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A blocking ELISA for the detection of antibodies to psittacine beak and feather disease virus (BFDV)

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Abstract

Currently, the only diagnostic test available routinely for the sero-diagnosis of BFDV is the haemagglutination-inhibition (HI) assay. This test, whilst useful and applicable to samples from a wide range of psittacine birds, is not an ideal assay; it requires erythrocytes from live animals, virus purified from the feathers of infected birds and polyclonal antibody preparations in order to perform the assay. Variations in these reagents make consistency between tests difficult to achieve, underscoring the need for a new test with standardised reagents for the sero-diagnosis of BFDV infection which has led to the development of an antibody response. The methods used to develop a novel “blocking” (or “competitive”) ELISA (bELISA) for the detection of anti-BFDV antibodies in psittacine sera are presented in this paper. The assay was developed using a baculovirus-expressed recombinant BFDV capsid protein and a newly developed monoclonal antibody raised against this protein. The assay was then validated with 160 samples from eastern long-billed corellas (*Cacatua tenuiostris*) vaccinated with the recombinant capsid protein and challenged with live virus and samples from 82 cockatiels known to be HI negative. The bELISA described in this study is a sensitive and specific diagnostic test and should have wide application for the sero-diagnosis of BFDV.

1 Introduction

Psittacine Beak and Feather Disease (PBFD) is the most common viral disease of psittacine birds and causes either a chronic debilitating feather disease in adult birds (Albertyn et al., 2004, Pass and Perry, 1985, Rahaus and Wolff, 2003, Ritchie et al., 1989b) or a severe, acute disease syndrome in nestlings and African Grey parrots (*Psittacus e. erithacus*) (Doneley, 2003, Raidal and Cross, 1995, Schoemaker et al., 2000). The causative agent, *beak and feather disease virus* (BFDV), is a circovirus with a single stranded DNA genome, approximately 1.7-2.0 kb in length (Bassami et al., 2001, Ritchie et al., 1989b) and is considered to have a worldwide distribution (Albertyn et al., 2004, Hsu et al., 2006, Kiatipattanasakul-Banlunara et al., 2002, Kock et al., 1993, McOrist et al., 1984, Pass and Perry, 1985, Rahaus and Wolff, 2003, Ritchie et al., 1989a). Surveys using PCR-based assay methods have found prevalence rates of between 8% (Bert et al., 2005) and 39% (Rahaus and Wolff, 2003). The reported seroprevalence varies between 16% and 62% (Khalesi et al., 2005, Raidal and Cross, 1994) among captive flocks and between 41% and 94% in wild flocks (Raidal et al., 1993a).

Currently, an haemagglutination-inhibition assay (HI) (Raidal et al., 1993b, Ritchie et al., 1991) is the only method available for the detection of anti-BFDV antibodies in psittacine sera. This assay is useful detects both IgM and IgG antibodies from a wide range of species of psittacine birds, but it suffers from an appreciable amount of inter-test variation due to the variability in quality and quantity between virus preparations and the sensitivity of the erythrocytes used in the test to the virus. To overcome these limitations, a

novel blocking (or competitive) ELISA has been developed which utilises a baculovirus-expressed recombinant BFDV capsid protein (Stewart et al., 2007) and a newly developed monoclonal antibody raised against this protein (Shearer et al., 2008b).

2 Materials and Methods

Insofar as was practical, the bELISA was developed and validated in accordance with guidelines established by the Office International des Epizooites (O.I.E.) for the development of serological assays for the diagnosis of infectious diseases (Jacobson, 2004).

2.1 Samples

Samples of blood, collected on filter paper and air-dried and serum or plasma from 5 species of psittacine birds (including sulphur-crested cockatoos (*Cacatua galerita*), galahs (*Eolophus roseicapillus*) rainbow lorikeets (*Trichoglossus haematodus*), eclectus parrots (*Eclectus roratus*) and gang-gang cockatoos (*Callocephalon fimbriatum*)) known to have naturally occurring anti-BFDV antibodies were used to optimise the blocking ELISA (bELISA). These were selected to include a range of HI antibody titres. A sample of serum from a chicken inoculated with a baculovirus-expressed recombinant BFDV capsid protein (Stewart et al., 2007) was used as a low-HI antibody sample and normal chicken serum was included as a known negative control.

