Assessment of recombinant beak and feather disease virus capsid protein as a vaccine for psittacine beak and feather disease

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Beak and feather disease virus (BFDV) is a significant pathogen of wild Australasian and African psittacine birds. We assessed the immunogenicity of recombinant BFDV capsid (recBFDVcap) to protect against the development of psittacine beak and feather disease (PBFD). Long-billed corellas (Cacatua tenuirostris) (n=13) received (by injection) 1 ml vaccine containing 10 μg recBFDVcap on day 0 and 0.4 ml vaccine containing 66.8 μg recBFDVcap on day 11. All vaccinated corellas and five non-vaccinated control corellas were given 0.4 ml BFDV suspension [titre=log12 haemagglutination units (HAU) 50 μl-1] intramuscularly and 0.1 ml orally 16 days after booster vaccination. Blood was collected during the vaccination period and blood and feathers were collected after BFDV administration. Testing of blood samples included BFDV DNA detection by PCR and quantitative PCR (qPCR) as well as antibody detection by haemagglutination inhibition (HI) and on feather samples, BFDV DNA and antigen was detected by haemagglutination (HA) and qPCR. Four of 97 blood samples collected from vaccinated birds after virus challenge tested positive by PCR, whereas 17 of 35 samples taken from non-vaccinated control corellas tested positive. Vaccinated birds did not develop feather lesions, had only transient PCR-detectable viraemia and had no evidence of persistent infection 270 days post-challenge using PCR, histopathology and immunohistochemistry. Non-vaccinated control corellas developed transient feather lesions and had PCR, HI and HA test results consistent with PBFD. They were BFDV PCR-positive for up to 41 days post-challenge and qPCR demonstrated reduced virus replication in vaccinated birds compared with non-vaccinated control birds.

INTRODUCTION

Psittacine beak and feather disease (PBFD) is a significant disease of both wild and captive psittacine birds in many parts of the world and has serious implications for the health of pet birds as well as for the conservation of threatened species (Albertyn et al., 2004; Bert et al., 2005; McOrist et al., 1984; Rahaus & Wolff, 2003; Raidal et al., 1993a). Vaccination against PBFD using inactivated virus purified from the feathers of chronically infected cockatoos has been shown to protect psittacine birds from developing PBFD (Raidal & Cross, 1994; Raidal et al., 1993b). However, the use of beak and feather disease virus (BFDV)-infected feathers or other tissues as a source of antigen is potentially hazardous due to an inability to assess the reliability of BFDV inactivation in vitro. The maintenance of birds infected with BFDV for the production of vaccine is impractical and expensive and the production and maintenance of birds infected with BFDV for the sole purpose of antigen production is ethically questionable. This has driven research efforts to find alternative methods for vaccine production. Many traditional cell culture systems have been tried for amplification of BFDV; however, these attempts have been unsuccessful (Pass & Perry, 1985). As an alternative, Stewart et al. (2007) described the use of a recombinant baculovirus for the production of the BFDV capsid protein and showed that the protein had properties similar to those of the native virus, including morphology, haemagglutinating activity and ability to react with anti-BFDV antibody.

Vaccine assessments are best done using susceptible or specific-pathogen-free animals, but these are difficult to obtain for wildlife species, particularly endangered ones. In this report, the vaccination and subsequent BFDV challenge of hand-raised long-billed corellas (Cacatua tenuirostris) collected from the wild by Western Australia Department of Environment and Conservation (DEC)

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officers is described. The aim of this experiment was to determine whether recombinant BFDV capsid protein, expressed using a baculovirus system, could cause seroconversion and prevent BFDV replication and excretion in susceptible psittacine birds. By documenting the effects of BFDV-challenge on vaccinated and control birds the results also provide important information a propos the pathogenesis of PBFD.

