Adaptations of a Competitive Enzyme-Linked Immunosorbent Assays for the Detection of Antibodies to *Influenza A Virus* in Horse Sera for Use in Wild Aquatic Birds

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Received: 14 August 2011 / Accepted: 26 June 2012 / Published online: 2 August 2012
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Abstract We applied a competitive enzyme-linked immunosorbent assay for the detection of antibodies for influenza A in equine sera to their detection in sera from wild aquatic birds. Suboptimal results were obtained for the optical density (OD) of the monoclonal antibody (MAb) control and reproducibility between duplicate analyses in the initial assessment. It was therefore necessary to modify the assay to deliver increased reliability and reproducibility while maintaining adequate sensitivity. We optimized reagent concentrations to obtain optimal OD values (close to 2) for the monoclonal antibody control and used 2, 2'-Azino-bis: 3-Benzthiazoline-6-Sulphonic Acid as an alternative chromogen to potentially reduce variability in duplicate analyses. The original assay was compared with the optimized versions, with and without post coating, for the detection of avian influenza viral antibodies in 240 sera obtained from wild plumed whistling ducks. A separate analytical sensitivity study on diluted positive field sera of

plumed whistling ducks and a test of antigen stability after post coating were also performed. Some quantitative differences were detected between the original and modified assays. The original assay recorded higher percentage inhibition results which were potentially indicative of increased sensitivity. However, when reagent concentrations were increased in the original assay to the same levels as used in the modified versions, there were no quantitative differences for practical purposes. The original assay produced a median (OD) value of 0.81 for the (MAb) controls that is at the limit of acceptability. By contrast, the modified assays always produced acceptable optical density values for MAb controls. Our overall results indicated the modified assays were potentially more reliable (OD values close to 2), and of adequate sensitivity compared to the original assay in the detection of avian influenza viral antibodies in wild bird sera. Although further optimization of antigen and MAb concentrations should also be considered to increase the sensitivity of a modified assay, while maintaining acceptable optical density values for the MAb control. Post coating had a minimal quantitative effect on the results and stabilized the plates for 214 days. We therefore recommend the incorporation of post coating.

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Keywords Competitive Enzyme-linked immunosorbent assay · Plumed whistling duck (*Dendrocygna eytoni*) · Avian Influenza serology · Australia

Introduction

Indirect enzyme linked immuno-sorbent assays (iELISAs) were the first technique to detect avian influenza viral (AIV) antibodies in poultry flocks [1, 2, 10]. The iELISA

requires species-specific conjugated antibodies; therefore a different conjugated antibody is needed for each host species tested. Competitive ELISAs (cELISAs) are not species-specific like iELISAs because the secondary antibody is specific for the MAb used, regardless of the species being tested [4, 8, 12]. In regards to other tests used to detect AIV antibodies, a cELISA was more sensitive and specific than the agar gel immuno diffusion (AGID) test [4, 9], and as or more sensitive and specific as the haemagglutination inhibition (HI) test [3, 4, 9, 12]. A cELISA was also able to detect antibodies at an earlier stage of infection compared with the AGID and HI tests [5, 7, 11, 13]. Therefore, cELISAs are useful multi species diagnostic tests.

The Australian Animal Health Laboratory (AAHL) developed a cELISA (designated here as AAHL-1) in order to detect equine influenza viral antibodies from horse sera in Australia [8]. The equine influenza virus is of avian origin (H3 subtype) and its nucleoprotein (NP) is conserved across the Type A influenza viruses. The MAb raised against the NP in the AAHL-1 test is in the conserved region and therefore will detect other Type A influenza viruses. For this reason the assay was adopted for the detection of AIV antibodies in chickens, followed by wild bird sera, in Australia although the ability of the test to detect AIV antibodies in bird sera had not been rigorously assessed or published.

AAHL provided its protocol (AAHL-1) and necessary reagents to screen AIV antibodies in serum samples from wild aquatic birds (WABs) in our large AI surveillance program in northern Queensland, Australia. As a starting point, we used the AAHL-1 test without any modification to screen AIV antibodies from 411 field serum samples from WABs and observed low and variable optical density (OD) values for the MAb control. In some instances, the OD values for the MAb did not meet the minimum quality control OD value of 0.8 [8]. It was demonstrated that the concentrations of the AIV antigen, MAb and conjugate as described by Selleck [8] were suboptimal and responsible for the low OD of the MAb.