To validate the bELISA, samples from eastern long-billed corellas (*Cacatua tenuiostris*) that were part of an experiment to assess a recombinant BFDV capsid protein-based vaccine (Bonne et al., 2008, Stewart et al., 2007) were tested using the optimised bELISA assay. Samples of blood and serum or plasma, as described above, were taken from vaccinated (n = 15) and non-vaccinated control (n = 5) birds on 8 separate occasions, including the time of vaccination and the time of challenge with live virus. In total, 160 samples were thus collected. In addition, samples from 82 cockatiels known to be HI negative (Shearer et al., 2008a) were included to evaluate the diagnostic specificity of the test. Samples included both dried blood on filter paper and plasma.

For blood collected onto filter paper, two 6mm diameter spots of blood on filter paper were excised using either scissors or a hole punch and placed into a microcentrifuge tube (Eppendorf, Hamburg, Germany). One hundred microlitres of ELISA blocking buffer (PBS, 0.05% (v/v) Tween 20, 5% (w/v) skim milk powder (Fonterra Foodservices, North Ryde, Australia)) was then added in order to make a 1:5 (w/v) suspension and serum eluted from the paper by incubating the mixture for 1 hour at room temperature. Serum or plasma samples were diluted 1:5 in blocking buffer.

2.2 Production of recombinant BFDV capsid protein

A full-length, baculovirus-expressed BFDV capsid protein was expressed and purified as described by Stewart *et al* (2007).

2.3 Optimisation of antigen and antibody dilutions using indirect Enzyme-Linked Immunosorbent Assay

The indirect ELISA was optimised as described by Shearer et al (2008b). The recombinant protein was diluted to 0.25, 0.5, 0.75, 1.0, 1.25, 1.5, 2.0 and 2.5 µg/ml in 0.05 M carbonate/bicarbonate buffer, applied to duplicate 12-well rows of two Microlon 600 ELISA plates (Greiner BioOne, Frickenhausen, Germany) and allowed to coat at 4 ºC overnight. The plate was then washed with wash buffer (PBS, 0.05% (v/v) Tween 20), blocking buffer was added to all wells and the plate incubated for 1 hour at room temperature. Two solutions of the monoclonal antibody were prepared; a 1:25 and a 1:30 dilution in blocking buffer. After washing the protein coated plates again, 50 µL of blocking buffer were added to all wells of each plate. Then 50 µL of a 1:25 dilution of the monoclonal antibody were added to the first well of the first row of each protein dilution and 50 µL of a 1:30 dilution added to the first well of the second row. The mixtures from the first well of each row were then serially diluted across the plates, leaving 50 µL of each dilution per well and the plates incubated for 1 hour at room temperature. After washing again, HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich, St. Louis, USA), at the manufacturer's recommended dilution in blocking buffer, was added to all wells of both plates and the plates incubated for 1 hour at room temperature. The plates were washed again, then 50 µL/well of a solution containing ABTS (BioRad Laboratories, Hercules, USA) was added and colour allowed to develop for 15 minutes at room temperature. The colour development reaction was stopped by the addition of 2% (w/v) oxalic acid and absorbance at 405nm measured using a spectrophotometer.

2.4 Optimisation of the Blocking Enzyme-Linked Immunosorbent Assay for the Detection of Specific Antibodies

Recombinant baculovirus-expressed BFDV capsid protein was diluted to 2.5 µg/mL in 0.05 M carbonate/bicarbonate buffer, added to all wells of a Microlon 600 ELISA plate (Greiner BioOne, Frickenhausen, Germany) and allowed to coat at 4ºC overnight. The plate was then washed with wash buffer, blocking buffer added to all wells and the plate incubated for 1 hour at room temperature. After another wash step, 50 µL of blocking buffer was added to all wells of the ELISA plate. Then 50 µL sera from each of the 5 psittacine birds described above, eluted as previously described, was added to the first well of each row and serially diluted across the plate. The plate was then incubated for 1 hour at room temperature. After removing the sera, the plate was washed again and 50 µL/well of a monoclonal antibody against the recombinant BFDV capsid protein , diluted 1:400 in blocking buffer, was added and the plate incubated for 1 hour at room temperature. After washing, 50 µL of HRP-conjugated polyclonal anti-mouse IgG (Sigma-Aldrich, St. Louis, USA), at the manufacturer's recommended dilution in blocking buffer, was added to each well and the plate incubated for 1 hour at room temperature. After washing, 50 µL/well of a solution containing ABTS (BioRad Laboratories, Hercules, USA) was added and colour allowed to develop for 15 minutes at room temperature. The colour development reaction was stopped by the addition of 2% (w/v) oxalic acid and absorbance at 405 nm measured using a spectrophotometer. The percentage inhibition (PI) of the test sera

