinically healthy birds in group 1A shed virus by day 84. Of the 5 survivors from groups 1B and 1C, all shed virus at one or more time points. Each bird was tested for the presence of PaBV- RNA in seven organs (brain, crop, proventriculus, ventriculus, heart, kidney and adrenal gland). The results are summarized in Table. 3. At least one organ from each bird was positive for PaBV-RNA except one bird (#65) from Group 1B (survived the study period of 200 DPC) in which all the examined tissue samples were negative. In contrast, although all birds in Group 1A (vaccinated) survived for 200 DPC (and 8 of 9 had no gross lesions) all had high levels of PaBV-RNA in all tested organs (Table. 3). With the exception of bird #65, the other 5 surviving birds from groups 1B and 1C had high levels of PaBV RNA in their organs. Tukey's HSD revealed significant differences at $p<0.05$ for the viral RNA load in the ventriculus between the vaccinated group and both the control and vaccinated post challenge groups and for the kidney, between vaccinated group and control group (Fig. 6). However, the results were not significant at $p<0.05$ for the vaccinated birds in comparison with other 2 groups when only the survivors of each group were compared.

Table 2. Results of qRT-PCR in cloacal swabs from pilot study.

Group	Bird #	Time point of cloacal swabs (DPC)				Time point of death/euthanasia (DPC)
		52	84	125	200	
1A	62	++	++++	++++	++++	200
	70	Neg.	+++	++++	++++	200
	38	++	++++	+++	+++	200
	40	+	++	++++	++++	200
	39	Neg.	+++	+++	++++	200
	69	Neg.	+	+++	++++	200
	34	Neg.	++++	++++	+++	200
	43	Neg.	+++	+++	+++	200
	37	+	+++	+++	+++	200
1B	48	++a				40
	60	++a				41
	57	+++a				49
	50	+	+++a			59
	45	Neg.	Neg.a			62
	64	+	Neg.a			69
	68	Neg.	+	+	+a	153
	65	Neg.	Neg.	+	Neg.	200
	61	Neg.	+	+	+++	200
1C	44	Neg.a				40
	54	+++a				41
	63	+++a				48
	59	Neg.	Neg.a			55
	51	Neg.	++	+++a		100
	58	++	+++	+++	+++	200
	36	++	++++	+++	+++	200
	49	Neg.	+++	++++	+++	200

Group 1A: vaccinated, Group 1B: unvaccinated control, Group 1C: vaccinated post challenge.
Scoring in term of threshold cycle values as follows: Neg., > 35; +, 35 to 30; ++ 30 to 25; +++, 25 to 20; ++++, 20 to 15; +++++, 15 to 10.
Neg.= negative. DPC, days post challenge. a= at death/euthanasia.

Table 3. Results of qRT-PCR and IHC in organs from pilot study.

Group	Bird #	Tissue							Time point of death/eutha-nasia (DPC)
		Brain	Crop	Provent-riculus	Ventric-ulus	Heart	kidney	Adrenal gland	
1A	62	++++s	+++++mo	+++++s	+++++s	++++nt	+++++nt	++++nt	200
	70	+++++s	+++++mo	+++++s	+++++s	++++nt	+++++s	++++nt	200
	38	++++s	+++++s	+++++s	+++++s	++++ng	+++++ng	+++++nt	200
	40	+++++s	+++++mo	+++++s	++++s	++++mo	+++++nt	++++nt	200
	39	+++++s	+++++mo	+++++s	+++++s	+++++s	++++s	++++nt	200
	69	++++s	++++mo	+++++mo	+++++mo	++++mi	++++nt	++++nt	200
	34	++++mo	++++nt	+++++mo	++++mo	++++s	+++++mi	++++nt	200
	43	+++++s	++++mo	+++++s	++++s	++++nt	+++++nt	++++nt	200
	37	+++++mo	+++++mo	+++++mo	++++mo	+++++mi	+++++mi	++++ng	200

		Brain	Crop	Provent-riculus	Ventric-ulus	Heart	Kidney	Adrenal gland	
1B	48	++mi	++mi	+++mi	+++mi	+++ng	++ng	+++nt	40
	60	+++s	+++mi	+++mo	+++mo	Neg. mo	+++nt	++++mo	41
	57	+++s	+++mi	++++mo	++++mo	+nt	++ng	++++ng	49
	50	+++++mo	+++mo	+++mo	+++mo	+++nt	+++ng	++++mi	59
	45	++ng	Neg.ng	Neg. ng	Neg. ng	++ng	Neg. nt	++nt	62
	64	+++mo	++ng	Neg. ng	++ ng	Neg. nt	++ nt	+++ nt	69
	68	++++mi	++ ng	++ ng	+++ ng	++ ng	+++ ng	+++ mi	153
	65	Neg. ng	Neg. ng	Neg. ng	Neg. ng	Neg. ng	Neg. nt	Neg. nt	200
	61	++++s	++++mo	++++s	++++s	++ nt	++++ nt	++++ nt	200

		Brain	Crop	Provent-riculus	Ventric-ulus	Heart	Kidney	Adrenal gland	
1C	44	++ ng	+++ ng	+++ ng	++ ng	Neg. ng	Neg. nt	Neg. nt	40
	54	+++s	+++ nt	+++s	+++s	Neg. nt	Neg. ng	+++ mi	41
	63	++++ mi	++ ng	++++ ng	+++ ng	Neg. nt	Neg. ng	++++ mi	48
	59	++++ mo	++ ng	Neg. ng	Neg. ng	Neg. ng	Neg. ng	+++ nt	55
	51	+++++mo	++++ ng	+++ mi	+++ mi	++ ng	+++ ng	+++ mo	100
	58	+++++ s	+++++mo	+++++ s	+++++ s	++++ mo	++++mo	+++++nt	200
	36	++++mo	++++mo	+++++mo	+++++mo	+++++nt	+++++nt	++++nt	200
	49	+++s	++++mo	+++++s	+++++s	++++nt	++++mo	+++++nt	200

Group 1A: vaccinated, Group 1B: unvaccinated control, Group 1C: vaccinated post challenge. Scoring in term of threshold cycle values as follows: Neg., > 35; +, 35 to 30; ++ 30 to 25; +++, 25 to 20; ++++, 20 to 15; +++++, 15 to 10.
Neg. = negative. IHC= immunohistochemical staining: ng = negative; mi = mild; mo = moderate; s = severe; nt = not tested. DPC, days post challenge.

Immunohistochemistry

As summarized in Table 3, PaBV-N antigen was detected by immunohistochemistry (IHC) in at least one of the following organs: Brain, crop, proventriculus, ventriculus, heart, kidney and adrenal gland from all the birds except two from Group 1B (#65 and 45) and one from Group 1C (#44), that were not tested for the presence of viral antigen in kidney and adrenal. Note that bird #65 was also negative for PaBV-RNA by PCR. Additional tissues from Group 1A birds were also examined. These included intestine, lung, liver, gonad, skin, cloaca, eye, peripheral nerves and skeletal muscle. One or more than one of these tissues were positive from each bird revealing, the widespread presence of viral antigen, *in the absence of lesions*. Some of these tissues were also positive in birds from Group 1B and 1C, in particular those that survived the challenge, (however not all birds were tested for these tissues). IHC staining for PaBV-N antigen was scored as mild, moderate or severe for each organ (Table 3). All vaccinated birds demonstrated mild to strong reactions for PaBV-N antigen in several organs. Severe IHC results were scored for the brain, proventriculus, and ventriculus in 6 of 9 vaccinated birds, despite the fact that they were negative for gross and/or histopathological lesions typical of PDD. The exception was bird #38. Consistent with the PCR results, large amounts of antigen were present in 44 of 47 PCR positive samples for Group 1A birds.

A

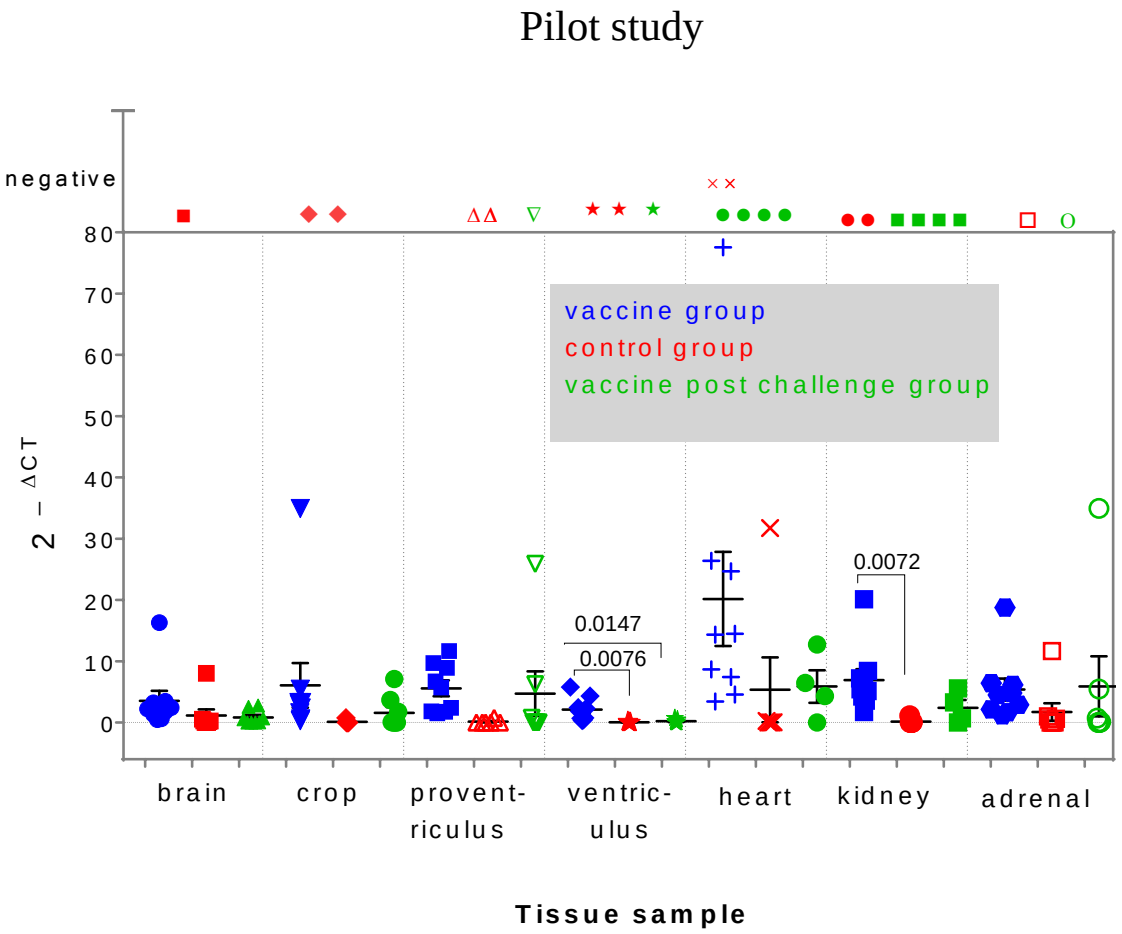


Figure 6. Results of qRT-PCR in organs from pilot study. A: Scatterplot of delta Ct values; horizontal lines represent the mean ± SE. for each organ. B: Fold changes. Differences were considered significant when $P < 0.05$ by ANOVA and Tukey's HSD.

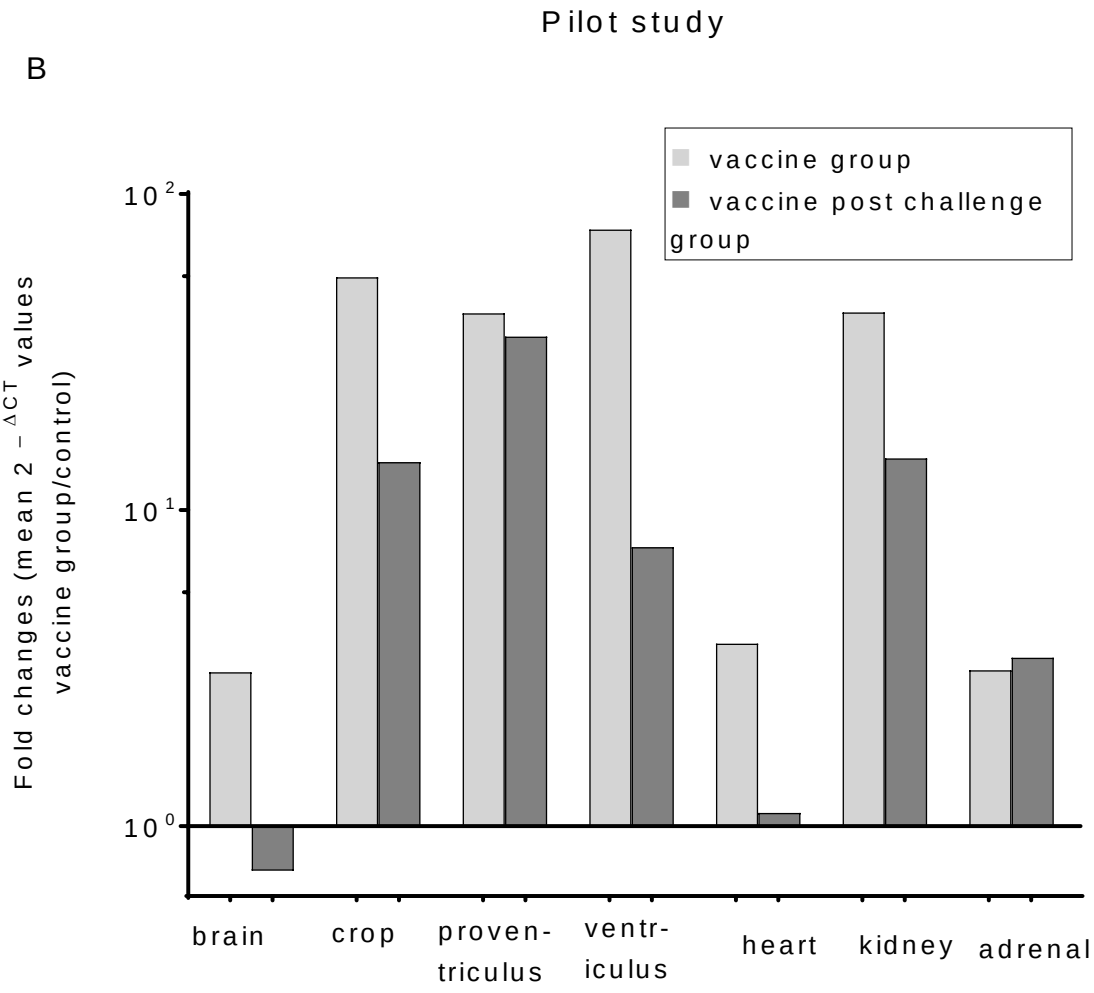


Figure 6. Continued.