In addition, the variation in percentage inhibition between duplicate wells exceeded $\pm 10\%$ which also did not meet quality guidelines [8]. The use of 3, 3', 5, 5'-Tetramethylbenzidine (TMB) as a chromogen with a short incubation period (e.g., 5 min in AAHL-1) can increase background noise, variability between duplicate analyses and the possible generation of false positive or negative results.

It was therefore necessary to modify the assay to ensure adequate sensitivity and reliability for the analysis of all samples collected in our AI surveillance program. We optimized reagent concentrations to obtain optimal OD values (close to 2) for the MAb control (this was done by

doubling the recommended reagent concentrations, data not shown) and used 2, 2'-Azino-bis: 3-Benzthiazoline-6-sulphonic acid (ABTS) as an alternative chromogen to potentially increase reliability. The chromogen ABTS has slower color development and less background staining potentially reducing variability. We also included an additional step of using post coating to have more stable AIV antigen in plates with the aim of avoiding non specific results and facilitating long term storage and reducing between-run variability.

In order to assess the effects of these modifications on the sensitivity and reliability of AIV antibody detection and to ensure that the modified assays were suitable for use in our study, we compared the AAHL-1 [8] technique with the modified assay, with and without post coating (referred to as James Cook University-1) (JCU-1 and JCU-2, respectively). For this comparison, we analysed 240 sera obtained from wild plumed whistling ducks (PWDs) (*Dendrocygna eytoni*). We concurrently compared the three assays with the AAHL-1 technique but using double the recommended reagent dilutions (now indicated as AAHL-2).

A separate study for the four assays (AAHL-1 and 2 and JCU-1 and 2) was performed on diluted positive field PWD sera to compare analytical sensitivity. The longitudinal stability of the AIV antigen following post-coating was assessed to determine if storage of large batches of plates was feasible in order to make the test more efficient and obtain more consistent results between sample batches. To summarize our aims are to assess the AAHL-1 cELISA for detecting AIV antibodies in wild bird sera and to determine whether our modifications affect reliability and sensitivity.

Materials and Methods

Sera

Plumed whistling ducks were caught from December 2007 to December 2009 at Billabong Sanctuary and Green Acres Lagoon, located south of Townsville, north Queensland, Australia. Whole blood samples (0.5–3 ml, $<1\%$ of body weight) were drawn aseptically from wing veins. The samples were transferred immediately to sterile 15 ml plastic tubes. Blood samples were refrigerated within 5 h of sampling. After over-night storage the samples were returned to air conditioned room temperature (22°C) before centrifugation at $317g$ for 10 min. Supernatant was decanted, transferred to a 1.5 ml microfuge tube and centrifuged at $2,348g$ for 2 min and stored at -20°C until analysed.

Competitive Enzyme-Linked Immuno Sorbent Assay

Four cELISAs were assessed under an equivalence study that included AAHL-1 [8] and three modified versions designated as AAHL-2, JCU-1 and JCU-2. We used U-bottom microtitre plates instead of the AAHL suggested Nunc maxisorp plates and H_3PO_4 (stop solution) in place of H_2SO_4 for this study. Modifications are described under the procedure of cELISA below.