samples and negative cut-off value (based on the PI of the known negative samples) were then calculated.

2.5 Validation of the bELISA

To validate the bELISA, 160 samples from eastern long-billed corellas (*Cacatua tenuiostris*) vaccinated with a recombinant BFDV capsid protein (Bonne et al., 2008, Stewart et al., 2007) and challenged with live virus were tested using the optimised bELISA. Samples included both dried blood on filter paper and plasma. After testing, the PI of the samples were compared to the corresponding HI titres. In addition, samples from 82 cockatiels that had been assessed as negative by HI (Shearer et al., 2008a) were also tested using the optimised bELISA.

For blood collected onto filter paper one spot of blood on filter paper was cut out using scissors or hole punch and collected into a microcentrifuge tube (Eppendorf, Hamburg, Germany). 100 µL of ELISA blocking buffer was then added (to make a 1:10 (w/v) suspension) and serum eluted from the paper by incubating the mixture for 1 hour at room temperature. Serum or plasma samples were diluted 1:10 in blocking buffer.

Tests to evaluate the precision, repeatability and accuracy of the assay were also conducted; however it was not practical to evaluate reproducibility between laboratories. Repeatability assays were conducted using pooled sera from 10 vaccinated birds. The intra-assay coefficient of variation was determined by performing 10 replicates of pooled sera and the inter-assay

coefficient of variation was determined by repeating this group of replicates 10 times. Data from the assay of intra-assay repeatability was plotted to determine the assay's precision. The linearity of the assay was also investigated using 10 replicates of the pooled sera that were serially diluted 1:2 and the PI values plotted against the $\log_2$ of the dilution factor.

3 Results

3.1 Indirect Enzyme-Linked Immunosorbent Assay

The optimal amount of protein used in the indirect ELISA was determined to be 250ng/well and the optimal dilution of monoclonal antibody was 1:400. The indirect ELISA performed well at many other combinations of protein and monoclonal antibody dilution, but the above protein amount and antibody dilution were selected as they had a good positive absorbance value and a useful dynamic range.

3.2 A Novel Blocking ELISA for the Detection of Antibodies to BFDV

Sera from each of the 5 species of psittacine birds with naturally occurring anti-BFDV antibodies tested positive by bELISA, as did the sera from the chicken inoculated with the recombinant BFDV capsid protein. The optimal dilution of these sera for use in the bELISA was determined to be 1:10. This gave a useful dynamic range and a lower limit of detection (greater analytical sensitivity) than the HI test.

All cockatiels that tested negative by HI were also negative by bELISA. The corellas vaccinated with the recombinant protein had peak mean PI levels of between 6.01% and 44.26% at challenge (after the second vaccination) and between 62.43% and 90.22% after challenge with live virus (Figure 1). Non-vaccinated control birds had peak antibody levels of between 55.25% and 83.26% after challenge with live virus. Importantly, although the samples from birds vaccinated with the recombinant protein were HI-negative at the time of the second vaccination, the bELISA detected low levels of antibodies in these samples. The correlation coefficient between antibody levels measured by bELISA and HI titres was high ($r^2 = 0.8156$, $p<0.05$) (Figure 2). Based on the results of the vaccine trial samples only, sensitivity was 99.09% and specificity was 71.43% when compared to the HI assay, with a positive and negative predictive value of 87.2% and 97.56% respectively. Using the results of the cockatiel sero-survey, the bELISA was 100% specific and combining these results with the results of the vaccine trial gave the same sensitivity and increased the specificity to 88.41% and the negative predictive value to 99.19%. The mean PI value of the pooled sera was 64.28% and almost all repeated samples fell within ± 2 standard deviations of the mean value (Figure 3a). The intra- and inter-assay coefficients of variation were 3.61% and 6.94%, respectively and the bELISA was determined to be linear within the working range of the samples tested (Figure 3b).