Detection of antibodies against PaBV

All of the vaccinated birds (Group 1A) had developed antibodies to PaBV N by the end of the experiment, as indicated by western blots using sera collected at necropsy. All of the vaccinated post challenge birds (Group 1C), both those that were euthanized due to PDD prior to 200 DPC and those that survived for 200 DPC were seropositive (Fig. 7). Among 7 tested Group 1B birds, all were also positive for antibodies to PaBV N. Therefore, in this experiment, development of an anti-N antibody response prior to challenge did not protect Group 1A birds from infection.

ImageJ software was used to analysis relative WB band density and the results were not significant at $p<0.05$ (ANOVA and Unpaired t tests) either between groups or for the birds which survived and were euthanized 200 DPC versus those euthanized before 200 DPC due to PDD (the control and vaccinated post challenge groups) (Fig. 8).
A paired *t* test also revealed that the level of antibodies to PaBV N 3 weeks after the recombinant N protein vaccine were not significantly different (at $p<0.05$) than levels at 200 DPC (Fig. 9) at the group level. However, it is interesting to note that 3 birds in the vaccinated group (#40, 43 and 34) had reduced levels of anti-N antibody compared to their pre-challenge level.

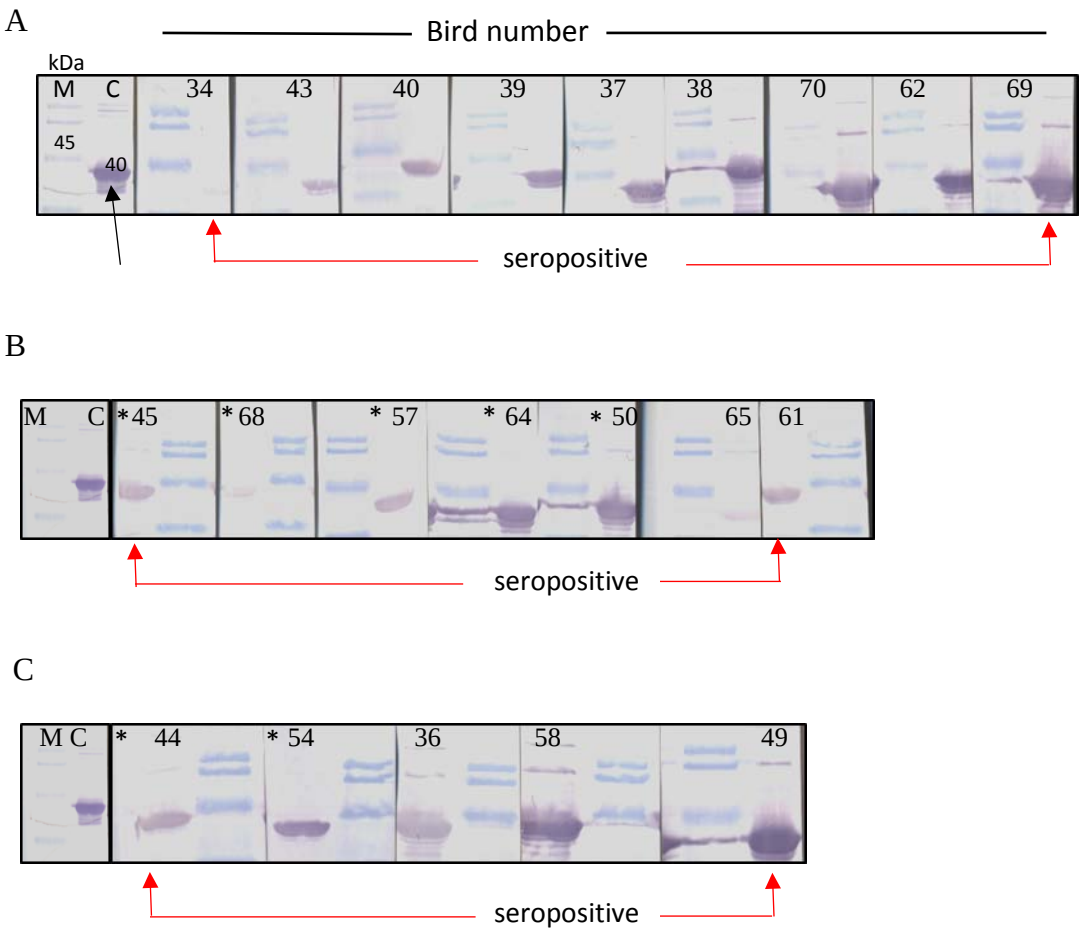


Figure 7. Western blot assay for the pilot study 200 DPC. (A) Vaccine group. (B) Control group. (C) Vaccine post challenge group. M= size marker. C = positive control serum. Black arrow = denotes the location of PaBV- N protein.
*= euthanized before the end of the experiment.

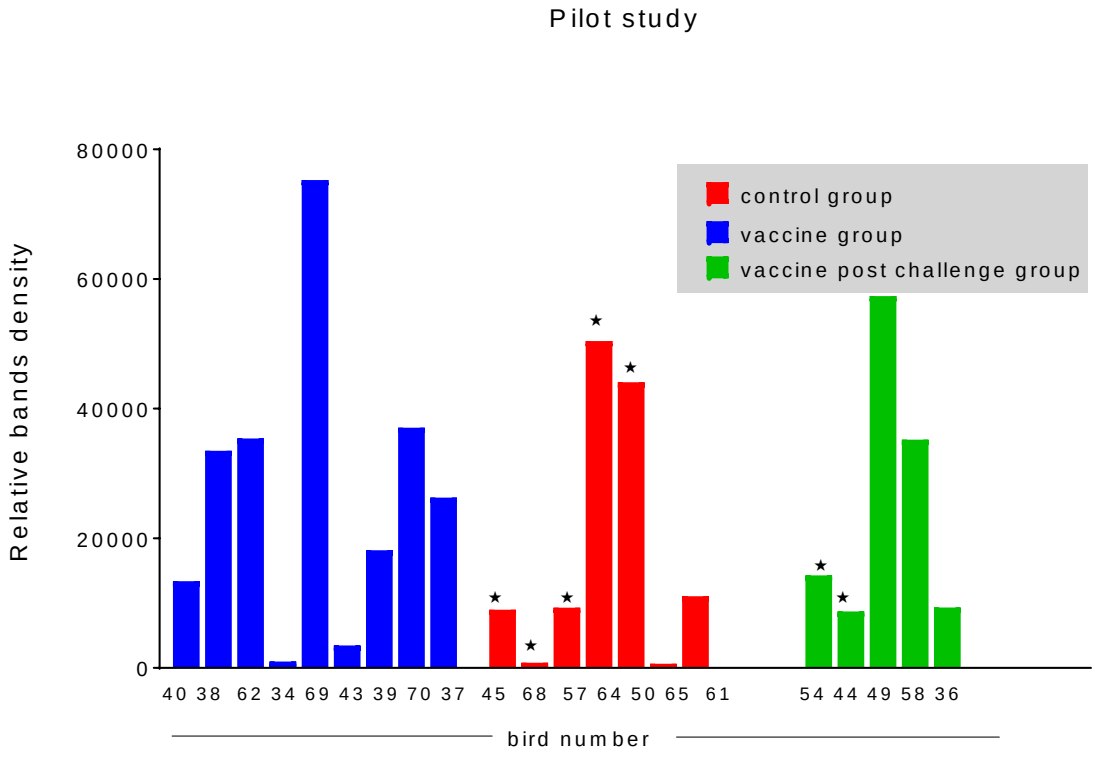


Figure 8. Semi quantification analysis of WB assays for the pilot study using ImageJ software. ★= euthanized before the end of the experiment.

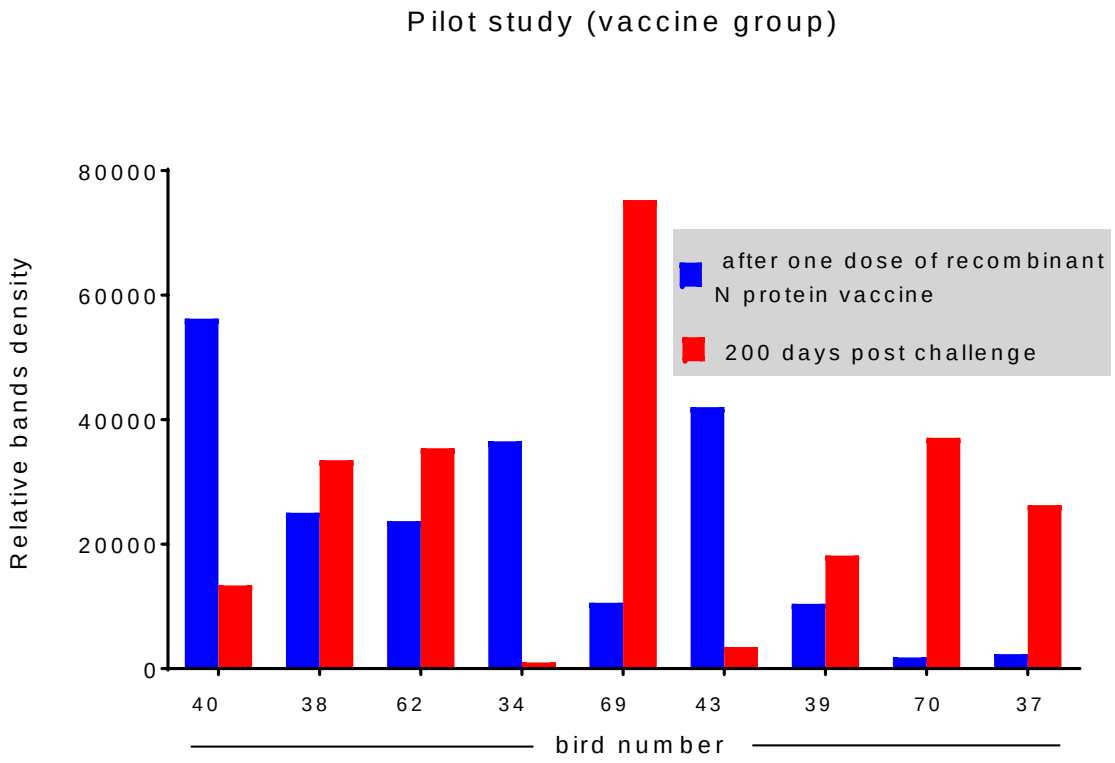


Figure 9. Semi-quantification analysis of WB assay for pilot study vaccinated group using ImageJ software.

Recombinant N Protein Vaccine

Based on results of the pilot experiment we next sought to determine if protection from disease (Group 1A birds in the pilot experiment) could be obtained using a recombinant N vaccine alone. This experiment used two groups: 2A, vaccinated (10 birds) and 2B, unvaccinated-control (10 birds). All the birds were seronegative by WB before starting this experiment. All tested negative for PaBV-RNA fecal shedding by PCR. Three weeks after receiving the first dose of recombinant N vaccine (25 µg per bird intramuscularly), birds were assayed for seroconversion and 4 of 10 were positive by WB (Fig. 10). A second dose (booster dose) of this vaccine was given to all birds in the N-vaccine group and they were tested after another 3 weeks. At this time, all birds were seropositive by WB against the recombinant N protein (Fig. 10).

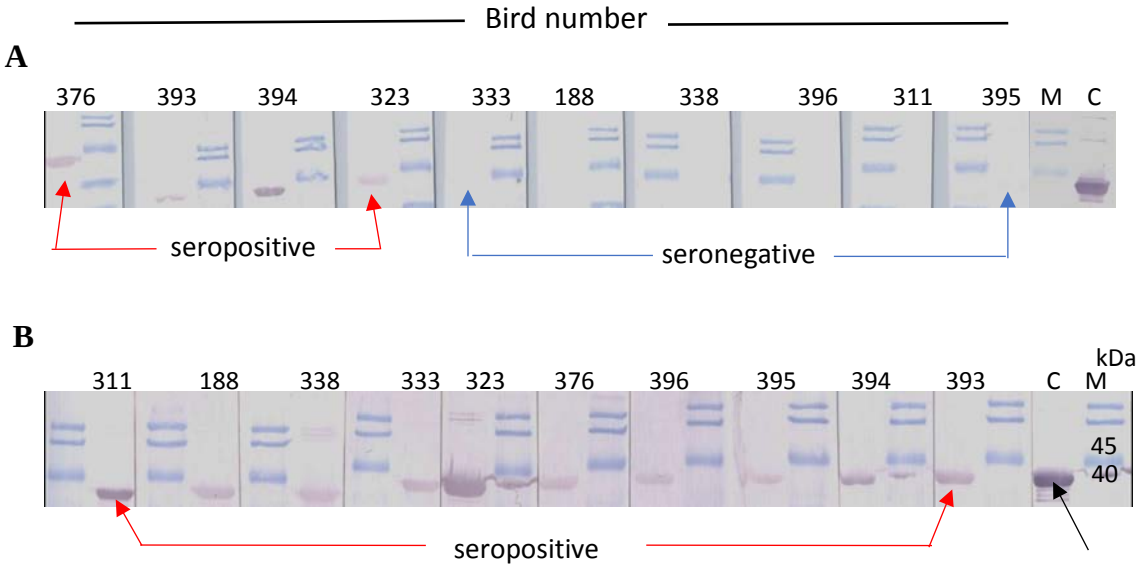


Figure 10. WB assay for vaccine experiment. (A) Vaccine group after first dose of recombinant N protein vaccine. (B) Vaccine group after second dose of recombinant N protein vaccine. M= size marker. C= positive control serum. Black arrow denotes the location of PaBV-N protein.