1. The recombinant AIV antigen (source-Paul Selleck, AAHL) was diluted at a rate of 1 in 800 (for AAHL-1) or 1 in 400 (for the AAHL-2 and JCU assays) in coating buffer (sodium bi-carbonate 3.11 mg, sodium carbonate 1.38 mg and distilled water 1,000 ml; source-TropBio Pty Ltd, JCU) and 50 μl was loaded into each well of a round (U) bottom 96-well-microtitre plate. The plate was covered and incubated overnight at 4° C for AAHL-1, AAHL-2 and JCU-1 and in a sealed humidified box for JCU-2.
2. One of the following steps was then followed: for the AAHL-1 and 2 and JCU-1 assays, the diluted coating buffer was removed and the wells were washed using wash buffer (Tris-technical grade-MP Biomedicals, sodium chloride-AR grade (Crown scientific), di-sodium salt-EDTA-AR grade, Pronelis, tween 20 (Sigma); Source-TropBio) with the aid of a squeeze bottle. Washing consisted of 4, 5-s rinses, with complete emptying of wells between rinses. After washing, the plate was inverted and tapped on a paper towel to remove any residual wash buffer. The plates were covered with lids immediately after washing to prevent drying; for the JCU-2, the diluted coating buffer was removed and 100 μl of post coating buffer (Proprietary, TropBio, Cat. No. 05-004-05) was dispensed into each well and kept in a humid box for 2 h. After incubation, the residual coating buffer was removed and the plate was tap dried with a paper towel and incubated at 37 °C for 2 h.
3. Each test serum was diluted at a rate of 1 in 10 with serum diluent (Tris-MP Biomedicals, sodium chloride, di-sodium salt-EDTA, casein, tween 20, bromophenol blue-BioRad-1.0 % in TEN buffer, distilled water; source-TropBio). Dilutions were made in a 96-wells transfer plate to facilitate mixing.
4. AIV antibody positive-control chicken sera provided by AAHL (Source, Paul Selleck, AAHL) was diluted in serum diluents at a rate of 1 in 50 and 1 in 500, whereas the AIV antibody negative-control chicken sera provided by AAHL (Source, Paul Selleck, AAHL) and AIV negative sera collected from JCU reared chickens were diluted at 1 in 10.
5. For the equivalence study, test sera and controls (positive, negative and conjugate) were tested in duplicate, whereas four wells were used for the MAb control. Fifty μl of diluted test serum and all controls were transferred from the dilution plate to the antigen coated plate. The plate was covered immediately with a lid and incubated at room temperature (22° C) for an hour.
6. Nucleoprotein mouse MAb (Source, Paul Selleck, AAHL) was diluted at 1 in 800 (for AAHL-1) or 1 in 400 (for the AAHL-2 and JCU assays) in serum diluent and 50 μl added to all wells immediately after the serum incubation step except for the wells specified for the conjugate control wells. The plate was covered and incubated for an hour at room temperature (22° C) and the plate was then washed as described in step 2.
7. Jackson goat anti-mouse immunoglobulin HRPO conjugate for AAHL-1 and 2 (Code #115-035-146) (Source, Paul Selleck, AAHL) and goat anti-mouse IgG (H + L) HRP conjugate for the JCU assays (Blotting grade, Cat. No. 170-6516, Bio-Rad Laboratories Pty Ltd, Regents Park, NSW) were diluted at a rate of 1 in 2,000 (AAHL-1) and 1 in 1,000 (AAHL-2 and JCU assays) in serum diluent just prior to addition to the plate and 50 μl of diluted conjugate was dispensed to each well. A lid was put on the plate and incubated at room temperature (22° C) for an hour.
8. During the conjugate incubation, the substrate buffer TMB (TMB Microwell peroxidase substrate system, KPL, Cat. No. 50-76-00) for the AAHL assays and ABTS (KPL, Gaithersburg, MD 20878, USA, 301.948.7755, product code: 50-66-06) for the JCU assays were removed from the fridge and left on the bench for 45–60 min before use. The plate was washed as previously described at the end of conjugate incubation. One hundred μl of substrate buffer was dispensed to each well of the plate. After the addition of TMB, the plate was incubated for 10 min at room temperature (22° C) followed by the addition of 100 μl of stop solution (1 M H_3PO_4) to each well. The OD of the reactions was read, within 5 min of adding the stopping reagent (Phosphoric acid (1 M H_3PO_4) add 16.88 ml of concentrated H_3PO_4 to 250 ml of distilled water) using an ELISA reader (Multiskan Ex, Labsystems Pathtech) at 450 and 650 nm. At the end of 1 h incubation with the ABTS substrate solution, the OD of the reactions was read at 414 and 492 nm using an ELISA reader (Multiskan Ascent Pathtech, Thermo Electron Corporation).
9. The results were expressed as a percentage inhibition relative to the OD of the MAb control using the

following formula: $100 - (100 * (\text{OD test serum mean} / \text{OD MAb control mean}))$. The calculation was performed using MS excel 2003.