METHODS

Acquisition of BFDV-free birds. The birds used for this experiment were 18 hand-raised long-billed corella (C. tenurostris) nestlings collected from the wild by DEC officers. This was approved under permit by the Animal Ethics Committee of Murdoch University. The birds were determined to be free of BFDV infection, as described below, and housed individually or in sibling groups and hand-reared using a hand-rearing formula (Roudybush formula 3, Kimani Aviaries) and temperature-controlled, air-filtered incubators. Once the birds reached weaning age, they were maintained in suspended wire cages or an aviary in a single climate-controlled quarantined room. Here the birds were maintained on a diet of commercial parrot pellets (Passwell), fresh fruit, vegetables and peanuts.

During the hand-rearing period, the birds were screened repeatedly for evidence of BFDV infection in the peripheral blood by PCR and once for evidence of pre-existing antibodies to BFDV by haemagglutination inhibition (HI). Blood samples were collected and tested for BFDV DNA on their first day of captivity and then on days 11, 18 and 25 after arrival. Anti-BFDV HI antibody was tested on blood samples collected on day 1. The vaccination trial commenced 41 days after the birds had arrived in captivity. Sampling continued during the vaccination and BFDV-challenge periods as described below.

Production of recombinant BFDV capsid protein. The baculovirus system used to express the recombinant capsid protein of BFDV was developed by Stewart et al. (2007) using the Bac to Bac (Invitrogen) baculovirus expression system. Briefly, PCR-amplified BFDV ORFC1 (which encodes the capsid protein) was excised from a pCR2.1 BFDV ORFC1 construct using an EcoRI/SalI double digest, ligated into the EcoRI/SalI site of the pFastBAC HTa (Invitrogen) baculovirus transfer vector and transformed into TOP10F’ chemically competent Escherichia coli (Invitrogen). The orientation of the gene in pFASTBAC was verified by restriction digestion with BamHI and sequence analysis. The recombinant bacmid was generated according to the manufacturer’s protocol (Invitrogen). The pFASTBAC HTa BFDV ORFC1 construct was transformed into chemically competent E. coli DH10Bac (Invitrogen) containing the bacmid and helper vector, where the BFDV ORFC1 was transposed into the bacmid. The recombinant bacmid containing the BFDV ORFC1 was purified and transfected using Cellfectin (Invitrogen) into SF9 insect cells to produce recombinant baculovirus (AcMNPV) containing the BFDV ORFC1 gene under the control of the polyhedrin promoter. The recombinant baculovirus was amplified until the titre was sufficient for high-level expression of the protein. For the production of recombinant BFDV capsid protein used in the vaccination trial, SF9 insect cells were cultured to a density of 2 × 10^6 cells ml^-1, infected with the recombinant baculovirus at an m.o.i. of 0.2 and then harvested 72 h post-infection. The cultures were then lysed and the protein purified as described previously (Stewart et al., 2007).

Western immunoblot detection of recombinant protein. The recombinant BFDV capsid protein was detected by Western immunoblot, using mouse antihistidine IgG (Serotec, diluted 1:5000) and alkaline-phosphatase-conjugated goat-anti-mouse IgG (Sigma Aldrich, diluted 1:30000) using previously described procedures (Stewart et al., 2007).

Quantification of expressed recombinant BFDV capsid protein for vaccination. The recombinant protein preparation intended for primary vaccination was quantified by densitometry (Proteomics International) and the recombinant protein preparation for secondary vaccination was quantified in our laboratory by the same method. Briefly, Samples were mixed with SDS loading solution without reducing agent and boiled for 5 min. Subsequently, the samples were loaded onto NuPAGE 4–12 % polyacrylamide mini-gels (Invitrogen) with MES electrophoresis buffer and electrophoresed at 200 V for 1 h. The gels were stained using colloidal Coomasie G250 (PI-Blue method). The gels were then scanned using a ProEXPRESS 2D Proteomic Imaging System (Perkin–Elmer). Quantitative band analysis was carried out using Totalall TL120 (Nonlinear Dynamics). The total stained material in each gel lane was determined and the quantity of the recombinant BFDV capsid protein in the sample was determined from the graph of known ovalbumin quantities (chicken lysozyme standard). The percentage of recombinant protein was measured using volume ratios from the recombinant BFDV capsid bands, where it was calculated as Rec/Rec BFDV CAPSID (total).