Experimental infection

Vaccinated and unvaccinated-control birds were challenged IM with 4 x 10^4 FFU of PaBV-2 and following challenge the birds were observed daily until the experiment was terminated at 200 DPC.

The first death occurred at 32 DPC (bird#341) in the unvaccinated control group. In contrast, none of the vaccinated birds died except bird #311 which died at 44 DPC (Fig. 11). Birds in the unvaccinated group lost a significant amount of weight (Fig. 12) and were in general less active than birds in the N-vaccine group. By 100 DPC, 3 more birds in the unvaccinated control group died. In all, 6 of 10 unvaccinated control birds died or were euthanized due to PDD before the end of the experiment, at 200 DPC. Within the vaccinated group the only death was at 44 DPC and the remainder of the birds remained clinically healthy. Mean body weights of all birds were recorded at the beginning of the challenge and every 10-14 days until 200 DPC. Unpaired *t* tests revealed that weight differences were significant (p=0.0009) between the vaccinated and the control group (Fig13).

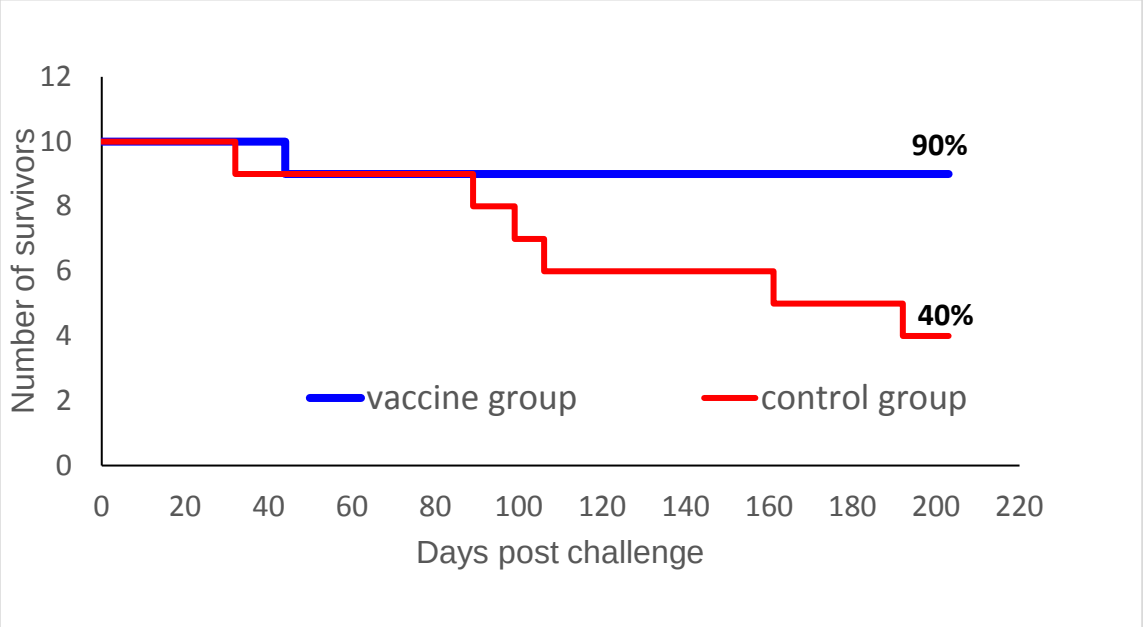


Figure 11. Survival curves for vaccine experiment. Percent survival was calculated for each group.

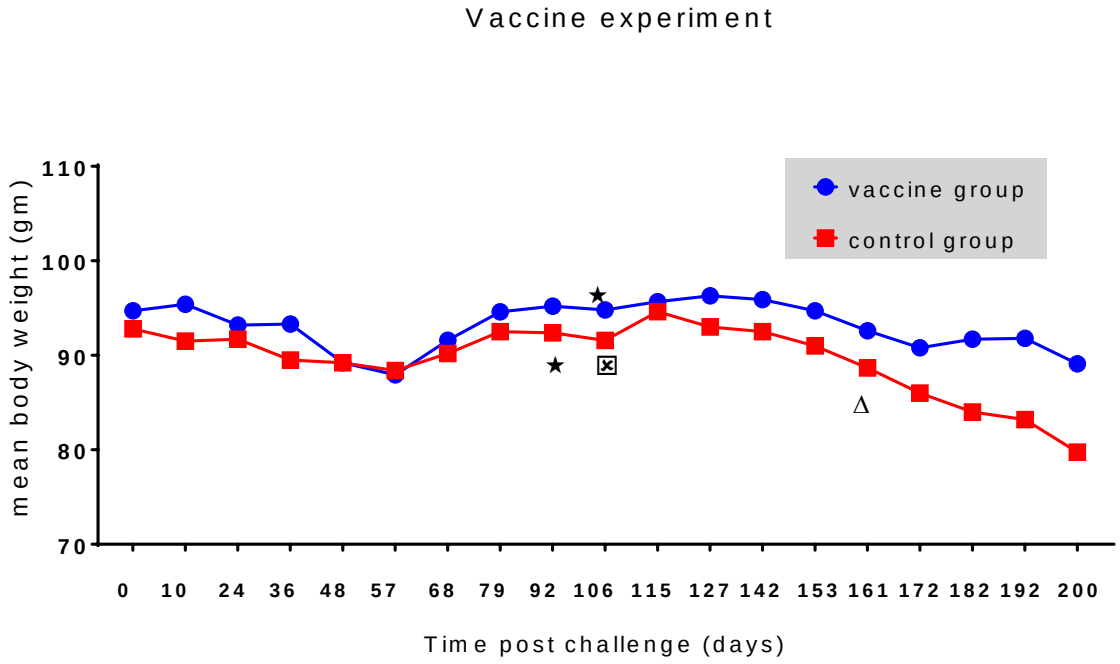


Figure 12. Mean body weight of vaccine experiment. ★ marks viral shedding in ≥ 50% of the birds. ☒ marks mortality in ≥ 40 % of the birds. Δ marks mortality in ≥ 50% of the birds.

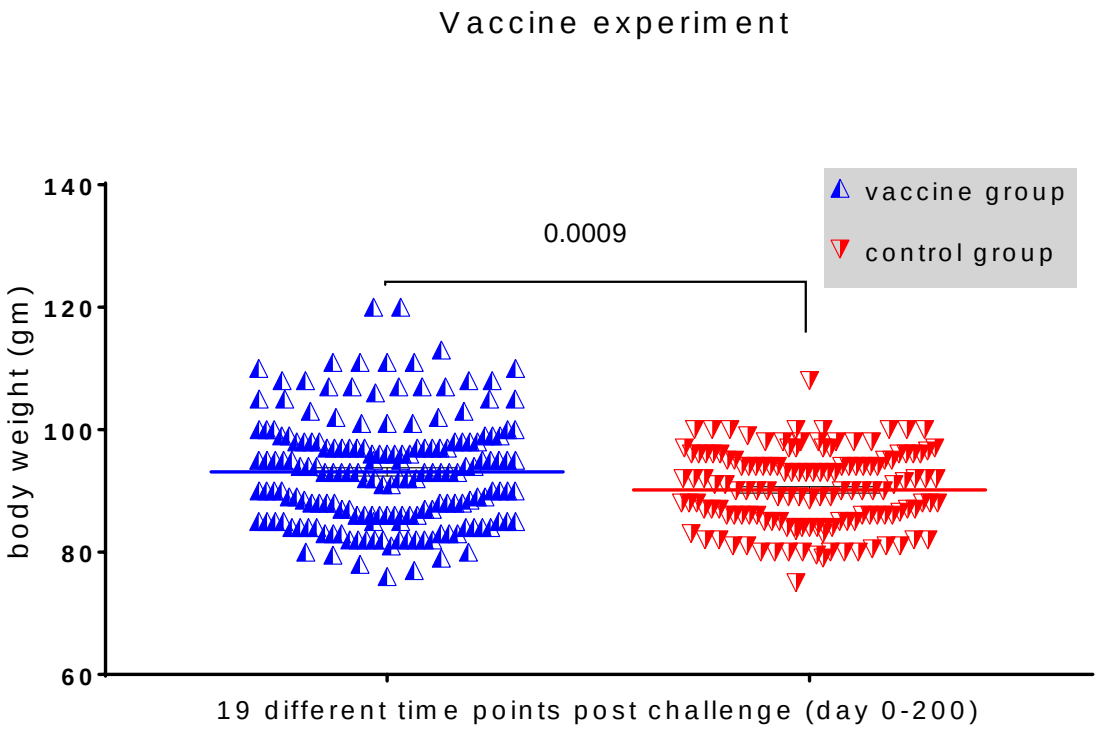


Figure 13. Scatterplot of individual body weights for vaccine study. Mean ± SE. *P* = 0.0009

Clinical observations and pathology

During the 200 day observation period, all vaccinated birds were clinically healthy except bird #311 (died at 44 DPC). This bird was emaciated and undigested seeds were found in its feces, thus PDD was-suspected. Indeed, necropsy revealed a mildly dilated proventriculus. All remaining vaccinated birds were necropsied at day 200 PC (Table 4). At this time, one bird (#333) had a mildly dilated proventriculus. In contrast, birds in the unvaccinated group (2B) showed a variety of signs of PDD including apathy, weight loss and undigested seeds in the feces. Upon necropsy, all birds in this group (with the exception of #318) revealed typical lesions of PDD (Table 4) including a dilated proventriculus (Fig. 14). In summary, macroscopic analysis revealed 2 of 10 vaccinated birds with mild PDD like symptoms or lesions while 9 of 10 unvaccinated birds had gross lesions consistent with a diagnosis of PDD.

Histopathological examination (Table 4) of birds from the vaccinated group showed no lesions suggestive of PDD (lymphoplasmacytic ganglioneuritis and encephalitis) (Fig. 15) except birds #311 and 333. Examination of tissues from bird #311 showed infiltration of a few lymphocytes and occasional plasma cells in and around the subserosal nerves/ganglia of the proventriculus and gizzard. Bird #333 had limited (one section) focal perivascular cuffing in brain and crop; the proventriculus and gizzard had mild to moderate infiltration of lymphocytes and a few plasma cells in the serosal and subserosal ganglia. In contrast, in the unvaccinated group, 8 of 10 birds showed different degrees of PDD- related microscopic lesions.

In brain sections, there were scattered mild to severe multifocal perivascular cuffing and gliosis (Fig. 15). Subserosal layers of the myenteric plexus in the proventriculus (Fig. 16) and gizzard (Fig. 17) also showed infiltration of lymphocytes mixed with a few plasma cells and some formation of lymphoid nodules.

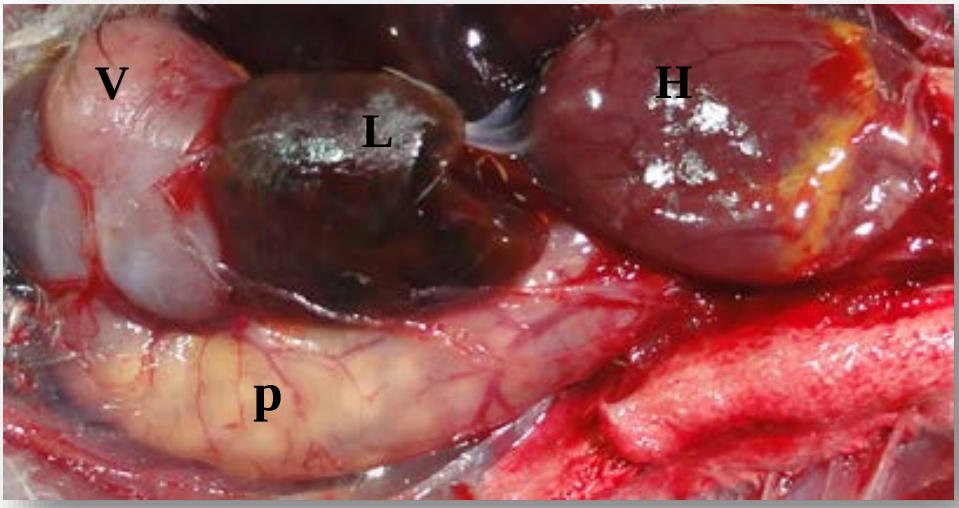


Figure 14. Proventriculus from control challenged bird. Severely dilated, thin proventriculus, indicative of PDD. P= proventriculus, V= ventriculus, H= heart, L= liver

Table 4. Macroscopic & Microscopic PDD-related lesions from vaccine experiment.

Group	Bird #	Macroscopic	Microscopic	Time point of death/euthanasia (DPC)
2A	311	Pos.	Pos.	44
	395	Neg.	Neg.	200
	396	Neg.	Neg.	200
	338	Neg.	Neg.	200
	188	Neg.	Neg.	200
	333	Pos.	Pos.	200
	323	Neg.	Neg.	200
	394	Neg.	Neg.	200
	393	Neg.	Neg.	200
	376	Neg.	Neg.	200
2B	341	Pos.	Neg.	32
	375	Pos.	Pos.	89
	336	Pos.	Pos.	99
	334	Pos.	Pos.	106
	317	Pos.	Pos.	161
	318	Neg.	Neg.	192
	339	Pos.	Pos.	200
	322	Pos.	Pos.	200
	335	Pos.	Pos.	200
	340	Pos.	Pos.	200

Group 2A: vaccinated, Group 2B: unvaccinated control.
Macroscopic lesion= Included not limited to enlarged dilated proventriculus. Microscopic lesion= marked lymphoplasmacytic ganglioneuritis and encephalitis or encephalomyelitis. Pos. = positive; Neg. = negative. DPC= days post challenge.