Equivalence Study

The study required 230 samples based on the formula below. It incorporated permitted differences between equivalent tests, their variance and levels of acceptable type 1 and 2 error: $[2 * (Z_{\alpha} + Z_{\beta})^2 * SD^2] \div \delta^2$ [6] where Z_{α} , value of the standard normal distribution at specified level of confidence = 1.96 (2-tailed), Z_{β} , power of the study = 1.28 (2-tailed), (Standard deviation, SD)² = the variance (0.02), δ (permitted difference) = 0.04. Variance estimates were obtained from previous serology results. The permitted level of difference was $\pm 10\%$ between wells within a plate [8].

Therefore, we conservatively assumed $\pm 4\%$ permitted difference between assays for sample size calculation.

Two hundred and forty serum samples were randomly selected from 500 samples that were initially screened by JCU-2 for the equivalence study comparing AAHL-1, JCU-1 and JCU-2. One hundred and sixty of the analysed serum samples were also concurrently tested using AAHL-2.

Analytical Sensitivity Study

Two pooled PWD sera samples were made to test the analytical sensitivity of assays AAHL-1 and 2 and JCU-1 and 2: one from 10 individual positive sera samples, each with 80–96 % inhibition and one from 10 individual negative sera samples, each with 10 % inhibition. The positive and negative sera were randomly selected from 15 positive and 120 negative sera as previously assessed by JCU-2.

A pooled sera sample was then diluted at 1 in 10 with serum diluent before making a dilution series of each. We tested the following eight dilutions, based on the results of two small prior experiments: 1 is to 0 (positive: negative) (0 % dilution, undiluted positive pooled sera), 1 is to 0.5 (33 %, mixed positive–negative pool), 1 is to 1 (50 %), 1 is to 2 (67 %), 1 is to 3 (75 %), 1 is to 4 (80 %), 1 is to 8 (89 %) and 0 is to 1 (100 %, undiluted negative pooled sera).

We tested 10 replications per dilution using the four assays. For quality assurance, we included the AAHL positive chicken sera at the dilutions of 1 in 50, 1 in 500 and 1 in 5,000, each consisting of two replications, and negative sera at the dilution of 1 in 10 and 1 in 100, each consisting of one replication. In addition, we included four conjugate and four MAb controls. We used the first two columns (16 wells) for all the controls and the remaining 10 columns (80 wells) of U-bottom ELISA micro-titre

plates for the analysis of the diluted sera. The sensitivity testing was performed concurrently for each assay.

The Longitudinal Effect of Post-Coating on AIV Antigen Stability in Plates

We prepared eight wells of 10 U-bottom microtitre plates, as previously described, and stored them at 4° C until testing. The plate coating was as described for the JCU-2 assay (step 1–2). The test procedure was as described for the JCU-2 assay (step 6–8). The only exception was that we dispensed serum diluents at 50 μ l per well instead of using test or control sera. The first two wells of a column of a plate were allocated for the conjugate controls and the remaining six wells for the MAb controls. Individual plates were tested at 0, 1, 2, 4, 7, 14, 28, 60, 79 and 214 days after preparation. The OD values for each plate were read at 414 and 492 nm (Multiskan Ascent Pathtech, Thermo Electron Corporation).

Statistical Analysis

Equivalence Study

Data obtained were entered in the MS Excel-2003, coded and sorted and then exported into STATA™ 11.0 (Stata Corporation, College Station, TX) for analysis. Data were cross-checked for validity and consistency.

We defined an acceptable difference in percentage inhibition between wells within a plate as 10 %, as indicated by the AAHL [8]. The average percentage inhibition of an individual sample was calculated. For equivalence testing we calculated the mean difference in percentage inhibitions (MDPI) with their corresponding 95 % confidence intervals (CIs) between the assays. For the equivalence experiment ($N = 240$), we compared the results between the following paired assays: AAHL-1 versus JCU-1; AAHL-1 versus JCU-2; JCU-1 versus JCU-2. For a subset of the results ($N = 160$), we also compared all four tests including AAHL-2. The assays were considered equivalent when the 95 % CI of the difference in average inhibition was within $\pm 5\%$.

Categories of negative and positive for AIV antibodies were used as a binary response variable. Sera that produced $< 40\%$ inhibition were negative and sera that produced $\geq 40\%$ inhibition were positive [8]. In addition, a study has found that the % inhibition values for AIV antibody negative control sera ranged from 0 to 27 % using cELISA for a range of bird species, further supporting the need for a threshold of $\geq 40\%$ inhibition [12]. We constructed two by two tables comparing the number of positive and negative results obtained for each assay, in pair-wise comparisons. McNemar tests were performed to compare the proportions

of two discordant pairs in each table as a qualitative measure of equivalence. We expressed and interpreted the results as a difference between the discordant paired proportions with the p value and 95 % CIs.