Vaccine preparation. Vaccine doses were prepared immediately before injection. Equal volumes of the recombinant proteins and Freund’s incomplete adjuvant (Sigma Aldrich) were mixed to form a stable primary emulsion using two sterile glass syringes connected together by a section of 16-gauge cannula.

Vaccination. Thirteen vaccinated corellas were given injections of 1 ml vaccine containing 10 µg recombinant BFDV capsid protein on day 0 and 0.4 ml vaccine containing 66.8 µg recombinant BFDV capsid protein on day 11 post primary vaccination. The vaccine was delivered by pectoral intramuscular injection. Non-vaccinated control birds (n=5) did not receive any injection. The birds were between 65 and 89 days old on the day of primary vaccination.

BFDV inoculum. Virulent BFDV inoculum, was produced by Raidal et al. (1993a) and had been stored at −80 °C until its use in this experiment. These stocks had a haemagglutination titre of log 12 haemagglutination units (HAU) 50 µl^-1 before the experiment, determined using haemagglutination assay (HA) as described by Raidal et al. (1993c).

BFDV challenge. Vaccinated birds and non-vaccinated control birds were challenged 16 days after the secondary injection (27 days after the primary injection) with 0.5 ml BFDV suspension, 0.4 ml of which was administered by pectoral intramuscular injection and 0.1 ml of which was administered orally.

Sampling. Blood was collected by jugular venepuncture from each of the 18 birds on days 0, 11 and 27 after primary vaccination. The BFDV suspension was administered 27 days after the primary vaccination (16 days after secondary vaccination). Then, on days 13, 20, 26, 41 and 97 after the birds were challenged with BFDV suspension (40, 47, 53, 68 and 124 days post primary vaccination), a blood sample was collected by jugular venepuncture, and a sample of powder-down or crest feather was collected from each of the birds. The birds had well-developed plumage, but frequently had developing powder-down and growing crest feathers emerging on the chest, neck and head and these were chosen for sampling when they were found. The blood was spotted onto Whatman filter paper no. 3, allowed to dry for at least 1 h at room temperature in an area free of environmental contamination and stored in individual zip-lock bags.
at 4 °C until used. Feathers were immediately placed in individual zip-lock bags and stored at 4 °C until used.

**PCR, HI and HA assays.** Dried blood spots were excised from the filter paper using a stationary hole puncher (OfficeWorks) according to the methods previously described by Bonne et al. (2008), deposited into 1.5 ml Eppendorf tubes and DNA extracted using a QIAamp DNA Blood Mini kit (Qiagen). PCR for detection of BFDV DNA was performed as described by Ypelaar et al. (1999). Anti-BFDV HI antibody detection was performed on dried blood spots as described by Riddoch et al. (1996) and HA testing was performed on feather extracts according to the protocol developed by Raidal et al. (1993c).

**Quantitative PCR (qPCR).** The above-mentioned DNA extracts were tested by qPCR and this was also performed on DNA extracts of feather samples. Primers were designed based on conserved regions of known BFDV sequences (Wishart & Fortin, 2001). Primers P5 (5'-GGACGCAAAATGAAGGAAG-3') and P6 (5'-TAGCGAGAGGTTATGCAAGCG-3') (GeneWorks) were designed to amplify an 81 bp fragment of ORF V1. Magnesium chloride concentrations and annealing temperatures were optimized using an Eppendorf Mastercycler Gradient thermocycler. The optimized PCRs consisted of 2 mM MgCl₂, 5 µl 5 × polymerization buffer containing dNTPs, 3.34 µM SYTO9 fluorescent dye (Invitrogen), 12.8 pmol each primer and 0.1 U Tth Plus DNA polymerase, in ultrapure water in a total volume of 25 µl (all reagents Fischer Biotec, except SYTO9), plus 2 µl extracted DNA. Reactions were carried out in a Corbett Rotor-Gene 3000 (Corbett Research) real-time thermocycler. Cycling conditions consisted of an initial denaturation at 95 °C for 5 min, then 40 cycles of 95 °C for 20 s, 58 °C for 30 s and 72 °C for 20 s, followed by a final extension step at 72 °C for 10 min. Known-copy-number DNA standards were included in each run for quantification of viral load. The cycle threshold (Cₚ) values obtained for these standards were plotted against time to construct a standard curve using the software supplied with the Rotor-Gene. The Cₚ of each of the samples was then compared against the graph to estimate the viral load. Melt curve analysis was performed after each run.