4 Discussion

At present, HI and HA assays are the best available assays for the detection of anti-BFDV antibodies and excreted virus, respectively. However, given the

amount of variation inherent in both the HI and HA assays, novel diagnostic tests for both the virus and the presence of anti-BFDV antibodies in serum are highly desirable. Presented in this paper are the methods used to develop a novel blocking ELISA for the detection of anti-BFDV antibodies in psittacine sera. Blocking ELISAs have been developed for the detection of antibodies to other avian viruses and have the distinct advantage over indirect ELISAs that secondary antibodies specific to the immunoglobulins of the species being tested are not required. For current large-scale screening and sero-surveillance, blocking ELISAs have largely replaced indirect ELISAs (Gorham, 2004)

The sensitivity and specificity of the blocking ELISA developed in this study were within values defined as acceptable by the OIE (Jacobson, 2004) and should prove to be a useful diagnostic test for BFDV. The analytical sensitivity was greater than the HI test, as it detected antibodies in vaccinated birds that were HI negative and the comparative diagnostic sensitivity and specificity was good. However, because of the greater analytical sensitivity of the bELISA, the absolute diagnostic sensitivity and specificity is likely to be higher. Given the calculated diagnostic sensitivity, 160 samples is adequate to validate the assay with 95% confidence of an accurate result (Jacobson, 1998). However, assuming the highest calculated diagnostic specificity of 88%, the number of known negative animals required is 1 014. The actual number of known negative animals required is likely to be much less, since the actual diagnostic specificity is likely to be higher. Since it is so difficult to

find psittacine birds that have not been exposed to the virus, ongoing testing with birds of known antibody status is desirable.

The precision, repeatability and accuracy of the assay were within values accepted by the OIE (Jacobson, 1998). The cut-off value was determined from a 95% confidence interval of the negative control wells (Coligan et al., 2001) since it was almost impossible to find a group of birds known to be truly seronegative. Assuming, though, that the cockatiels were truly seronegative, the cut-off value calculated from a 95% confidence interval of the mean PI of those 82 samples was similar to that calculated using the negative control wells only. As more samples are tested using this assay, the methods of calculating a cut-off value can be compared with receiver-operator curves to see which method is more appropriate. The positive and negative predictive values were good and given the high seroprevalence of BFDV, should translate well to samples from naturally infected birds.

The correlation coefficient between bELISA values and HI titres from the vaccinated corellas (Bonne et al., 2008) was high, which is somewhat surprising given the differing nature of the two tests. The HI assay gives a defined cut-off, whereas the bELISA allows for continuous readings of PI values for samples with antibody levels which fall in between HI end-points. This difference means that although the correlation coefficient was high no direct inference between bELISA PI values and HI titres can be made as each HI end-point titre can be associated with a range of bELISA PI values (Figure 2). Thus, the bELISA is the preferred antibody test, as it allows more precise

characterisation of antibody levels. The intra- and inter-assay coefficients of variation were good and in line with OIE assay validation guidelines (Jacobson, 2004). Absorbance values fell within the linear range of the assay for the samples tested, however evaluation of the assay's behaviour for samples with PI values greater than 64.28% was not possible.

Only one other ELISA has been developed to test for the presence of anti-BFDV antibodies in psittacine sera (Johne et al., 2004). This was an indirect ELISA, utilising a truncated recombinant BFDV capsid protein and a secondary antibody directed against psittacine IgY. This ELISA tested 11 serum samples from 7 different psittacine species and thus has yet to be validated with a large number of samples of birds with known antibody status. Additionally, no studies have yet been conducted investigating the cross-reactivity of psittacine IgY and given that there are 78 genera and 332 species within the *Psittacidae*, such studies may be prohibitively difficult. This means, though, that a sample from a rare species of psittacine bird which tested negative by indirect ELISA could not be guaranteed to be truly negative. Consequently, we believe that a blocking ELISA, as described in this article, is likely to be a more reliable diagnostic test. The blocking ELISA also has the advantage that serum containing both IgM and IgY can be reliably tested using the one assay, as it does not rely on secondary antibodies directed against either class of immunoglobulin.

