Evaluation of Avian Influenza Serologic and Virologic Diagnostic Methods in Wild Anseriformes and Charadriiformes

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Received 19 March 2013; Accepted 26 September 2013; Published ahead of print 30 September 2013

SUMMARY. Evaluation of avian influenza virus (AIV) diagnostic methods, including a nucleoprotein (NP) competitive enzyme-linked immunosorbent assay (c-ELISA), hemagglutination inhibition (HI) test, type A real-time reverse transcription polymerase chain reaction (RRT-PCR), and embryonating chicken egg (ECE) virus isolation (VI), suggested validity of these tests in wild birds comparable to that reported in poultry. This was determined by analyzing the results from experimental inoculation of three species of wild birds with a low-pathogenicity AIV and from field surveillance data. The NP c-ELISA in a high–AIV prevalence setting had 100% diagnostic sensitivity (Se; 95% confidence interval [CI]: 81.5%–100%) and 91% diagnostic specificity (Sp; 95% CI: 70.8%–98.9%) in negative controls compared with the RRT-PCR. In low–AIV prevalence flocks using a >60% inhibition positivity threshold, relative to the HI test, c-ELISA performed with 90.5% Se (95% CI: 86.2%–93.8%) and 41.2% Sp (95% CI: 38.1%–44.5%). Assessment of HI suggests a titer ≥8 is a positive test result in wild-bird sera, and using this titer had 83.3% Se (95% CI: 58.6%–96.4%) in experimentally infected birds. The RRT-PCR diagnostic performance compared with VI in cloacal swabs varied over 2–6 days postinoculation, having high Se (83.3%–100%) and Sp (94.1%–100%) with substantial agreement (kappa = 0.8). The cycle thresholds (C_t) for the RRT-PCR of $C_t < 37$ for positivity and $C_t = 37$–40 as indeterminate were found to be valid for the species included in this study. In view of the interpretative diagnostic difficulties in heterogeneous populations of wild birds, this evaluation in three species of wild birds and in surveillance data should provide greater confidence in the application of these methods routinely used in poultry.

RESUMEN. Evaluación de los métodos de diagnóstico serológicos y virológicos para influenza aviar en Anseriformes y Charadriiformes silvestres.

La evaluación de los métodos de diagnóstico para la influenza aviar, incluyendo un ensayo por inmunoabsorción competitivo (C -ELISA) para la nucleoproteína (NP), una prueba de inhibición de la hemaglutinación (HI), un método de reacción en cadena de la polimerasa en tiempo real (RRT- PCR) para el tipo A y el aislamiento viral en embriones de pollo, sugirió que la validez de estas pruebas en las aves silvestres es comparable a la reportada con las aves comerciales. Esto se determinó mediante el análisis de los resultados de la inoculación experimental de tres especies de aves silvestres con un virus de la influenza aviar de baja patogenicidad y de los datos de vigilancia de campo. La prueba de ELISA para la nucleoproteína mostró una sensibilidad diagnóstica del 100% en un entorno de alta prevalencia de la influenza (Se; 95% intervalo de confianza: 81.5% a 100%) y una especificidad diagnóstica del 91% (Sp; 95% intervalo de confianza: 70.8%–98.9%) en controles negativos en comparación con la RRT- PCR. En parvadas de baja prevalencia de influenza aviar, utilizando un umbral de positividad por inhibición > 60%, relativo a la prueba HI, la prueba de ELISA competitiva mostró una sensibilidad diagnóstica (Se) de 90.5% (95% IC: 86.2%–93.8%) y una especificidad diagnóstica de 41.2% Sp (95% IC: 38.1%–44.5%). La evaluación de la prueba de inhibición de la hemaglutinación sugiere que títulos ≥ 8 sean considerados como positivos en sueros de aves silvestres, y el uso de este título mostró una sensibilidad de 83.3% (95% IC: 58.6%–96.4%) en las aves infectadas experimentalmente. El rendimiento diagnóstico RRT- PCR en comparación con el aislamiento viral con hisopos cloacales mostró variación de 2–6 días después de la inoculación, mostrando una sensibilidad (83.3%–100%) y especificidad (94.1%–100%) elevadas, con una concordancia significativa (kappa = 0.8). Los ciclos umbrales (C_t) para la prueba de RRT-PCR de $C_t < 37$ considerados positivos y los ciclos $C_t = 37$–40 considerados como indeterminados resultaron ser válidos para las especies incluidas en este estudio. En vista de las dificultades diagnósticas interpretativas en poblaciones heterogéneas de aves silvestres, esta evaluación en tres especies de aves silvestres y los datos de vigilancia deben proporcionar una mayor confianza en la aplicación de estos métodos utilizados rutinariamente en aves comerciales.