**Extraction of DNA from feathers.** Viral DNA was extracted from feathers using the methods described by Ypelaar et al. (1999). Five millimetre feather calamus was cut on a sterile surface and placed into a microcentrifuge tube (Eppendorf). To this, 200 µl 70% (v/v) ethanol was added and the tube vortexed briefly, the ethanol was removed and 200 µl sterile distilled water were then added and the tube vortexed again. The sterile water was removed and 500 µl lysis buffer [50 mM KCl, 10 mM Tris/HCl (pH 8.0), 2.5 mM MgCl₂, 0.005% (v/v) Tween 20, 0.005% (v/v) Nonidet P40] containing 250 µg proteinase K ml⁻¹ (Qiagen) was added. The feather in lysis buffer was incubated at 37 °C for 1–2 h, before being heated to 95 °C for 10 min. The solution was centrifuged and DNA was extracted from the supernatant with the Qiagen blood mini kit, using the blood and body fluid spin protocol.

Crude DNA extracts of feather eluates prepared for HA testing were also made. Feathers were incubated with 100 µl PBS at 60 °C for 1 h in a microcentrifuge tube (Eppendorf). The solution was centrifuged briefly after incubation and 10 µl supernatant transferred to another microcentrifuge tube and then boiled for 10 min.

**Statistical analysis.** One-way ANOVA with Tukey’s multiple comparison post test was performed using SigmaPlot 9.01 for Windows (Stystat Software) to test for significant differences in mean HI titres between the two groups (vaccinated and non-vaccinated) and between time points within each group.

**Clinical examinations.** Clinical examinations were performed weekly and the birds were inspected for evidence of individual developing feathers with thickening of the feather sheath, feather constrictions and other clinical signs consistent with PBFD, previously described by others (Jacobson et al., 1986; Jergens et al., 1988; McOrist et al., 1984; Pass & Perry, 1984).

** Necropsy and pathological examinations.** All birds were killed and necropsied 270 days post BFDV challenge. At this time blood and tissue samples were collected for serology, virus detection and histopathology. Feather samples were collected for HA and PCR assay. Liver was collected and stored at −20 °C for PCR and blood was collected for HI antibody and PCR.

Tissue samples collected post mortem included skin and coelomic visceral organs, which were fixed in buffered formalin for 24 h and then processed by routine histology methods, stained with haematoxylin and eosin and examined by light microscopy. Immunohistochemistry (IHC) using primary monoclonal antibody (Shearer et al., 2008a) to recombinant BFDV capsid protein and a horseradish peroxidase (HRP)-conjugated secondary antibody were performed on tissue sections as described by Shearer et al. (2008b).

**RESULTS**

**PCR**

Samples collected from the birds during the hand-rearing period, the day of primary and secondary vaccination and the day of live BFDV challenge were all negative for BFDV DNA by PCR. Thirteen days post-challenge with BFDV, all five non-vaccinated control birds had become PCR-positive for BFDV DNA and none of the vaccinated birds had detectable BFDV DNA. Similarly, at 20 days post-challenge, all non-vaccinated control birds were still positive, and still none of the vaccinated birds had PCR-detectable BFDV DNA. In contrast, at 26 days post-challenge one vaccinated bird had become positive and four non-vaccinated control birds remained positive. Forty-one days post-challenge, three non-vaccinated control birds tested PCR positive and three vaccinated birds had positive results (Fig. 1). Ninety-seven days post-challenge all birds were PCR negative. Table 1 summarizes the PCR results from the day of challenge until the end of the experiment.