Although the ELISA described in this article has been tested with sera from 6 different species of psittacine birds and validated with 160 samples, further

testing is necessary to ensure that the assay performs well with sera from as many species of psittacine birds as possible. Any potential problems with consistency of the test between species would theoretically be the result of differing cross-reactivities between the test serum and the recombinant protein. Stewart et al. (2007) failed to find any differences in cross reactivity between the recombinant protein and a number of psittacine anti-BFDV antisera in both western blotting and HI. Additionally, cross-reactivity work using sera from rainbow lorikeets, short-billed corellas, a sulphur-crested cockatoo, a red lory (*Eos bornea*) and a galah-corella hybrid and virus from rainbow lorikeets, a red lory, two swift parrots (*Lathamus discolor*), a sulphur-crested cockatoo and a scarlet-chested parrot (*Neophema splendida*) failed to find evidence of antigenic serotypes (Khalesi et al., 2005). However, a similar experiment using sera from the above birds and virus eluted from the feather of a cockatiel found that sera from some of the birds did not inhibit agglutination by the cockatiel isolate, suggesting that the cockatiel isolate may be sufficiently different antigenically to be considered a separate serotype (Shearer et al., 2008a).

The monoclonal antibody used in this study recognised BFDV from a cockatiel, sulphur-crested cockatoo and a rainbow lorikeet (Shearer et al., 2008b). This indicates that there is some antigenic homology between isolates, even if the cockatiel BFDV isolate is indeed a separate serotype. Whilst it is possible that the epitope detected by this monoclonal antibody is not present in some BFDV isolates, the fact that no serotypes have been discovered to date suggests that enough major epitopes should be conserved

between isolates that polyclonal anti-BFDV sera will react with the recombinant protein. Bound sera will thus block the monoclonal antibody from binding, even if the test serum does not react with the exact epitope detected by the monoclonal antibody. Thus, the bELISA should be reliable for use with sera from many species of psittacine birds, unless experimental evidence comes forth that novel BFDV serotypes exist or that the epitope detected by the monoclonal antibody is not immunodominant. Answers to questions about the existence of a cockatiel-adapted BFDV serotype and the relative infectivity and antigenic characteristics of various virus isolates remain to be discovered. These answers may only be found by conducting HI assays using sera from many cockatiels, tested against virus eluted from the feathers from a range of psittacine birds.

The bELISA has a number of advantages over the HI test. First and most importantly, the bELISA is much more easily standardised as the amounts of both the recombinant protein and monoclonal antibody used in the assay can be quantified more accurately. Even though both the recombinant protein and monoclonal antibody are initially expensive to develop and produce, they are a much more reliable and consistent source of reagents than the virus preparation, polyclonal antibodies and erythrocytes used in HI assays. Virus used in the HI assay must be purified from the feathers from persistently infected birds. This process is expensive, ethically questionable, time consuming, results in low yields of virus and the extraction procedure can be contaminated with host proteins. The sensitivity of erythrocytes to BFDV may vary between individuals within a species (Sanada and Sanada, 2000) and

the sensitivity of an individual bird's erythrocytes may also vary over time. Variation in the amount of virus between preparations and decreased HA activity of the virus over time compounds this problem. Even though this variation may be standardised to an extent, HI assays are still prone to an appreciable amount of inter-test variation.

Other advantages of the bELISA over the HI assay are reduced sample preparation time and the potential to shorten the time taken to perform the assay if plates are pre-coated and blocked in bulk, or if the monoclonal antibody is directly conjugated to HRP. However, the effect of these modifications on assay performance would need to be assessed. The bELISA could also be adapted for the performance of cross-reactivity assays; however possible differences in affinity of the monoclonal antibody for the different virus samples used as antigen in the assay would need to be determined and taken into account.

Overall, the bELISA described in this paper should be a useful tool for the sero-diagnosis of BFDV infection. It is more readily standardised, simpler to perform, more repeatable and has a greater analytical sensitivity than the HI assay. In the future it should also provide valuable information in subsequent studies on the pathophysiology of the virus, such as the fluctuations in antibody levels at various stages of disease and the transfer of maternal antibodies and their effect on infection and immunity. In this experiment it was able to provide the valuable information that the vaccinated corellas had developed antibodies against the recombinant protein after the first

vaccination and it should also prove to be very useful in the further optimisation of BFDV vaccination protocols.