We also compared the MAb control results between the assays using the Wilcoxon rank sum test (unpaired test) followed by a median test (unpaired test). The results were expressed as mean and median OD values with the p values.

Analytical Sensitivity Study

For the sensitivity study we tested a dilution series of pooled PWD sera. A descriptive statistic was carried out on the mean percentage inhibition in relation to different dilutions of serum samples and the results were presented in a line graph.

In the dilution series we had 0 % dilution (undiluted positive pooled sera), 33–89 % (mixed positive–negative sera) and 100 % (undiluted negative pooled sera). Therefore, we fitted three linear models. For the 0 % dilution, we performed the one-way-ANOVA (Model-A) on the mean percentage inhibition between assays. For the 33–89 % dilutions, we performed two-way-ANOVA (Model-B) on the mean percentage inhibition for the variables of assay and dilution factors. For the 100 % dilution, once again we used the one-way-ANOVA (Model-C) on mean percentage inhibition between assays.

Contrasts were carried out to determine the differences between cELISA types within each dilution of 0 and 100 % and to explain the interaction between dilutions (33–89 %) and ELISA types on mean percentage inhibition.

The Longitudinal Effect of a Post-Coating Buffer on AIV Antigen Stability in Plates

In the post-coating buffer experiment, the OD values of paired wells were used to calculate mean OD values. To test for differences in mean OD of the MAb, we used a one-way analysis of variance. The results were expressed as a mean, 95 % CI and p value.

Results

Equivalence Study

Mean Difference in Percentage Inhibition Between the Assays

The 95 % confidence limits (CIs) of the difference in percentage inhibition between the modified versions of cELISAs JCU 1 and 2 ($N = 240$) were 4.5–7.2 % (Table 1) and therefore it is ambiguous whether the assays are equivalent given that we regard a difference ± 5 % as acceptable. The results for AAHL-1 and JCU-1 were similar having a 95 % CI of 4.8–6.8, therefore, also ambiguous with regard to equivalence. The CI for differences between the AAHL-1 assay and JCU-2 assay were greater than the allowable range of ± 5 % (9.8–13.3 %) and the assays were therefore not quantitatively equivalent (Table 1).

When only 160 samples were tested, incorporating the testing of AAHL-2, the data showed no quantitative difference between the AAHL-2 and JCU assays. The 95 % CIs were 1.2–3.8 for the AAHL-2 versus JCU-1; -0.02 to 0.5 for AAHL-2 versus JCU-2; 1.4 – 4.1 for JCU-1 versus JCU-2.

However, the CIs were higher than the acceptable limit ± 5 % when we compared the results between the AAHL-1 and the other assays (5.6 – 8.9 for AAHL1 versus AAHL-2; 3.6 – 5.9 for AAHL-1 versus JCU-1 and 5.8 – 9.2 for AAHL-1 versus JCU-2).

Qualitative Analysis of Percentage Inhibition Between the Assays

Qualitative analysis of the results showed significant differences between AAHL-1 and JCU-1 and between AAHL-1 and JCU-2 assays, but no difference between the JCU assays based on the McNemar test (Table 2) ($N = 240$). However, the criterion for equivalence was again assumed to be within ± 5 %, in this case the proportion of discordant pairs. Therefore, it was ambiguous whether AAHL-1 and JCU-1 and AAHL-1 and JCU-2

Table 1 Differences in mean percentage inhibition for pair-wise comparisons between the AAHL-1, JCU-1 and JCU-2 assays in the analysis of plumed whistling duck sera ($N = 240$)

Assay	Mean	Min/Max	Comparative assays	Mean difference (MD)	95 % CI of MD
AAHL1	27.9	–10/99	AAHL1–JCU1	5.8	4.8, 6.8
JCU1	22.1	–7/98	AAHL1–JCU2	11.6	9.8, 13.3
JCU2	16.3	–19/98	JCU1–JCU2	5.8	4.5, 7.2