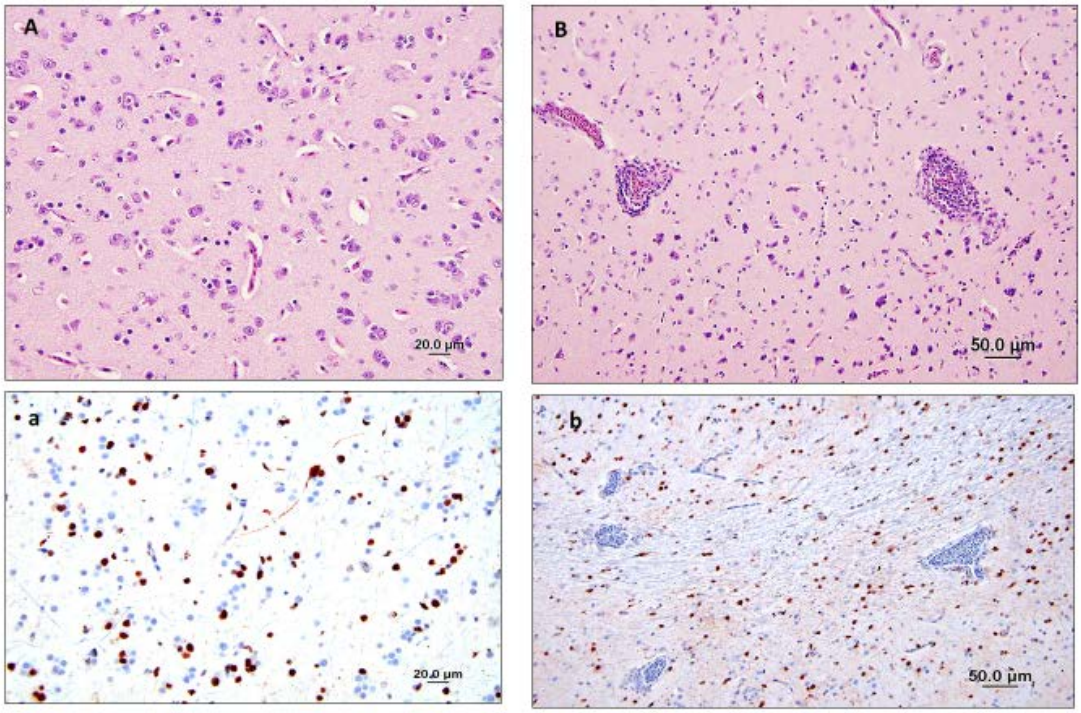


Figure 15. Histological and immunohistochemical staining findings in brains of vaccine experiment birds. A: Brain from vaccinated bird with no significant PDD- related lesions revealed by H&E staining. B: Brain from control bird with extensive perivascular mononuclear cuffing revealed by H&E staining. a: Positive IHC staining for PaBV antigen in brain from A. b: Positive IHC staining for PaBV antigen in brain from B.

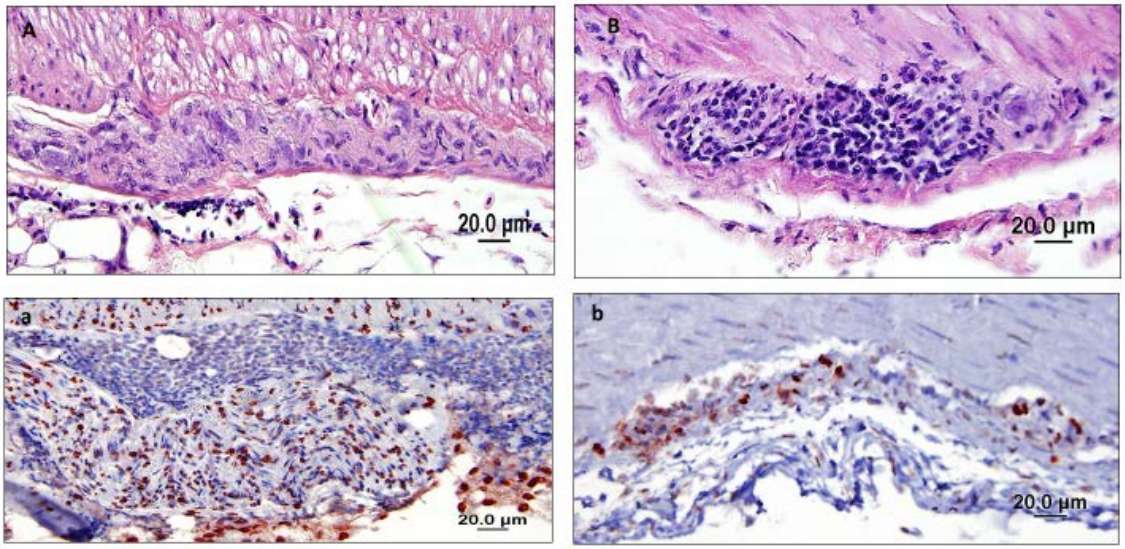


Figure 16. Histological and immunohistochemical staining findings in proventriculus of vaccine experiment birds. A: Proventriculus from vaccinated bird with no significant PDD- related lesions revealed by H&E staining. B: Proventriculus from control bird with myenteric ganglioneuritis revealed by H&E staining. a: Positive IHC staining for PaBV antigen in proventriculus from A. b: Positive IHC staining for PaBV antigen in proventriculus from B.

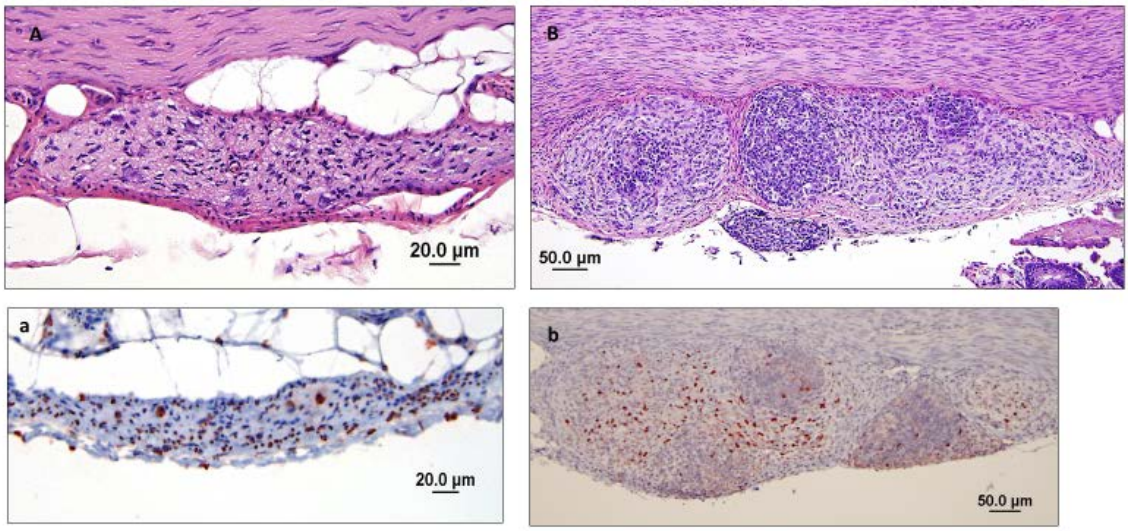


Figure 17. Histological and immunohistochemical staining findings in ventriculus of vaccine experiment birds. A: Ventriculus from vaccinated bird with no significant PDD-related lesion revealed by H&E staining. B: Ventriculus from control bird with myenteric ganglioneuritis and formation of lymphoid nodules revealed by H&E staining. a: Positive IHC staining for PaBV antigen in ventriculus from A. b: Positive IHC staining for PaBV antigen in ventriculus from B.

Detection of PaBV-RNA in cloacal swabs and organs

qRT-PCR was used to detect the presence of the PaBV-RNA in cloacal swabs (Table 5) and several organs (Table 6). Cloacal swabs were collected at 6 time points (Fig.18). Most of the birds shed the virus at least once during the study period of 200 DPC. Bird #341 from the unvaccinated group, who died, at 32DPC, was the only negative individual in the group. Bird #311 from the vaccinated group died at 44DPC before a fecal swab was collected. Bird #340 in the unvaccinated group was also negative throughout the 200 days of the study.

Birds were also tested for the presence of PaBV-RNA in tissue samples. Seven organs were tested (brain, crop, proventriculus, ventriculus, heart, kidney and adrenal gland). Among the unvaccinated group, no birds were entirely free of PaBV. However, #339 and #340, which survived the challenge, had only 1 positive organ each. Other birds in this group had high levels of viral RNA.

In the vaccinated group, #311 (died at 44 DPC) had a positive brain and ventriculus. Five additional birds were strongly PCR positive at 200 DPC. Bird # 395 had one organ positive, its brain. Three birds (#338, #394 and #393) were PCR negative for all examined tissues. A *t* test was performed and revealed significant differences at $p<0.05$ between vaccinated and control groups for crop and heart tissues (Fig. 19).
While no other tissues showed a significant difference between the two groups, the clear trend was that the vaccinated birds had higher levels of viral RNA in their tissues.

Immunohistochemistry

Six of 10 of vaccinated birds were IHC positive (moderate to severe) for PaBV-N antigen (Table 6) (Figs. 15-17), of which, 4 had severe levels of staining in the brain. Five of 6 positive birds showed no gross and/or histopathologically lesions normally attributed to PDD. A sixth bird, #333, had such lesions. Four birds in the vaccinated group (#311, #338, #394, #393) were IHC negative for all organs tested. In all birds except #311, PCR and IHC results were well correlated. In the unvaccinated control group (Fig. 15-17) birds #341 and #340 were IHC negative for all tissues tested; however, both birds were PCR positive in 1 or 2 tested organs.

Table 5. Results of qRT-PCR in cloacal swabs from vaccine experiment.

Group	Bird #	Time point of cloacal swabs (DPC)						Time point of death/euthanasia (DPC)
		49	98	106	153	183	200	
2A	311	nt. a						44
	395	Neg.	Neg.	Neg.	Neg.	++++	Neg.	200
	396	Neg.	++++	++++	++++	Neg.	+++	200
	338	Neg.	Neg.	Neg.	Neg.	Neg.	++	200
	188	Neg.	Neg.	Neg.	Neg.	Neg.	++	200
	333	Neg.	++	+	+++	+++	++	200
	323	Neg.	Neg.	+	++++	Neg.	+++	200
	394	Neg.	Neg.	Neg.	Neg.	++++	++	200
	393	Neg.	Neg.	+	Neg.	++++	Neg.	200
	376	++++	++++	++++	+++	Neg.	+++	200
2B	341	Neg. a						32
	375	Neg.	+ a					89
	336	+	++	++++a				99
	334	+	+++	+++	+++ a			106
	317	Neg.	++	+++	+++	+++ a		161
	318	Neg.	Neg.	Neg.	++	Neg.	+++ a	192
	339	Neg.	Neg.	+	Neg.	+++	Neg.	200
	322	Neg.	+	++	+++	++++	++++	200
	335	Neg.	++	++	+++	++++	+++	200
	340	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.	200

Group 2A: vaccinated, Group 2B: unvaccinated control.
Scoring in term of threshold cycle values as follows: Neg., > 35; +, 35 to 30; ++ 30 to 25; +++, 25 to 20; ++++, 20 to 15; +++++, 15 to 10. Neg.= negative. DPC, days post challenge. a= at death/euthanasia. nt= not tested.

Table 6. Results of qRT-PCR and IHC in organs from vaccine experiment.

Group	Bird #	Tissue							Time point of death/eutha-nasia (DPC)
		Brain	Crop	Proventriculus	Ventriculus	Heart	kidney	Adrenal gland	
2A	311	++ ng	Neg. nt	Neg. ng	++ ng	Neg. ng	nt	nt	44
	395	++++ mo	Neg. ng	Neg. ng	Neg. ng	Neg. nt	Neg. nt	Neg. nt	200
	396	+++ s	++++ mo	++++ mo	++++ mo	+++ nt	++++ nt	+++ nt	200
	338	**Neg. ng**	**Neg. ng**	**Neg. ng**	**Neg. ng**	**Neg. nt**	**Neg. nt**	**Neg. nt**	200
	188	++++ s	+++ s	+++ s	+++ s	+++ mi	+++ mi	++++ nt	200
	333	++++ s	++++ mo	++++ mo	++++ mo	+++ mi	++++ nt	++++ nt	200
	323	+++++mo	+++++ nt	+++++mo	++++mo	+++ nt	++++ nt	++++ nt	200
	394	**Neg. ng**	**Neg. ng**	**Neg. ng**	**Neg. ng**	**Neg. nt**	**Neg. nt**	**Neg. nt**	200
	393	**Neg. ng**	**Neg. ng**	**Neg. ng**	**Neg. ng**	**Neg. nt**	**Neg. nt**	**Neg. nt**	200
	376	+++++ s	++++ mo	++++ s	++++ s	+++ nt	++++ nt	+++ nt	200
2B	341	+++ ng	Neg. nt	++ ng	Neg. ng	Neg. nt	Neg. ng	+ nt	32
	375	+++++ s	++ mo	+++ mo	+++ mo	++ nt	+++ nt	+++ nt	89
	336	++++ s	++++ mo	++++mo	+++++mo	+++ mi	++++ nt	++++ nt	99
	334	+++++ s	++++ mo	++++ s	+++++ s	+++ nt	++++ nt	+++ nt	106
	317	+++++ s	++++ mo	++++ mo	++++ mo	++++ nt	++++ nt	++++ nt	161
	318	++++ s	++++ mi	++++ mi	++++ mi	++ nt	++++	++++ nt	192
	339	Neg. ng	Neg. ng	Neg. ng	Neg. ng	Neg. nt	mo	+ mi	200
	322	++++ s	+++ mi	++++ mo	+++ mo	++ nt	Neg. nt	++++ nt	200
	335	+++++ s	++++ mo	+++ mo	+++ mo	++ nt	+++ nt	++ mi	200
	340	++ ng	Neg. ng	Neg. ng	Neg. ng	Neg. nt	++++ nt Neg. nt	Neg. nt	200

Group 2A: vaccinated; three birds were negative. Group 2B: unvaccinated control.
Scoring in term of threshold cycle values as follows: Neg., > 35; +, 35 to 30; ++ 30 to 25; +++, 25 to 20; ++++, 20 to 15; +++++, 15 to 10.
Neg. = negative. IHC= immunohistochemical staining: ng = negative; mi = mild; mo = moderate; s = severe; nt = not tested. DPC, days post challenge.