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Figures

Figure 1: Blocking ELISA (bELISA) mean percentage inhibition values of vaccinated (○—○) and control (●…●) birds after vaccination and challenge. Vaccinations are indicated by an arrow (↑) and challenge with a triangle (Δ). Bars indicate standard error.

Figure 2: Correlation of haemagglutination inhibition (HI titres) with blocking ELISA (bELISA) percentage inhibition (PI)

Figure 3: A: Precision assay, note that almost all values fall within ±2 standard deviations from the mean; B: Linearity assay using sera pooled from 10 vaccinated birds. The percentage inhibition (PI) values are linear within the dynamic range of the test. Bars indicate standard error.

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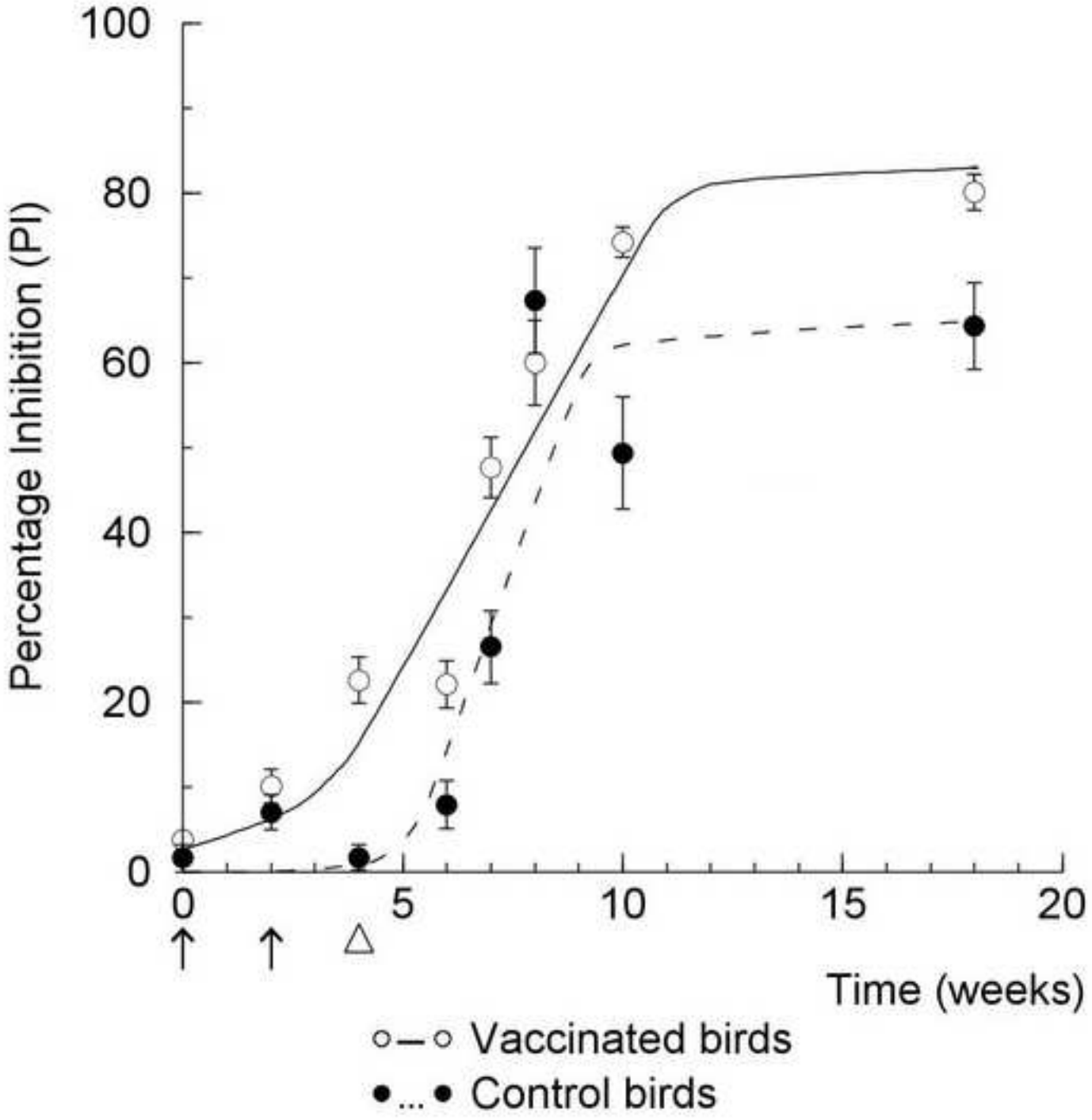