**qPCR**

The assay successfully detected BFDV DNA in the blood of all control (non-vaccinated) corellas (Fig. 2a). In non-vaccinated control birds the viral load ± SEM was estimated at 1.358 473 ± 1.113 226 copies µl⁻¹ (range 14 478–5 768 973 copies µl⁻¹) at 2 weeks post-challenge and rose to a peak of 4 850 482 ± 4 775 008 copies µl⁻¹ (range 1709–23 949 983 copies µl⁻¹) by 4 weeks post-challenge before dropping to 575 486 ± 551 069 copies µl⁻¹ (range 823–2 779 419 copies µl⁻¹) at 6 weeks post-challenge. Transient low-level viraemia of between 58 and 4057 copies µl⁻¹ was detected in six vaccinated birds at various time points, but all birds were seropositive at the times when viral DNA was present in blood samples. The viral load in blood samples of vaccinated birds followed a similar pattern to that of the
non-vaccinated control birds, with mean viral loads increasing in a curvilinear fashion from 58 copies mL\(^{-1}\) at 2 weeks post-challenge to a peak of 1177 ± 723 copies mL\(^{-1}\) (range 209–4057 copies mL\(^{-1}\)) by 6 weeks post-challenge (Fig. 2a). Specific peaks were present in the melt curves of BFDV-positive samples between 82 and 84.5 °C.

The assay also detected BFDV DNA in feather preparations (Fig. 2b). The mean viral load ± SEM in feather extracts of control birds was estimated at 8831 ± 4662 (range 262–20 639 copies mL\(^{-1}\)) at 2 weeks post-challenge, rose to a peak of 852 500 ± 681 941 583 copies mL\(^{-1}\) (range 2591–3 409 766 576 copies mL\(^{-1}\)) at 4 weeks post-challenge, then decreased to 380 734 071 ± 380 729 701 copies mL\(^{-1}\) (range 430–1 903 652 876 copies mL\(^{-1}\)) by 6 weeks post-challenge. Virus was detectable by HA in the feather samples of only one control bird at 4 and 6 weeks post-challenge, which were also the samples that had the greatest amount of viral DNA present. Viral DNA was present in the feather extracts of three vaccinated birds 2 weeks post-challenge, six vaccinated birds at 4 weeks post-challenge and 10 vaccinated birds 6 weeks post-challenge. However, none of the three birds with viral DNA in feather extracts at 2 weeks post-challenge had detectable viral DNA in the equivalent blood sample. At 4 and 6 weeks post-challenge, only one out of six and four out of ten birds, respectively, had detectable amounts of viral DNA in the equivalent blood sample (between 193 and 420 451 copies mL\(^{-1}\)). The samples which were positive for qPCR of feather extracts but negative for blood samples were considered to be false positives.

### Serology

Screening the birds for anti-BFDV antibody after arrival did not detect any HI-positive serum. The development of antibodies to BFDV during the vaccination and challenge period, as determined by HI, is illustrated in Fig. 1 and summarized in Table 2.

### Detection of virus excretion by HA

Excretion of virus from feathers could not be detected in any samples from vaccinated birds using HA. Of five non-vaccinated control birds, one bird tested positive by HA for excretion of virus in feathers. This bird was HA negative on

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**Table 1.** Summary of PCR results for vaccinated and non-vaccinated control birds from 0 to 270 days post-challenge.