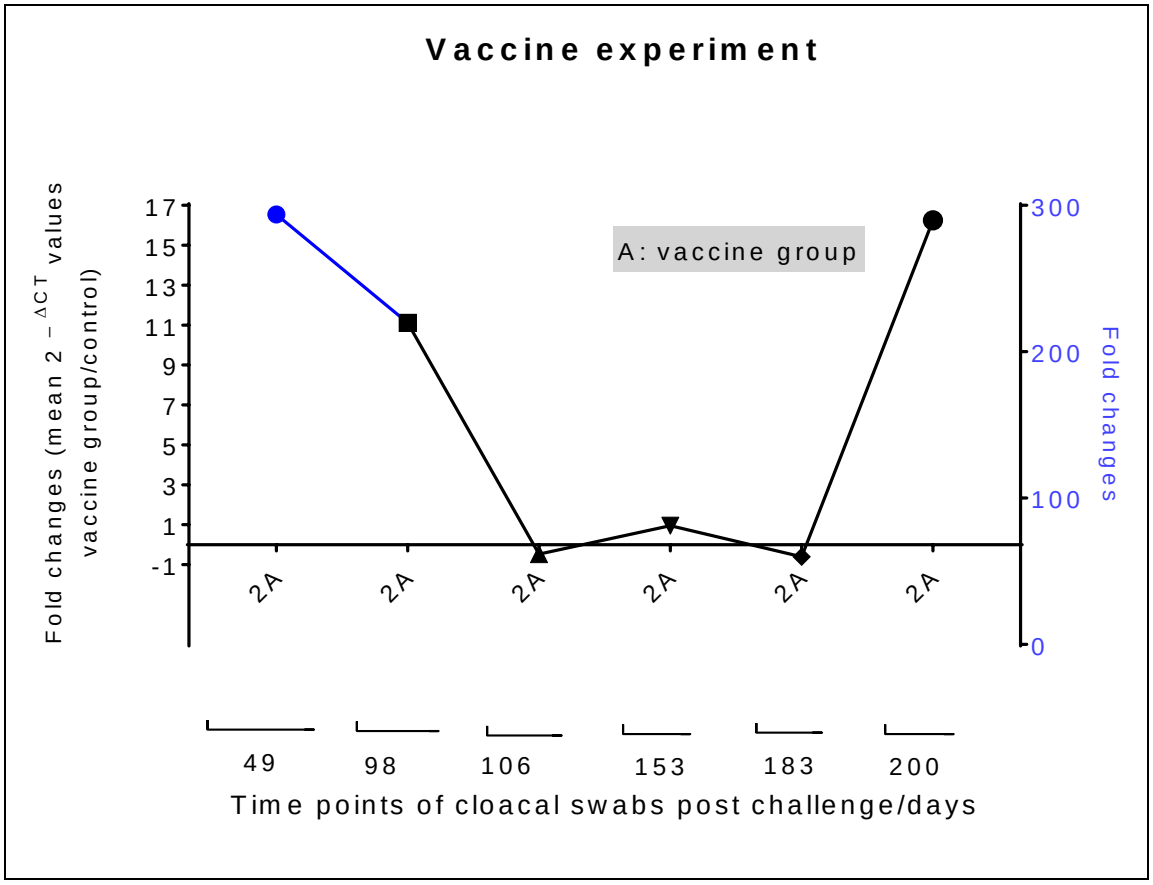


Figure 18. Fold changes in virus shedding, qRT-PCR of cloacal swabs, at different time points post challenge from vaccine experiment. Right Y axis represents results of the first time cloacal swabs were taken. Vaccine group shed less virus than control group at day 106 and 183 post challenge.

A

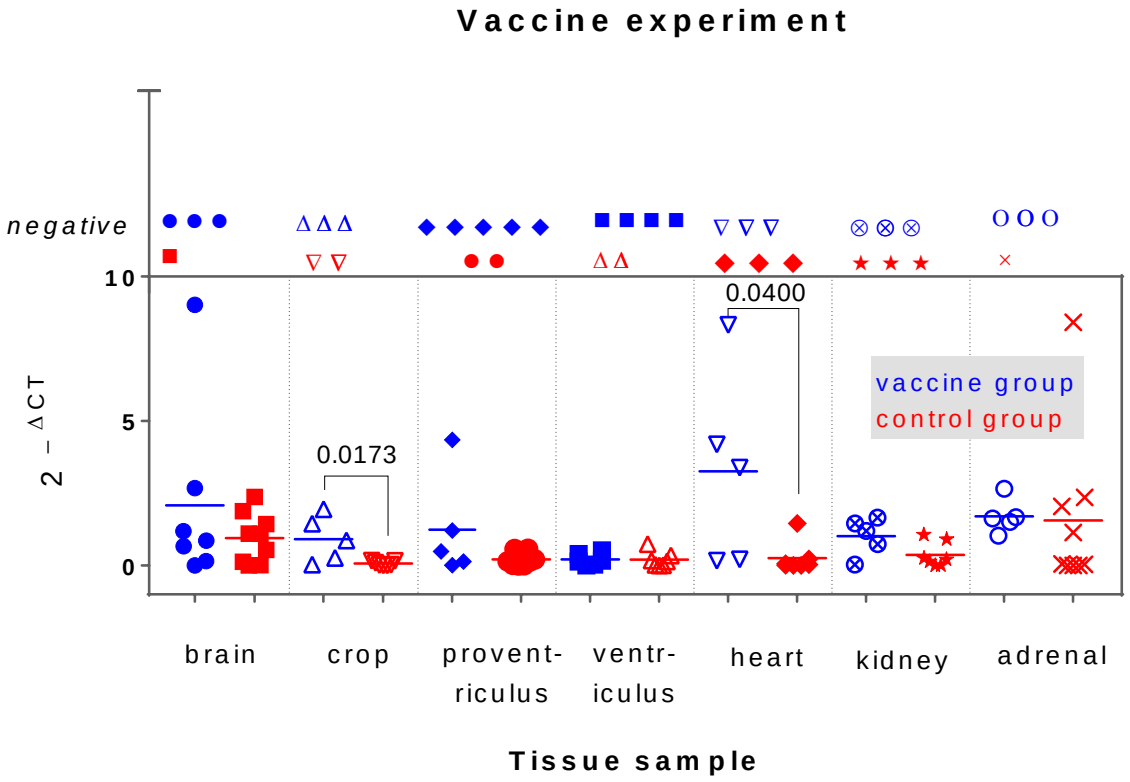


Figure 19. Results of qRT-PCR in organs from vaccine study. A: Scatterplot of delta Ct values; horizontal lines represent mean for each organ. B: Fold changes. Difference is significant when $P < 0.05$. *t* test

B

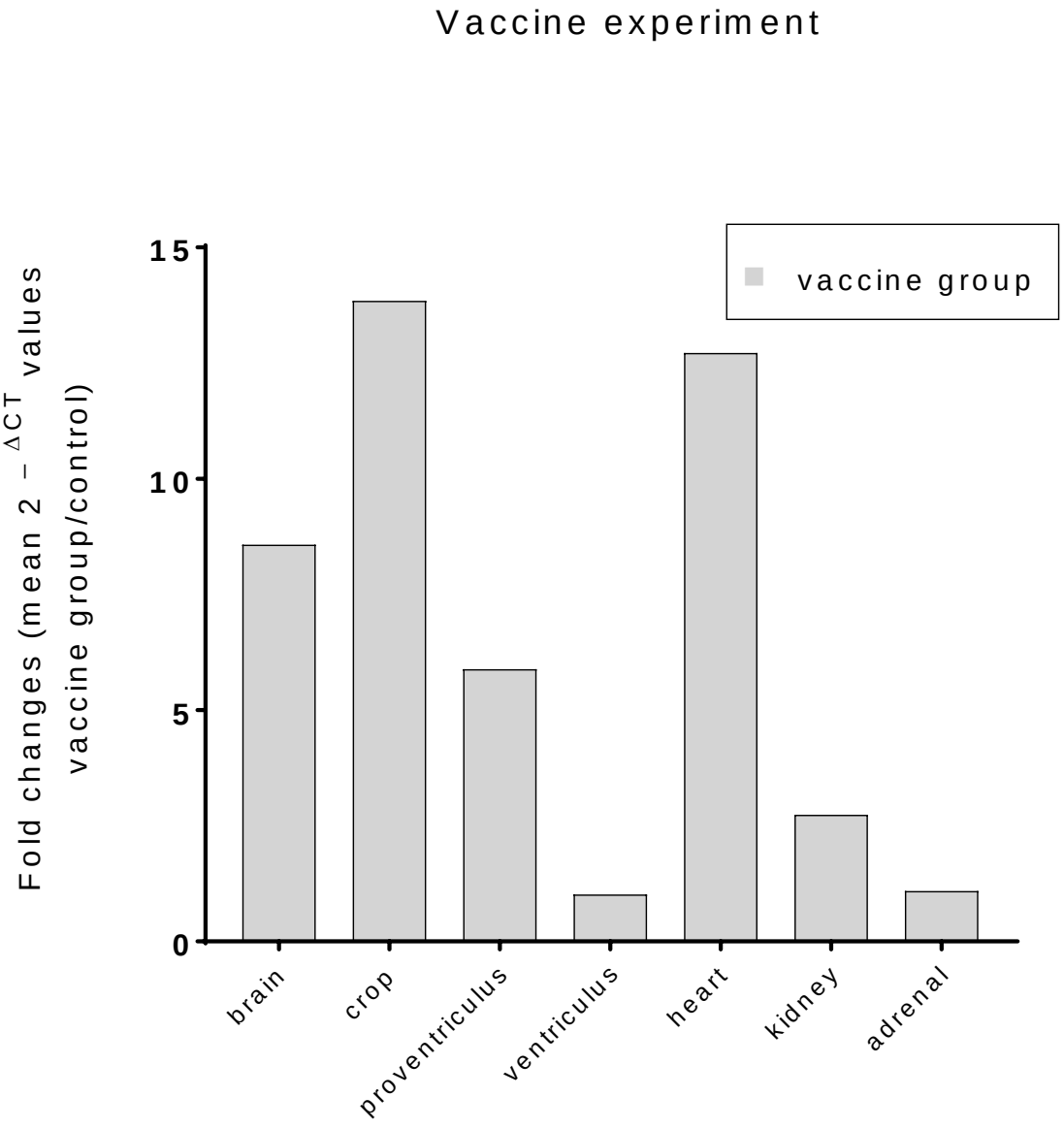


Figure 19. Continued

Detection of antibodies against PaBV

All vaccinated birds were seropositive after two doses of recombinant N vaccine (Fig. 10) and the results of WB analysis using ImageJ software were analyzed using a paired *t* test. There was a significant difference ($p<0.05$) in WB reactivity between first and second dose of recombinant N vaccine.

All vaccinated birds remained seropositive, by WB, on day 200 PC (Fig. 20). All tested control birds (four euthanized after 200 DPC and three euthanized before 200 DPC) became seropositive by WB (Fig. 20). The results were not significant at $p<0.05$ (Unpaired *t* test) between the two groups. Moreover, there was no significant difference within control birds that survived 200 DPC or were euthanized before 200 DPC due to PDD (Fig. 21).

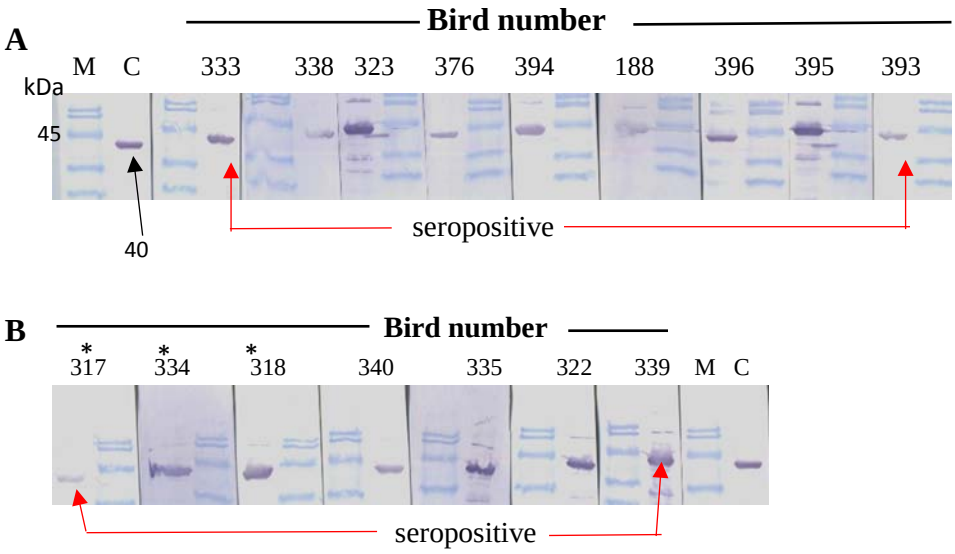


Figure 20. WB assay for vaccine experiment 200 DPC. (A) Vaccine group. (B) Control group. * denotes birds euthanized before the end of the experiment. M= size marker. C = positive control serum. Black arrow denotes the location of PaBV- N protein.