Figure2

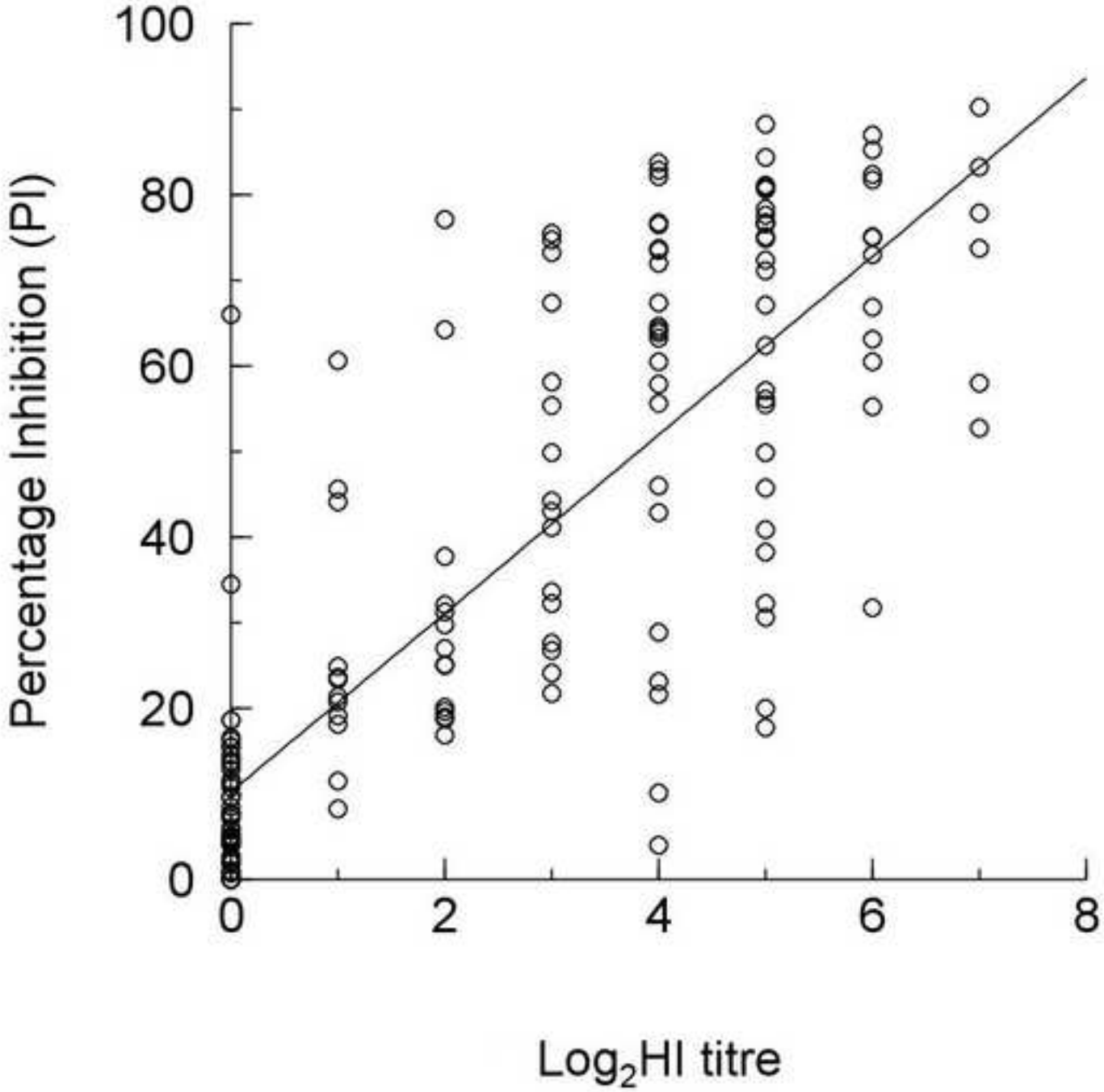
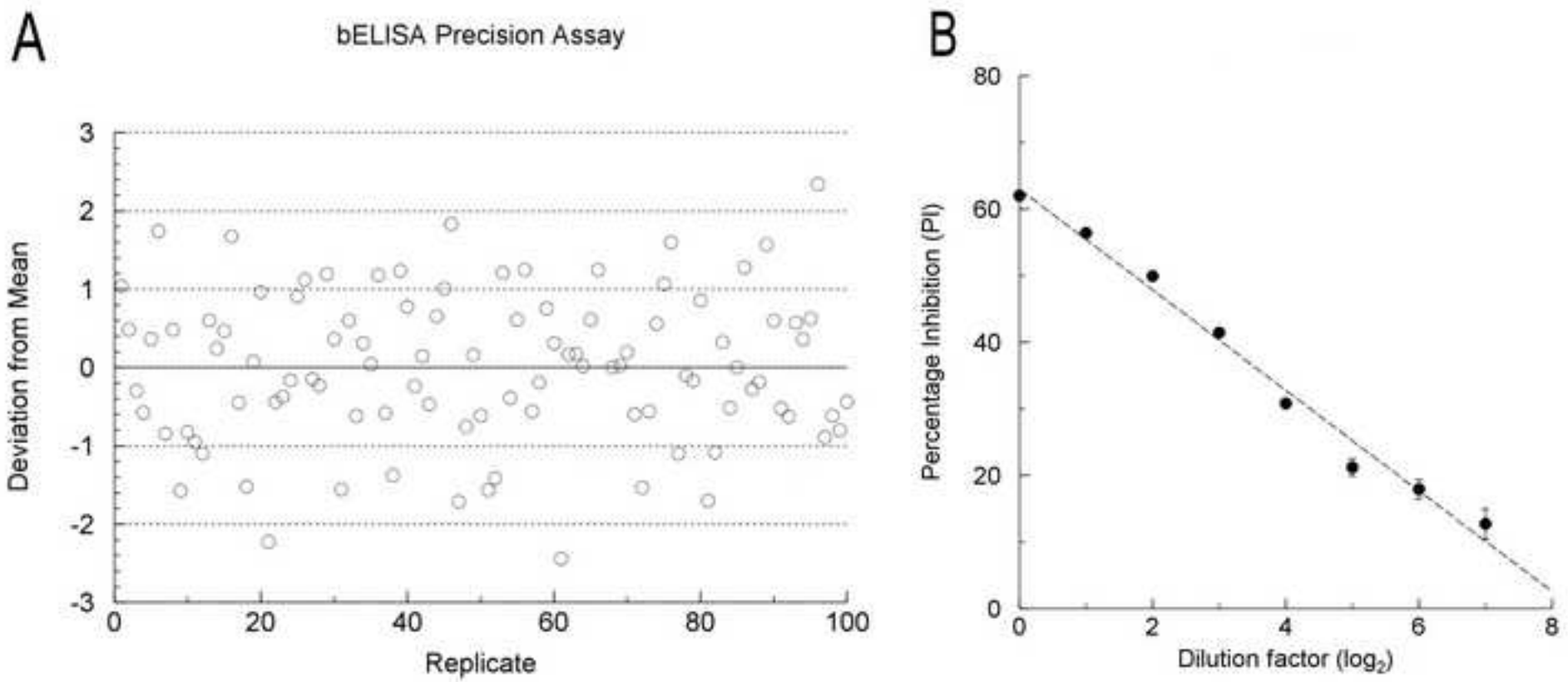

Percentage Inhibition (PI)
100
80
60
40
20
0
0
2
4
6
8
Log2HI titre

Figure3


A
bELISA Precision Assay
Deviation from Mean
3
2
1
0
-1
-2
-3
0
20
40
60
80
100
Replicate
B
Percentage Inhibition (PI)
80
60
40
20
0
0
2
4
6
8
Dilution factor (log2)