Table 2 The assessment of agreement between AAHL and JCU assays in the proportions of samples in the categories of ≥ 40 % inhibition (positive) and < 40 % inhibition (negative)

	Number of sera in % inhibition categories as produced by the AAHL-1 assay		
	≥ 40 %	< 40 %	Total
Number of sera in % inhibition categories as produced by the JCU-1 assay ^a			
≥ 40 %	30	2	32
< 40 %	13	195	208
Total	43	197	240
Number of sera in % inhibition categories as produced by the JCU-2 assay ^b			
≥ 40 %	28	1	29
< 40 %	15	196	211
Total	43	197	240
	Number of sera in % inhibition categories as produced by JCU-1 assay		
	≥ 40 %	< 40 %	Total
Number of sera in % inhibition categories as produced by the JCU-2 assay ^c			
≥ 40 %	27	2	29
< 40 %	5	206	211
Total	32	208	240

AAHL Australian Animal Health Laboratory, JCU James Cook University, CI confidence interval, *p* probability value

^a Difference between the discordant paired proportions: -0.1 ; 95 % CI: -0.1 , -0.01 ; *p* (Exact McNemar test): 0.007

^b Difference between the discordant paired proportions: -0.1 ; 95 % CI: -0.1 , -0.02 ; *p* (Exact McNemar test): < 0.001

^c Difference between the discordant paired proportions: -0.01 ; 95 % CI: -0.04 , 0.01 ; *p* (Exact McNemar test): < 0.453

Table 3 Comparative OD values for the monoclonal antibody controls from 3 different versions of c-ELISA (AAHL-1, JCU-1 and JCU-2) (n = 12)

Assay	Mean	Median	Range	Comparative assays	<i>p</i> (Wilcoxon rank sum test)	<i>p</i> (continuity corrected) (Median test)
AAHL1	0.9	0.8	0.6/1.2	AAHL1–JCU1	< 0.001	< 0.001
JCU1	1.9	2.0	1.2/2.3	AAHL1–JCU2	< 0.001	< 0.001
JCU2	1.8	1.8	1.2/2.1	JCU1–JCU2	0.133	0.414

OD optical density, AAHL Australian Animal Health Laboratory, JCU James Cook University, *p* probability value

assays were equivalent as the 95 % CI of the differences overlapped with 5 %.

When a subset of the samples was analysed, incorporating the testing of AAHL-2, the results showed no difference in the number of positive sera determined between all pair-wise comparisons except between AAHL-1 and JCU-1 (-0.06 to 0.01) which was again ambiguously equivalent at a 5 % level.

Comparison of Monoclonal Antibody Controls

The OD values obtained for the MAb controls differed significantly between the AAHL-1 and JCU-1 and the AAHL-1 and JCU-2, but there were no significant differences between the JCU assays (Table 3) (N = 240). When a subset of the samples were analysed (N = 160), incorporating the testing of AAHL-2, the OD values obtained

for the MAb controls also differed significantly between the AAHL-1 and other assays, but were statistically equal between the AAHL-2 and JCU assays (Table 4).

Analytical Sensitivity Study

Descriptive Results of Sensitivity Testing

The results of the mean percentage inhibition in relation to different dilution of serum samples are presented in Fig. 1.

Model-A One-Way-ANOVA Testing on Mean Percentage Inhibition with 0 % Dilution (Undiluted Positive Pooled Sera)

The one-way-ANOVA testing between cELISA types for mean percentage inhibition values of the undiluted positive

Table 4 Comparative OD values for the monoclonal antibody controls from 4 different versions of c-ELISA (AAHL-1, AAHL-2, JCU-1 and JCU-2) (n = 8)

Assay	Mean	Median	Range	Comparative assays	<i>p</i> (Wilcoxon rank sum test)	<i>p</i> (Continuity corrected) (Median test)
AAHL-1	0.7	0.7	0.6/0.9	AAHL-1 vs. AAHL-2	<0.001	<0.001
				AAHL-1 vs. JCU-1	<0.001	<0.001
AAHL-2	2.1	2.0	1.9/2.3	AAHL-1 vs. JCU-2	<0.001	<0.001
JCU-1	1.8	1.9	1.2/2.3	AAHL-2 vs. JCU-1	0.528	0.617
				AAHL-2 vs. JCU-2	0.015	0.134
JCU-2	1.9	1.9	1.7/2.1	JCU-1 vs. JCU-2	0.793	0.617