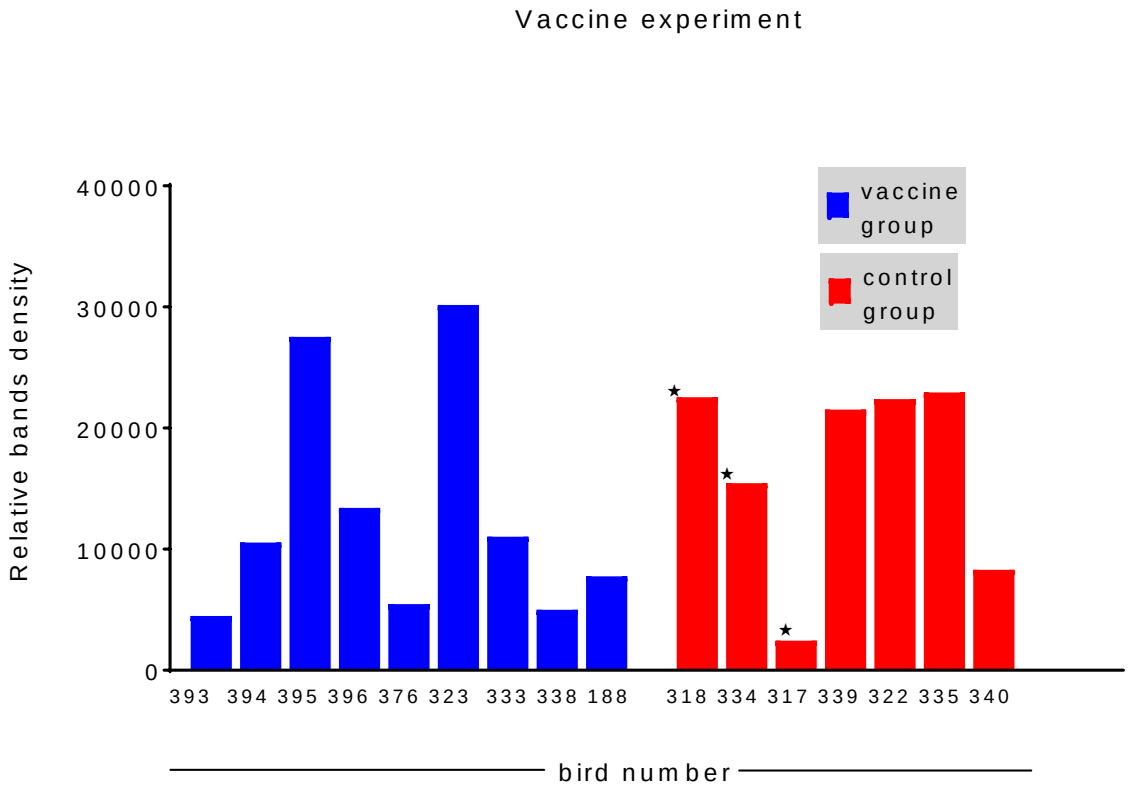


Figure 21. Semi quantification analysis of WB assay for the vaccine experiment.
Using ImageJ software. ★= euthanized before the end of the experiment.

Cyclosporine A Experiment

Based on the result of N protein vaccination, we performed this experiment to investigate the possibility that PDD might be an immune mediated disease. In particular, we were interested in the role of T cells in the development of PDD. Therefore, we used cyclosporine A, administered daily, to suppress T cell responses. Two groups of 8 cockatiels each were challenged with virulent PaBV-2. Group 3A birds were treated daily with cyclosporine A and Group 3B birds were given the vehicle alone (sesame oil). WBC differential counts, done with blood collected 29 days after the experiment began, revealed that mean lymphocyte counts were lower in birds in the cyclosporine A treated group in comparison with the control group. However, the difference was not significant at $p<0.05$ (Fig.22).

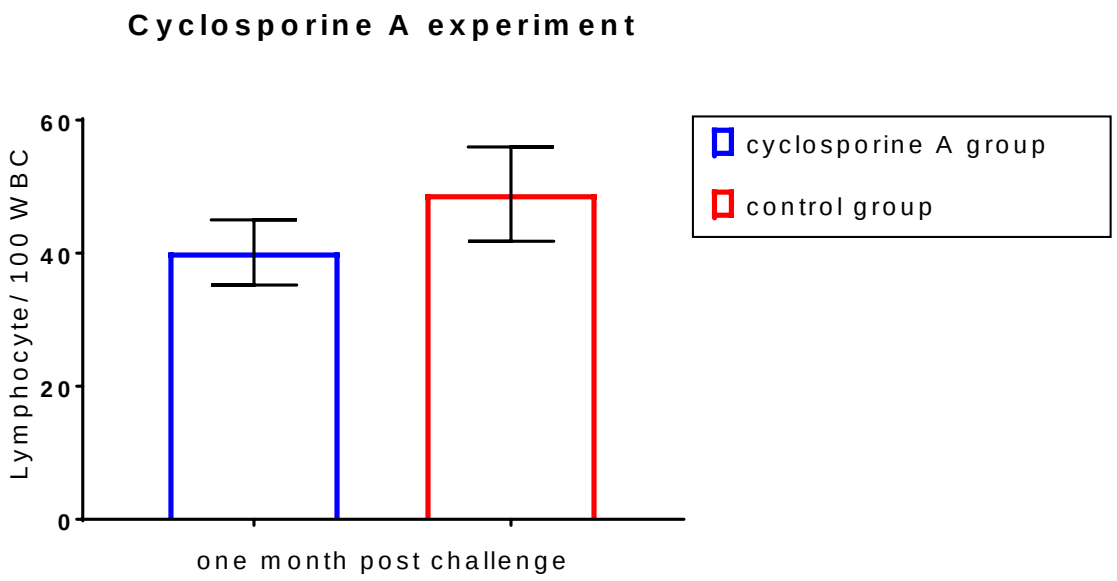


Figure 22. Cyclosporine A experiment, comparing percent blood lymphocytes.

Clinical observations and pathology

All the birds were challenged with 8 x 10^5 FFU of PaBV-2 24 hours after the cyclosporine A treatment began. Through the study period of 70 DPC, none of the cyclosporine A treated birds (Group 3A) showed any clinical signs related to PDD and all these birds survived the challenge (Fig. 23). On the other hand, birds in Group 3B were less active and the majority lost weight in comparison to Group A (Fig.24). Weight differences were compared using an unpaired *t* test and were significant $p<0.05$ between the two groups (Fig.25).

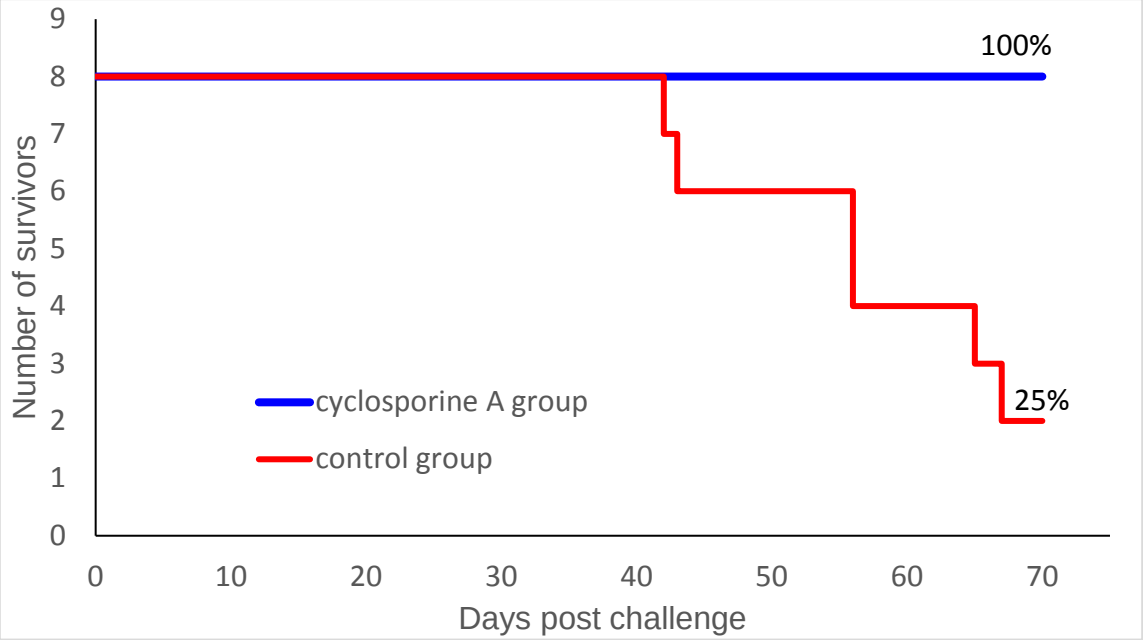


Figure 23. Survival curves for the cyclosporine A experiment. Percent survival was calculated for each group.

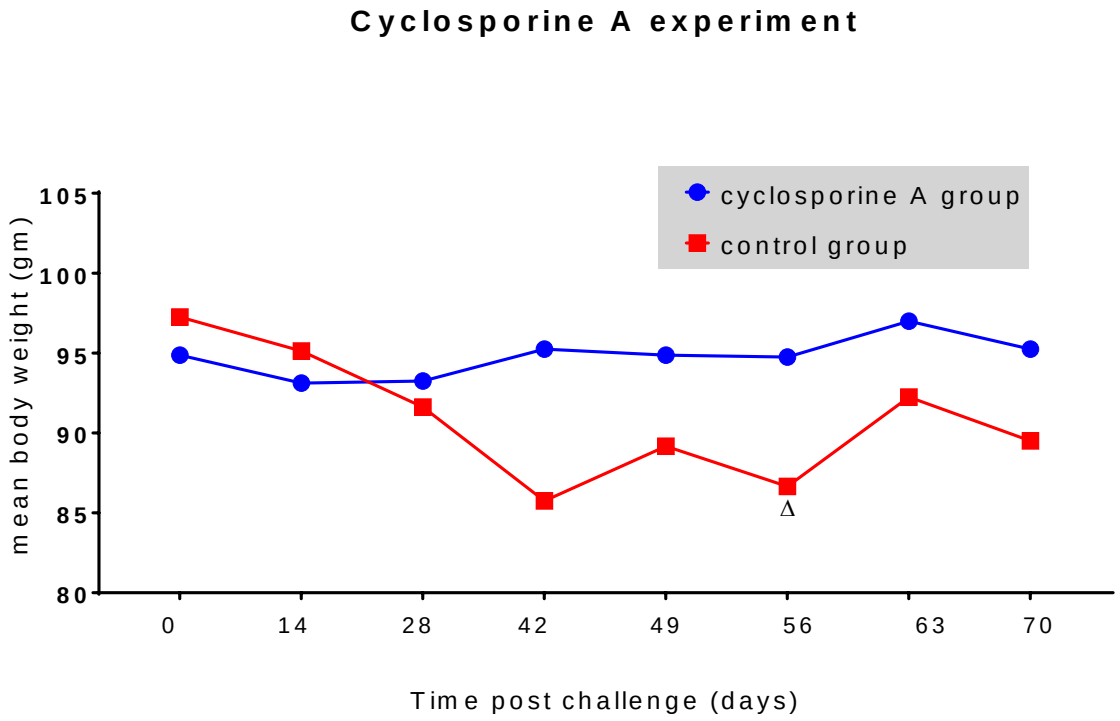


Figure 24. Mean body weights of cyclosporine A experiment groups. Δ marks mortality in ≥ 50% of the birds.

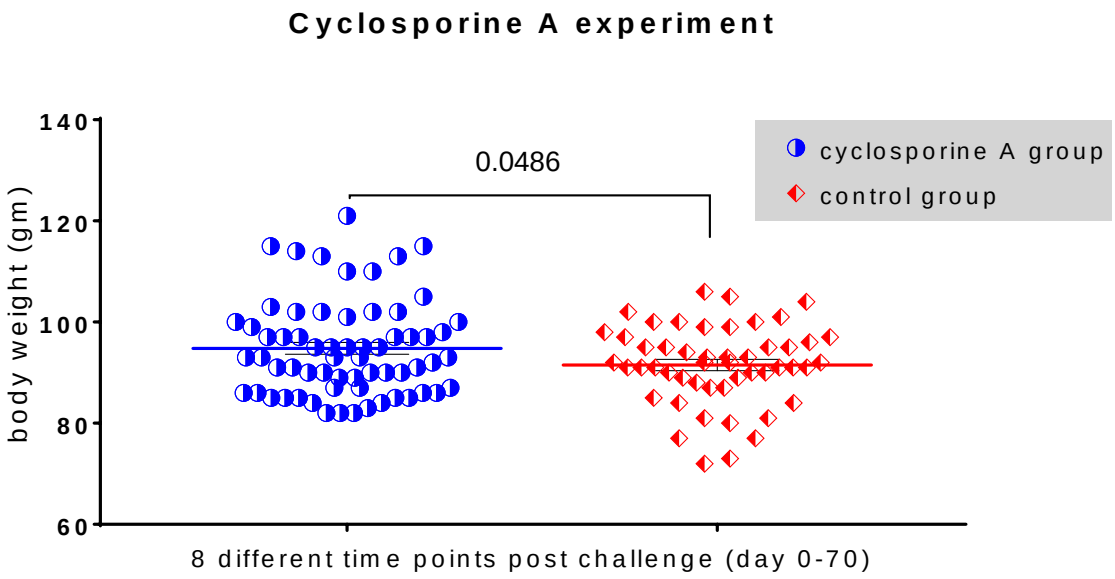


Figure 25. Scatterplot of body weights for cyclosporine A experiment. Mean ± SE. *P* = 0.0486

In Group 3B, 6 of 8 birds died or were euthanized before the end of the experiment; upon necropsy, 7 of 8 birds had a dilated proventriculus. In contrast, no Group 3A birds showed gross lesions related to PDD (Table 7).

Histopathological examination revealed that only one bird (#388) from Group 3A had microscopic lesions. Bird #388 had marked lymphocytic inflammation within the nerve ganglia in the muscular tunics of the proventriculus and ventriculus in addition to perivascular lymphocytic encephalitis in brain (Table 7). In contrast, 6 of 8 birds from Group 3B showed microscopic PDD lesions which included lymphocytic ganglioneuritis in the proventriculus and ventriculus as well as lymphocytic perivascular encephalitis.