<table>
<thead>
<tr>
<th>Days post-challenge</th>
<th>Vaccinated birds</th>
<th>Non-vaccinated control birds</th>
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<tbody>
<tr>
<td></td>
<td>Number of positive birds</td>
<td>Number of negative birds</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>13</td>
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<tr>
<td>13</td>
<td>0</td>
<td>13</td>
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<tr>
<td>20</td>
<td>0</td>
<td>13</td>
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<tr>
<td>26</td>
<td>1</td>
<td>12</td>
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<tr>
<td>41</td>
<td>3</td>
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<td>97</td>
<td>0</td>
<td>13</td>
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<tr>
<td>270</td>
<td>0</td>
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**Fig. 1.** Comparison of vaccinated (n=13) and non-vaccinated (n=5) corellas showing seroconversion after vaccination and post BFDV challenge. Serum HI antibody titres as mean ± SD serum log₂ HI titres for each group and the number of seropositive birds are shown for each time point. The individual feather HA and serum HI antibody results for one non-vaccinated control bird (52A5) are shown as bars along with the time points (asterisks) when this bird was PCR-positive.
days 0, 11, 27, 40, 47 and 124. However, on day 53 (26 days post-challenge) and day 68 (41 days post-challenge) this bird had a HA titre of $\log_2 12$ HAU $50\,\mu l^{-1}$ and $>\log_2 12$ HAU $50\,\mu l^{-1}$, respectively. This bird’s HA and HI developments are illustrated in Fig. 1.

**Statistical analysis**

The Tukey's multiple comparison post test did not find any significant difference between group mean HI titres at each time point after challenge. However, vaccinated birds had significantly different HI antibody titres on day 27 compared with day 68 ($P<0.01$). Similarly, there were statistically significant differences between vaccinated birds on day 27 compared with day 124 ($P<0.01$); vaccinated birds on day 27 compared with non-vaccinated control birds on day 124 ($P<0.01$); vaccinated birds on day 40 compared with day 68 ($P<0.01$); vaccinated birds on day 40 compared with day 124 ($P<0.01$); vaccinated birds on day 40 compared with non-vaccinated control birds on day 53 ($P<0.05$); vaccinated birds on day 40 compared with day 124 ($P<0.001$); vaccinated birds on day 47 compared with non-vaccinated control birds on day 124 ($P<0.05$); and non-vaccinated control birds on day 40 compared with non-vaccinated control birds on day 124 ($P<0.05$).

**Clinical and post-mortem observations**

Non-vaccinated control birds developed feather sheath thickening and dysplasia of varying severity in individual developing powder-down and crest feathers 2–4 weeks post-challenge. This included pinching-off of the developing calamus, which is a pathognomonic sign for PBFD. No other clinical signs were observed in non-vaccinated control birds or vaccinated birds.

Compared with a geometric mean HI antibody titre of $\log_2 4.48$ (combined results) at 97 days post-BFDV challenge, at 270 days post-challenge the geometric mean HI antibody titre for vaccinated birds was $\log_2 4.38$ and for non-vaccinated control birds was $\log_2 4.40$.

**Table 2. Mean log₂ HI titres ± SD versus days post-challenge**

<table>
<thead>
<tr>
<th>Days post-challenge</th>
<th>Vaccinated birds</th>
<th>Non-vaccinated control birds</th>
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<tr>
<td></td>
<td>Mean log₂ HI titre</td>
<td>SD</td>
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<tr>
<td>0</td>
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<tr>
<td>11</td>
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<tr>
<td>27</td>
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<td>68</td>
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<tr>
<td>124</td>
<td>5.00</td>
<td>1.15</td>
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$n$: The number of birds that had detectable HI titre in that group. The vaccinated birds received the primary vaccine dose on day 0 and secondary dose on day 11. The live BFDV suspension was administered to all vaccinated and non-vaccinated control birds on day 27.
titre was log$_2$ 4.47 and all birds were blood-, feather- and liver-PCR-negative. All birds appeared clinically normal with no evidence of gross or histological lesions detected in skin or visceral organs. There was a range in size of the bursa of Fabricius, but in the majority the bursa was approximately of equal size to the spleen and both of these organs contained evidence of normal lymphoid follicles at different stages of development or involution and there was no evidence of intracytoplasmic BFDV inclusions. Also, there was no evidence of intracytoplasmic BFDV antigen in any tissues from IHC.