OD optical density, AAHL Australian Animal Health Laboratory, JCU Jame Cook University, *p* probability, vs. versus

Fig. 1 The results of the sensitivity testing for different versions of competitive enzyme-linked immunosorbent assays (Australian Animal Health Laboratory-1, Australian Animal Health Laboratory-2, James Cook University-1 and James Cook University-2) showing mean percentage inhibition versus percentage dilution. AAHL-1 Australian Animal Health Laboratory c-ELISA-1, AAHL-2 Australian Animal Health Laboratory c-ELISA-2, JCU-1 James Cook University c-ELISA-1, JCU-2 James Cook University c-ELISA-2

PWD sera showed significant differences among the assays ($p < 0.001$). The predicted means (95 % CIs) for the assays were as follows: AAHL-1 95.6 % (94.1–97.2), AAHL-2 89.1 % (87.6–90.6), JCU-1 89.5 % (88–91.1) and JCU-2 90.2 % (88.7–91.7).

Post hoc analysis showed that there was no significant difference between the AAHL-2, JCU-1 and the JCU-2 assays ($p \leq 0.420$). The AAHL-1 assay, however, produced a higher mean percentage inhibition for the pooled positive sera compared to the other assays ($p < 0.001$).

Model-B Two-Way-ANOVA Testing on Mean Percentage Inhibition with 33–89 % Dilutions

The two-way-ANOVA testing on mean percentage inhibition for the variables of assay and dilution factor (33–89 %, mixed positive–negative pool) found a significant interaction effect between the cELISA types and the dilution factor ($p = 0.018$). The AAHL-2, JCU-1 and JCU-2 assays were not significantly different at 33, 80 and 89 % ($p > 0.05$) dilutions, however, the AAHL-1 assay gave significantly higher percentage inhibitions than the other assays ($p < 0.001$) at all dilutions (Table 5).

The AAHL-2 and JCU-1 assays were not significantly different at 50, 67 and 75 % dilutions ($p \geq 0.21$), but the JCU-2 assay differed significantly from them ($p \leq 0.04$) at these dilutions. The AAHL-1 produced a significantly higher mean percentage inhibition than the other assays (Table 5).

Model-C One-Way-ANOVA Testing on Mean Percentage Inhibition with 100 % Dilution (Undiluted Negative Pooled Sera)

The one-way-ANOVA testing on the mean percentage inhibition values of the undiluted negative PWD sera in different cELISA types showed significant differences between all assays ($p < 0.001$). The predicted means (95 % CIs) were as follows: AAHL-1 11.5 % (9.2–13.8), AAHL-2 7.4 % (5.1–9.7), JCU-1 –3.4 % (–5.7 to –1.1)

Table 5 Predicted means of percent inhibition (95 % CI) of the interaction between different versions of c-ELISA and dilution factors

Dilutions (%)	AAHL-1	AAHL-2	JCU-1	JCU-2
33	90.9 (87.5, 94.4)	79.1 (75.7, 82.6)	80.4 (76.9, 83.8)	83.9 (80.5, 87.4)
50	83.0 (79.6, 86.4)	67.4 (64.0, 70.9)	67.3 (63.9, 70.8)	74.2 (70.8, 77.7)
67	70.4 (66.9, 73.8)	49.5 (46.0, 52.9)	52.6 (49.1, 56.0)	55.6 (52.1, 59.0)
75	60.2 (56.8, 63.7)	38.2 (34.7, 41.6)	40.4 (37.0, 43.9)	44.6 (41.1, 48.0)
80	58.2 (54.7, 61.6)	33.1 (29.7, 36.5)	37.5 (34.0, 40.9)	38.9 (35.5, 42.4)
89	41.3 (37.8, 44.7)	20.2 (16.8, 23.6)	21.0 (17.5, 24.4)	23.0 (19.6, 26.4)

c-ELISA competitive enzyme-linked immunosorbent assay, AAHL Australian Animal Health, JCU James Cook University

and JCU-2 2.5 % (0.2–4.9). Post hoc analysis also showed significant differences between the assays ($p \leq 0.015$).