Table 7. Macroscopic & Microscopic PDD-related lesions from cyclosporine A experiment.

Group	Bird #	Macroscopic lesion	Microscopic lesion	Time point of death/euthanasia (DPC)
3A	3703	Neg.	Neg.	70
	W	Neg.	Neg.	70
	388	Neg.	Pos.	70
	390	Neg.	Neg.	70
	666	Neg.	Neg.	70
	384	Neg.	Neg.	70
	36	Neg.	Neg.	70
	381	Neg.	Neg.	70
3B	626	Pos.	Neg.	42
	378	Pos.	Pos.	43
	387	Pos.	Pos.	56
	386	Pos.	Pos.	56
	385	Pos.	Neg.	65
	3750	Pos.	Pos.	67
	389	Pos.	Pos.	70
	22	Neg.	Pos.	70

Group 3A: Cyclosporine A treated, Group 3B: Untreated control. Macroscopic lesion= Included not limited to enlarged dilated proventriculus. Microscopic lesion= marked lymphoplasmacytic ganglioneuritis and encephalitis or encephalomyelitis. Pos. = positive; Neg. = negative. DPC= days post challenge.

Detection of PaBV-RNA in organs

qRT-PCR results of brain, proventriculus and ventriculus showed that the viral load was higher in treated birds (Group 3A) in comparison with control birds (Group 3B) (Table 8). The results were analyzed using an unpaired *t* test and were significant $p<0.05$ between these two groups for brain and proventriculus only (Fig. 26).

Detection of antibodies against PaBV

WB assays were performed on all cyclosporine A treated birds (sera collected upon necropsy at 70 DPC) and on 5 of 8 control birds (three of these were euthanized before 70 DPC due to PDD and two survived through 70 DPC). Among 8 tested Group 1A birds, only three were seropositive. In contrast, all tested Group 1B birds were seropositive (Fig. 27). The results were not significant at $p<0.05$ (*t* test) between cyclosporine A treated birds and the controls, however the results were significant between birds that survived the challenge and those euthanized before the end of this experiment for the control group (Fig.28).

Vaccine Safety Trial

Two doses of recombinant N vaccine were given for 50 cockatiels without subsequent challenge to assess any potential risk.

All 50 cockatiels were clinically healthy through the six months observation period and none of them died or showed any adverse reaction due to two doses of recombinant N vaccine. Western blot assays revealed that 77-80% of those birds were seropositive after the second dose of recombinant N protein vaccine.

Table 8. Results of qRT-PCR in organs from cyclosporine A experiment.

Group	Bird #	Tissue			Time point of death/euthanasia (DPC)
		Brain	Proventriculus	Ventriculus	
3A	3703	++++	+++++	+++++	70
	W	++++	+++++	+++++	70
	388	+++++	++++	++++	70
	390	+++++	+++++	+++++	70
	666	++++	+++++	++++	70
	384	++++	+++++	++++	70
	36	+++++	+++++	++++	70
	381	++++	++++	++++	70
		Brain	Proventriculus	Ventriculus	
3B	626	++	Neg.	Neg.	42
	378	++++	+++	+++	43
	387	++++	++	+++	56
	386	++	Neg.	++	56
	385	++	+++	+++	65
	3750	+++	++	Neg.	67
	389	+++	Neg.	Neg.	70
	22	+++	+++	+++	70

Group 3A: Cyclosporine A treated, Group 3B: None treated control.
Scoring in term of threshold cycle values as follows: Neg., > 35; +, 35 to 30; ++ 30 to 25; +++, 25 to 20; ++++, 20 to 15; +++++, 15 to 10.
Neg. = negative. DPC, days post challenge.

A

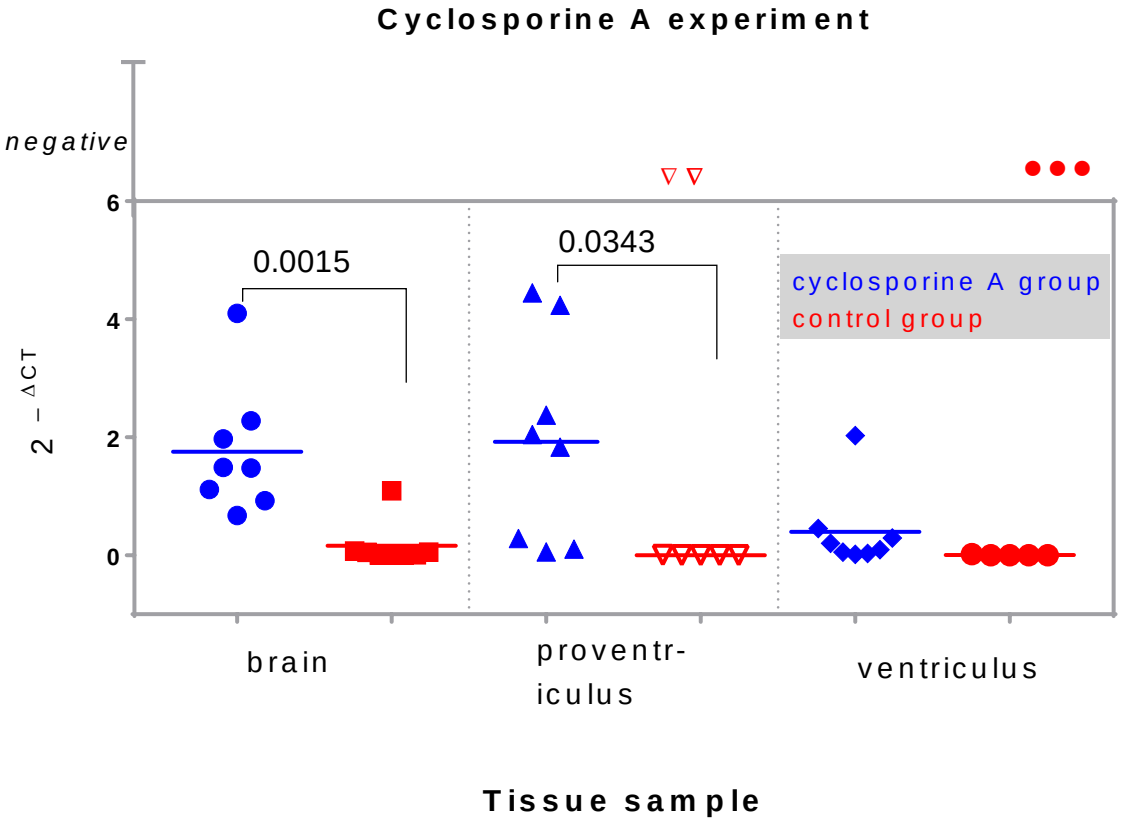


Figure 26. qRT-PCR in organs from cyclosporine A experiment. A: Scatterplot of delta Ct values; horizontal lines represent mean for each organ. B: Fold changes. Difference is significant when $P < 0.05$. *t* test

B

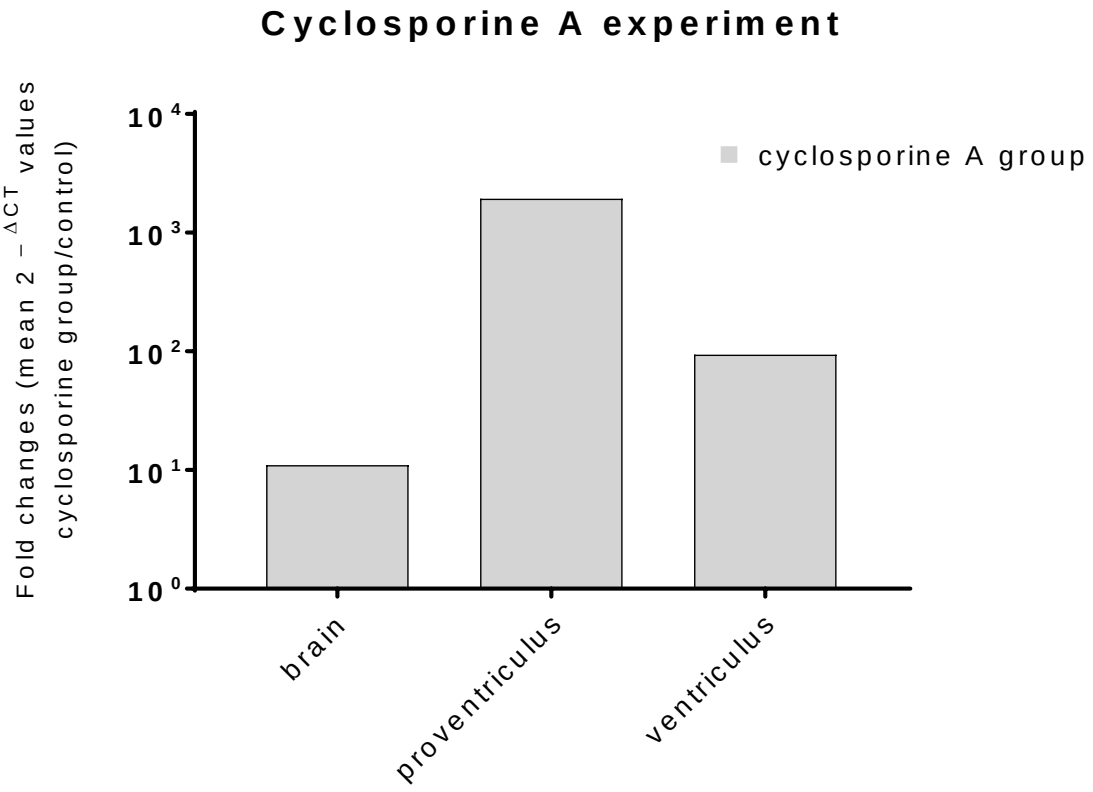


Figure 26. Continued.

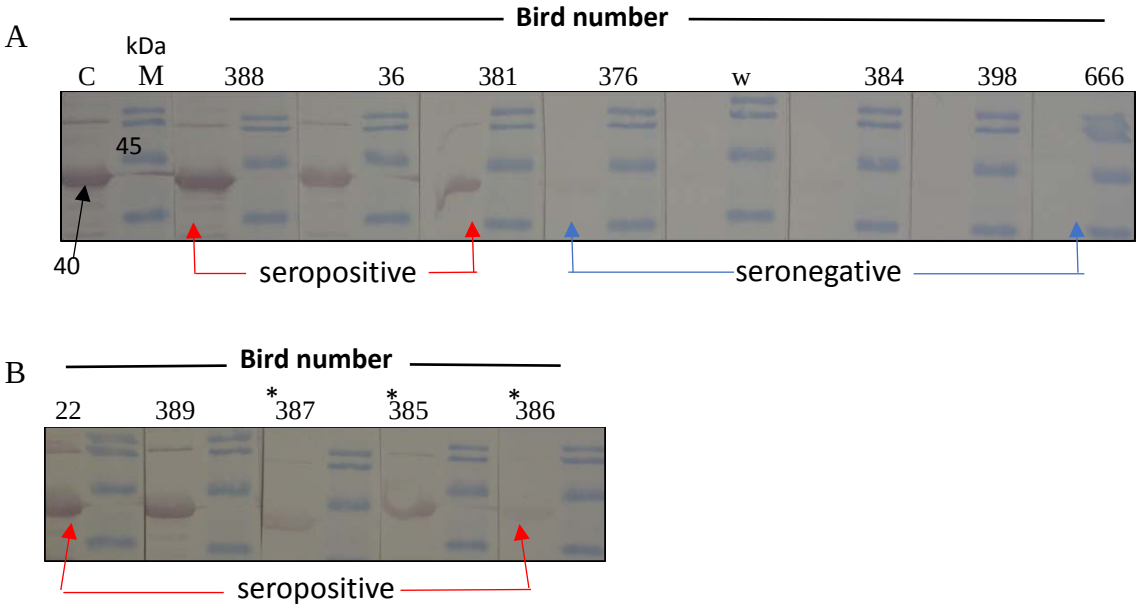


Figure 27. WB assay for cyclosporine A experiment. Unless noted, sera were collected at 70 DPC. (A) Cyclosporine A treated. (B) Untreated control. M= size marker. C = positive control serum. Black arrow denotes the location of PaBV- N protein. *= euthanized before the end of the experiment.

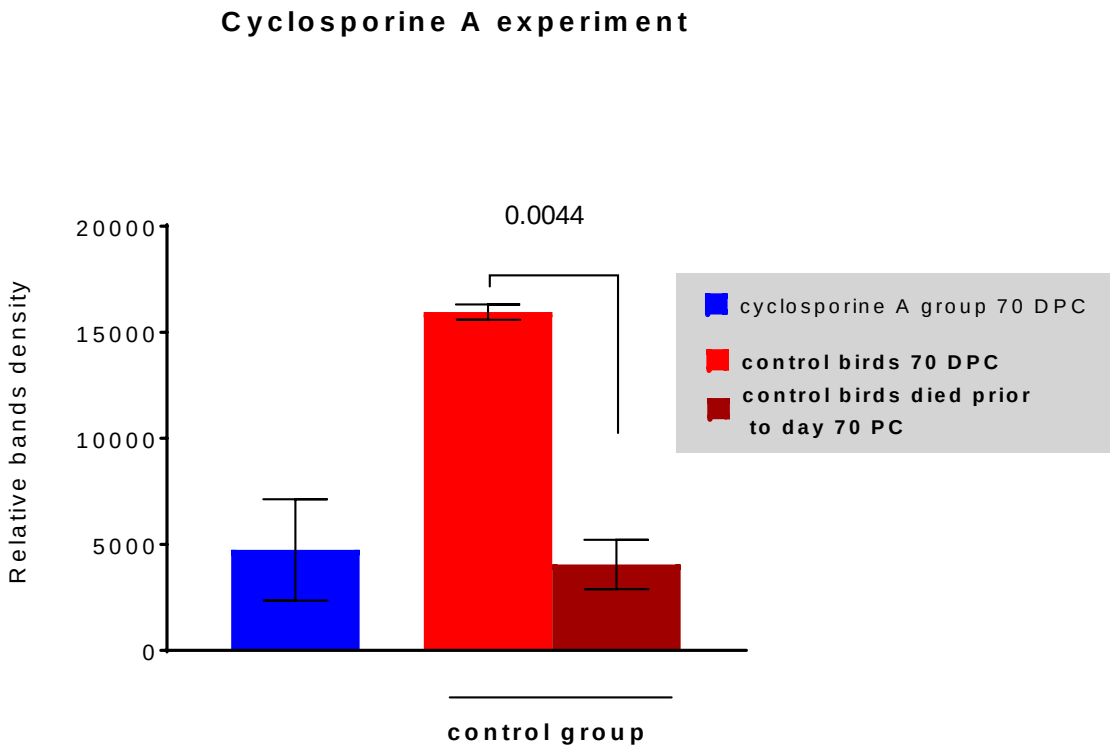


Figure 28. Semi quantification analysis of WB results for the cyclosporine A experiment using ImageJ software.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Even though PDD was first described as a fatal disease of captive parrots more than 40 years ago, and the first PaBVs were identified more than 8 years ago [1, 12, 13], many questions remain about the disease and the virus. Many infected birds are clinically healthy carriers and the triggers of disease are unknown; natural routes of transmission are suspected to be fecal-oral but this has not been demonstrated experimentally; vertical transmission of PaBV is suspected but has not been formally demonstrated either; it is not known with what frequency infected birds clear an infection. In addition, detecting infection is difficult as some birds shed virus very infrequently and many birds fail to produce antibody. Finally, the pathogenesis of PDD is suspected to be immune-mediated, as is well-established for mammalian bornavirus infections [31, 119, 182], however this has not been formally determined.