**DISCUSSION**

The results obtained in the current study indicate that the use of recombinant BFDV capsid protein is a potentially viable option for vaccination to promote an adaptive immune response to BFDV. All vaccinated corellas developed HI antibody titres after vaccination and did not develop clinical signs of PBFD after BFDV challenge. In contrast, non-vaccinated control corellas developed evidence of mild feather lesions in developing powder-down and growing crest feathers consistent with clinical PBFD after BFDV challenge.

The frequency of PCR- and HA-positive time points was less in vaccinated birds compared with non-vaccinated control birds. Vaccinated birds were never PCR-positive in blood at more than one time point and did not have detectable HA feather excretion, whereas non-vaccinated control birds remained PCR-positive for more than 3 weeks and HA feather excretion was detected twice. Excretion of virus in feathers is strongly associated with the presence of disease (Raidal et al., 1993c; Khalesi et al., 2005) and viraemia, detected by PCR on blood, was evident in non-vaccinated control birds and only transient viraemia could be detected in vaccinated birds.

The qPCR results indicated that there was reduced virus replication in vaccinated corellas compared with non-vaccinated control corellas. The qPCR assay detected viral DNA in birds that tested negative by the standard PCR and demonstrated that the viral load in both vaccinated and control birds increased for the first 4 weeks after challenge. That this occurred despite vaccinated birds having detectable anti-BFDV antibodies before being challenged indicates that vaccination does not prevent viral replication. This is a common scenario with other vaccines (Opriessnig et al., 2006) and is not surprising in this case. This evidence of viral replication may not have been detected if a standard PCR assay alone had been used. Given that vaccination does not prevent viral replication, it is likely that the chicks of vaccinated birds will still be susceptible to transient infection and virus replication in the presence of maternal antibodies, as occurs with porcine circovirus type 2 and chicken anemia virus (Brentano et al., 2005; Larochelle et al., 2000). This has implications for the management of the disease in infected flocks.

As in the earlier vaccination and challenge experiment by Raidal (1994), where vaccinated sulphur-crested cockatoos (Cacatua galerita) remained clinically normal after challenge and developed high HI titres, in the current experiment, none of the vaccinated corellas developed clinical signs of PBFD and all developed HI titres, albeit of a lesser magnitude than those observed by Raidal (1994). In contrast to the study by Raidal (1994), where non-vaccinated control birds developed acute PBFD, the non-vaccinated control birds showed only mild clinical signs, and only one bird was found to have BFDV excretion in the feathers. These differences between the two experiments could be due to either the storage of the inoculums, shown by HA titration to have resulted in a 2 log$_2$ decrease in viral titre, or to the lesser amount of inoculum delivered to each bird in the current experiment (approximately half of that used by Raidal, 1994). In the current literature, there is debate about the existence of species-specific or species-adapted genotypes of BFDV (Bassami et al., 2001; Khalesi et al., 2005; Phenix et al., 2001; Raue et al., 2004; Ritchie et al., 2003). Thus the species of bird may also affect the susceptibility to infection. However, in pathogenesis studies by Raidal (1994), 18 adult galahs (Eolophus roseicapillus), three nestling galahs and six nestling sulphur-crested cockatoos (6–7 weeks of age) were challenged with the previously mentioned inoculum. Only one adult galah had clinical signs indicative of PBFD, whereas the nestling galahs had a gradual onset of PBFD clinical signs and the nestling sulphur crested–cockatoos developed acute PBFD within 4 weeks and died.