The Longitudinal Effect of Post Coating Buffer on the Stability of AIV Antigen in Plates

Mean OD values for the MAb at 10 different points during a 214 day period in the post coating buffer experiment were as follows 1.8 (95 CI 1.6–1.9, Day 0), 1.9 (1.8–2.0, 1), 1.9 (1.9–2.0, 2), 1.9 (1.8–2.0, 4), 1.9 (1.7–2.0, 7), 2.0 (1.7–2.2, 14), 1.9 (1.7–2.0, 28), 1.8 (1.6–2.0, 60), 1.8 (1.7–1.9, 79) and 1.5 (1.4–1.6, 214). There was no difference in the mean OD values for the MAb over eight time points and a 60 day period ($p > 0.05$), however there was a difference in OD values over 10 time points and a 214 day period ($p < 0.001$).

Discussion

The AIV cELISAs used in this study did not perform equally in the quantitative detection of AIV antibodies in the sera of PWDs. Unequivocal differences were measured between AAHL-1 and JCU-2 where the AAHL-1 assay produced a higher mean % inhibition value in the analysis of 240 serum samples. Higher percentage inhibition values for AAHL-1 over the other assays were also evident in the sensitivity testing.

The discrepancy of the results between the AAHL-1 and either of the JCU techniques corresponds to the use of different reagent dilutions (antigen, antibody and conjugate) along with the type of chromogen (TMB or ABTS) used in the assays. The JCU assays used reagent dilutions optimised for OD (close to 2) for the MAb control, having twice the concentration of antigen and MAb as the AAHL-1 assay. Reduced levels of antigen and MAb in the AAHL-1 procedure would increase the percentage inhibition values for a given serum sample in comparison to other assays by reducing the amount of MAb available to compete with antibodies in the test sera, thus increasing the difference in OD between the MAb control and that obtained for the test sera. The higher percentage inhibition values observed for AAHL-1 are probably indicative of increased sensitivity although the overall quality of the AAHL-1 cELISA can be considered in doubt due to unacceptable OD values (< 0.8) for the MAb control [8]. We did not have adequate time and resources to test various combinations of reagent concentrations and chose to test modified versions having a single combination of reagent concentrations optimised for an OD of 2 for the MAb control.

The OD values for the MAb controls in the JCU assays were satisfactory (median OD close to 2). However, it appears that when the same reagent dilutions are used

(AAHL-2) all three assays have equal performances for practical purposes. In regards to quantitative differences between the assays our sensitivity testing also showed a higher sensitivity for AAHL-1 assay over others but this assay once again failed to meet the test quality guideline of 0.8 OD value for MAb control (data are not presented here). Analytical sensitivity of the other assays (AAHL-2, JCU-1 and JCU-2) appeared to be identical with satisfactory OD values for the MAb controls (Fig. 1; Table 5). Moreover, these modified assays showed an adequate relative sensitivity compared to AAHL-1 to detect AIV antibodies field sera for practical purposes as differences were not unambiguously more than 5 % (Table 2).

Our study to investigate the longitudinal effect of a post coating treatment on AIV antigens in cELISA plates demonstrated antigen stability in plates over a 214 day period. This result clearly indicated the benefit of using the post-coating buffer where a large number of plates can be prepared and stored, minimising variation between batches of plates. Therefore, the addition of the post coating step to cELISA appears to be beneficial.

In conclusion, our study is the first to improve the AAHL cELISA using wild bird sera in Australia. Further optimization of antigen and MAb concentrations should be considered to increase the sensitivity of a modified assay, compared to JCU-2, while maintaining acceptable OD values for the MAb control. In addition, the performance of these versions of cELISAs needs to be compared with commercially available cELISAs to determine their absolute and relative sensitivity and specificity.

Acknowledgments We are very grateful to the Australian Biosecurity Cooperative Research Centre (ABCRC) and Australian Department of Agriculture, Forestry and Fisheries (DAFF) for providing project financial support. We thank AusAID for providing an Australian Leadership Award (ALA) and the ABCRC for a top-up PhD scholarship to MAH. We extend our thanks to the scientific and ethics permit authorities for providing the following project approvals, Eco-access permit No. WISp04374507, Queensland Parks and Wildlife Service, northern Region and ethics permit No. A 1175, JCU. Finally, we extend our heartfelt thanks to the members of our AI research team and other contributors to our research for their excellent co-operation during the study period.

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