In studies using Lewis rats, immunization with recombinant N protein resulted in exacerbation of Borna disease [183]. This result was despite the fact that BoDV-1 N protein is the most abundantly expressed viral protein in infected cells and triggers strong immune responses [184, 185]. This result could potentially confound vaccine development. However, the results of this project show, for the first time, evidence for the protective efficacy and effectiveness of a recombinant N protein vaccine to prevent

development of PDD in challenged birds. These studies, further indicate that PDD is indeed, an immune mediated disease.

Two vaccine experiments performed in cockatiels in which inactivated PaBV and/or PaBV recombinant N protein vaccines were used. Following challenge with virulent PaBV, the disease course was monitored over the period of 200 days. Additionally, a study using cyclosporine A as an immunosuppressive agent was done to investigate the potential pathogenesis of this disease. Finally, a safety study for the recombinant N protein vaccine was also performed.

In the first (pilot) experiment, all the birds were seronegative for anti PaBV-N antibodies as tested by WB assay before started this experiment. All the vaccinated birds (group 1A) remained seronegative after an initial dose and two booster doses of inactivated whole virus vaccine. It should be noted that the titer of PaBV in DEF is low, even when most cells in the culture are positive for PaBV antigen. In addition, little virus is released into the supernatant in this noncytopathic infection [48, 186, 187]. The low quantity of viral antigen, in comparison to cellular antigens likely contributed to the lack of a detectable anti-N response. The ideal for an inactivated whole virus vaccine is a high titer virus preparation, harvested from culture supernatant, purified and inactivated [188]. However, three weeks after administration of a single dose of recombinant N protein vaccine, over 70% (7/9) of cockatiels previously given 3 doses of inactivated vaccine were positive for anti-N antibodies providing some evidence that the inactivated

virus preparation had indeed primed the immune response. Another contributing factor to the failure of the inactivated vaccine to induce detectable antibodies might be loss immunogenicity due to the use formalin for inactivation. This has been reported for Rift Valley fever virus and bovine herpes virus-1 when formalin was used to inactivate these agents [189, 190]. None of the vaccinated birds (9/9) showed any clinical signs suggestive of PDD throughout the period of 200 DPC. Only one bird, #38, had a mildly dilated proventriculus upon necropsy, and histopathological examination revealed mild to moderate perivascular cuffing in the brain. In contrast, most birds in the unvaccinated and vaccinated post-challenge groups developed clear signs of PDD.

The most surprising result of both vaccine trials was that, despite lack of clinical disease, all vaccinated, infected, birds had levels of viral RNA and antigen comparable to the control (unvaccinated) group suggesting that protection from disease was in fact due to administration of recombinant N. Of the 19 vaccinated birds of two experiments, 3 from the second experiment appeared to have been protected from infection, as evidenced by virus negative tissues at necropsy. In contrast, all of the unvaccinated birds had some evidence of infection. Thus, it is possible that the N vaccine protected some birds against infection. Perhaps more important and encouraging was the observation that the vaccinated, infected birds remained healthy, suggesting that the anti-N response was not immunopathogenic. In contrast, several rodent trials were failed to generate protection against Borna disease by using N protein viral vector vaccines and sometimes the

clinical disease was exacerbated as it is difficult task to generate protective immunity against an immune- mediated disease without exacerbating it [123, 183].
These findings suggest that a better vaccine protocol might be developed, with the aim of achieving higher levels of protection against infection and with little risk of immunopathogenesis in those birds that are virus positive prior to vaccination or in instances where protection from infection is not complete.

It should also be noted that vaccinated birds received a 'relatively high' challenge dose of virus (delivered by the IM route) in comparison to another study in which successful persistence infection and development of clinical disease was achieved in 2 of 4 cockatiels by giving them as little as $10^{2.7}$ FFU of PaBV-2/ bird via parenteral route[145]. Moreover, the investigators failed to achieve persistence infection via the mucosal route (peroral and oculonasal) even when 500-fold dose of the same challenge was used [145]. Therefore, we may have seen better protection from infection were the challenge dose lower, and administered by a more 'natural' route. Unfortunately, other groups have failed to reliably infect cockatiels by oro-nasal routes [145]. Thus, defining a biologically relevant challenge remains problematic. Future vaccine trials with recombinant N would also benefit from development of an anti-N ELISA with high specificity and sensitivity so that levels of vaccine induced antibodies might be correlated to protection against challenge. It is clear however that in *infected* birds, an anti-N response does not protect from disease as there was no correlation between antibody levels and survival or disease development.

The results of a safety trial of the recombinant N vaccine also encourage further development, as 50 cockatiels previously exposed to PaBV in an aviary setting, remained healthy after receiving 2 doses of the recombinant N vaccine.

The induction of protection from the development of PDD by using recombinant N protein vaccine was unexpected as in essence, most PDD- affected birds (natural or experimental infection) have anti N antibodies, indicating that this disease can develop despite an antibody response [18, 25, 110, 146, 161]. Moreover, it has been demonstrated that even high levels of neutralizing antibodies do not prevent persistent infection and the development of Borna disease in rodents models [121, 182]. Bornaviruses are non-cytopathic both in vivo and in vitro [176, 182, 191]. Borna disease in mammals is well known as an immune mediated disease caused by cellular immune responses, in particular CD8+ T cells [182]. Both, humoral and cellular immune responses target the N protein of this virus [184, 185]. However, several rodent vaccine studies failed to generate protection against Borna disease using N protein viral vector vaccines and some cases clinical disease was exacerbated [123, 183]. In contrast however, Henkel *et al* [117] demonstrated that Lewis rats were protected from the challenge with BoVD-1 by vaccination with a recombinant parapoxvirus vector expressing BoVD-1- N. Furthermore, it seems that induction of immunity against BoVD-1 depends on anti-viral mechanisms of non-cytolytic CD8+ T-cells as MRL mice (wild type and perforin-deficient) can be protected by vaccination with a recombinant parapoxvirus vector expressing BoVD-1 N followed by a vaccinia virus vector

expressing BoVD-1 N [122]. It appears that cytokines produced by brain- infiltrating CD8+ T cells, in particular IFN- gamma and TNF-alpha, are responsible for virus clearance, without causing severe neuronal damage [122, 192, 193]. So, it is clear that virus-specific CD8+ T cells can mediate either viral clearance or the immune mediated pathogenesis typical of this disease [122]. A delicate balance must be maintained regarding virus replication, vaccination and responses of virus- specific T cells in order achieve protection [122].

The results presented here support a role for immune- mediated pathology in the development of PDD. We propose that vaccination with recombinant N in alum favored the development of Th2 humoral responses that protected these birds from the development of clinical PDD. Use of recombinant PaBV N protein eliminates any immune responses to a vector, which might be interfere with protective immune responses directed against N [117]. Use of alum as an adjuvant may have induced Th2 humoral immune responses in the absence of IL-4- or IL-13-mediated signaling, but may also have suppressed Ag-specific Th1 responses [194, 195]. Our proposal is supported by results of BoDV-1 infections of Lewis rats [196] where authors suggested that a switch from Th1 cellular immune responses in the acute phase of BoDV1- infected Lewis rats, to a Th2 humoral immune response in chronic phase of disease was correlated with survival in association with decreased inflammation in the CNS [196]. Subsequent studies also revealed that IgG locally produced in CNS of experimentally infected Lewis rats in the chronic phase of Borna disease influences BoDV1- gene

expression and pathogenesis [197]. Based on these results we hypothesize that our vaccine might have suppressed the cytolytic and the non-cytolytic activities of PaBV-specific CD8+ T cells as disease did not develop in vaccinated birds, despite the presence of viral RNA and viral antigen in numerous organs.

At the current time, the reagents to dissect immune responses in cockatiels are not available. However, cyclosporine A is well known to inhibit cytotoxic T lymphocytes responses [198, 199]. Therefore, we assessed the ability of cyclosporine A to prevent PDD in birds challenged with virulent PaBV. Indeed, the cyclosporine A treated birds remained healthy during the observation period. As was observed with vaccinated birds, the cyclosporine A treated birds were infected and produced virus. In fact, their viral load was higher than control (challenged) birds. These results are the strongest indication to date that PDD may develop as a result of T cell- mediated immunopathogenesis and that the same cells might be responsible for restricting virus replication.

Given variable results with vaccination of rodents against BoDV-1 and the likelihood of immune mediated pathogenesis of PDD, we expected to see enhanced disease among cockatiels immunized with recombinant PaBV N protein. However, we saw the opposite effect; protection against disease in conjunction with increased virus replication and distribution. Therefore, we designed and performed a vaccine experiment using only recombinant N protein vaccine. The experiment confirmed that protection against the development of clinical PDD did not require priming by the inactivated whole virus

vaccine administered in the pilot study. In general, the results of the N vaccine experiment were similar to the first experiment as regarding protection against the development of PDD. However, three vaccinated birds were negative for viral RNA and viral antigen in their organs, an indication that there may have been a protective (against infection) component provided by the N vaccine.

At the end of the N vaccine experiment, tested control birds (7/9) were seropositive regardless of different time points of death or euthanasia. The majority of these birds suffered from PDD in contrast to the vaccinated birds. Together, these results indicate that there is no correlation between the humoral immunity and the protection from PDD.

Intermittent viral RNA shedding also was noted for birds in both vaccine studies, however, in the second experiment, viral shedding was lower in vaccine group than in the control group in two of six times points (Fig.18). This might be due to the fact that two doses of recombinant N vaccine were not only enough for the protection from clinical PDD but also lower viral load in cloacal swabs by stimulating the noncytolytic CD8+ T cells. This was demonstrated for other neurotropic viruses like Sindbis virus and flaviviruses where antiviral non-neutralizing antibodies clear the virus from the brain by prevention or restriction gene expression of the virus [200-203]. Moreover, binding of antibodies to the surfaces of infected cells might lead to activation of intracellular signaling pathways which in turn attracted T- cells and activate microglia

which might lead to viral clearance as result of IFN-γ or TNF-α production [200, 201, 204, 205].

In all, the present study provides for the first time that the recombinant N protein vaccine can protect against the development of clinical PDD as a result of a switch from a Th1 cellular immune response to a Th2 humoral immune response.

Conclusions and Future Studies

Conclusions of the studies presented herein include:

- A vaccine prepared from recombinant PaBV N protein in alum is safe and elicits a specific antibody response.
- A recombinant N vaccine, used as described herein, does not efficiently prevent infection with an IM challenge of virulent PaBV.
- The recombinant N vaccine, used as described herein provides effective protection from clinical disease. Use of this vaccine reduced both morbidity and mortality among experimentally infected cockatiels. This result was supported by a lack of microscopic lesions in the vaccinated, challenged cockatiels. However, vaccinated birds became persistently infected.
- In vaccinated, PaBV challenged cockatiels, there was no apparent correlation between anti-N antibody titers and susceptibility to infection and/or disease development.

- There is no indication that the recombinant N vaccine, used as described herein, enhances disease in PaBV infected cockatiels.
- Unvaccinated, PaBV challenged cockatiels developed variable antibody responses, with no apparent correlation with morbidity or mortality.
- The presence of viral RNA and antigen in vaccinated, challenged cockatiels, along with a lack of immune infiltrates in tissues, suggested an immune-mediated component to PDD.
- Cyclosporine A treatment of cockatiels effectively prevents clinical PDD in PaBV challenged individuals. The lack of macroscopic and microscopic lesions in cyclosporine A strongly supports an immune mediated pathogenesis for PDD.

Many questions remain regarding protection against the disease and/or infection with PaBV. We suggest the following studies:

- Development of a more natural PaBV infection model, mimicking conditions of natural infection, to provide a more realistic test of vaccine efficacy.
- Changing vaccine dose and administration schedule in an effort to improve protection against infection.
- Producing a vaccine with additional viral proteins, such as G, alone or in combination with N to achieve greater protection from PaBV infection.
- Maintaining vaccinated, PaBV infected birds for longer periods (up to a year) to monitor disease development and/or clearance of the virus.

- Maintaining cyclosporine A treated, challenged birds, for longer periods (up to a year) to monitor disease development and/or clearance of the virus.
- Determine the most efficient, yet effective cyclosporine A treatment schedule for experimentally infected birds.
- Determine the effects of cyclosporine A treatment using controlled trials with naturally infected birds.
- Develop reagents to identify T cell subsets in cockatiels and other parrots in order to determine the mechanism(s) of immune-mediated disease development.

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