The results also provide important information concerning the pathogenesis of PBFD as the PCR data show that not all BFDV-infected birds develop fulminant disease. Whilst non-vaccinated control birds developed mild clinical signs, only one bird (non-vaccinated control) had HA excretion in the feathers. This bird had test results typical of PBFD. Thus the results obtained here and those by Raidal (1994) are similar, even though Raidal (1994) observed a higher frequency and more severe clinical disease in non-vaccinated birds. One explanation for this is that the chicks used in the current paper received a lesser challenge dose of BFDV inoculum, but it is more likely to be due to their older age when challenged. It is well accepted that there is an age-related susceptibility to BFDV. In the present experiment it was very important to first confirm that the chicks were truly negative for BFDV infection and this required repeated PCR testing. This is the reason why they were first vaccinated at 65–89 days of age and not earlier. Indeed the results indicate that, at this age, corella fledglings may well be naturally resistant to developing PBFD but not to BFDV infection. This is supported by observations made in pathogenesis and epidemiological studies by Raidal (1994), where galah and sulphur-crested cockatoo chicks developed acute PBFD, whereas adult birds of these species remained clinically normal.

In the present experiment, vaccination with recombinant BFDV capsid protein elicited an immune response that could be detected by HI assay. The observation of mild clinical signs and detection of consistent viraemia by PCR
in non-vaccinated control birds shows that the challenge inoculum was infective and disease-causing, even after approximately 15 years of storage. Detection of only occasional transient viraemia in vaccinated birds, as opposed to the extended period of viraemia in non-vaccinated control birds, provides evidence that vaccination may be useful for preventing persistent viraemia and virus shedding, but not necessarily in completely preventing BFDV replication.

The small decline in HI antibody data between days 97 and 270 post BFDV-challenge indicates that anti-BFDV HI antibody persists in immune birds and may well do so for many years. Epidemiological data supports this observation (Raidal et al., 1993b). An annual rate of decline of \( \log_2 0.22 \) can be calculated from the data, because between these two time points we are confident that antibody titres were not being maintained by ongoing immune stimulation. The birds were held as a closed flock and no evidence of persistent BFDV infection or excretion was detected in blood samples collected on day 97 and blood, feathers and liver collected at post-mortem on day 270 post-challenge. At this time point they were all at least 1 year old and all, including the control birds, had normal lymphoid tissue in the bursa of Fabricius and spleen.

These results are significant for understanding the pathogenesis of PBFD, since it has been previously hypothesized that infected birds might have a long latency period of up to 2 years, whereby they remain clinically normal until the progression of the disease is accelerated following stress, or when moulting is commenced. It is also well accepted that birds with acute or chronic PBFD lesions suffer from a compromised immune system with the presence of characteristic intracytoplasmic viral inclusions readily identifiable in the bursa and the presence of chronically persistent viral excretion. As far as we are aware this is the first report that demonstrates the relatively long retention (greater than 1 year) of bursal activity in any \textit{Cacatua} species. The involution of the bursa of Fabricius in chickens occurs much earlier than this and it is completely atrophied by 24 weeks of age (Naukkarinen & Sorvari, 1984). Given the data presented in this present paper it seems likely that the bursa of Fabricius of \textit{C. tenuirostris} retains significant function as a peripheral lymphoid organ until at least 1 year of age. It may be that primary immunosuppression is first required to permit establishment of chronic BFDV infection and excretion and the pathogenesis of chronic feather and lymphoid lesions, at least in fledgling corellas up to the age of 2 years.

The present paper provides evidence that baculovirus-expressed recombinant BFDV capsid protein is immunogenic in \textit{C. tenuirostris} fledglings and may be a suitable candidate vaccine to prevent PBFD in psittacine birds. Further work is needed to determine an optimal vaccination regime to protect nestling birds, which are presumably more susceptible to infection. This would include determining the optimum antigen dosage per vaccination, the amount of time between primary and secondary vaccinations, the appropriate ages of birds that could be vaccinated, the positive and negative effects of passive transfer of maternal antibody, the choice and effect of a suitable adjuvant, and the safety of various routes of administration.

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