Key words: antibody, avian influenza virus, ELISA, evaluation, HI, RRT-PCR, sensitivity, specificity, virus isolation, wild birds

Abbreviations: AAHL = Australian Animal Health Laboratory; AD = average deviation; AGID = agar gel immunodiffusion test; AI(V) = avian influenza (virus); AUC = area under the curve; b-ELISA = blocking enzyme-linked immunosorbent assay; c-ELISA = competitive enzyme-linked immunosorbent assay; CI = confidence interval; C_t = cycle threshold; DAFWA = Department of Agriculture and Food Western Australia; DPI = days postinoculation; ECE = embryonating chicken eggs; EID_{50} = 50% egg infectious dose; GMT = geometric mean titer; H/HA = hemagglutinin; HI = hemagglutination inhibition; HP = highly pathogenic; ID_{50} = 50% infectious dose; IgY = immunoglobulin Y; K = Kappa statistic; LP = low pathogenicity; LR = likelihood ratio; N = neuraminidase; NP = nucleoprotein; NPV = negative predictive value; OIE = World Organisation for Animal Health; OP = oropharyngeal; PPV = positive predictive value; RDE = receptor-destroying enzyme; ROC = receiver-operating characteristic; RRT-PCR = real-time reverse transcription polymerase chain reaction; Se = diagnostic sensitivity; Sp = diagnostic specificity; VI = virus isolation; WA = Western Australia

The call for better understanding of the epidemiology of avian influenza virus (AIV), combined with the urgency to diagnose and respond to highly pathogenic AI (HPAI) H5N1 infection in poultry and wild birds has given impetus to developing improved AIV diagnostic capability. Methods such as ELISA, real-time reverse transcription PCR (RRT-PCR), and commercial immunoassay kits have been refined or developed as an adjunct to conventional AIV

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diagnostics that can be costly, can be time consuming, or can lack sensitivity. These methods, developed primarily for gallinaceous poultry are assumed to perform similarly in other birds; however, few performance evaluations have been undertaken in wild birds.

The application of a suite of AIV diagnostic methods is supported by a reasonably good understanding of the immune system in gallinaceous poultry (27), since host immune response is not only important for protection from disease but is also integral to the diagnosis of infection. However, little is known about the immune system and host responses of wild birds to AIV infection. Many experimental AIV inoculation studies in wild birds have discounted the value of serologic testing because of poor test performance, such as with the agar gel immunodiffusion test (AGID), which has reported poor sensitivity (1,24). Various nucleoprotein (NP) ELISA formats, including blocking and competition ELISA protocols (b-ELISA; c-ELISA) that are semiautomated and inexpensive, have enabled more sensitive measurement of NP antibody responses. In one study, evaluation of b-ELISA and AGID in experimentally infected wild birds, including 18 species of waterfowl and two gull species, found b-ELISA to be more sensitive than AGID for detection of AIV (sensitivity [Se]: 82% and 67.4%, respectively), with both tests having perfect specificity (Sp) (2). In other studies, commercial ELISAs have been assessed in wild birds with varied performance results depending on the test and species (3,22). The hemagglutination inhibition (HI) test is generally considered the reference serologic test for anti-HA antibody; however, based on a number of studies in which poor HI results were observed in ducks (14,24), it was concluded that ducks develop poor antibody responses compared with poultry. In ducks, test sensitivity issues for the AGID and HI are likely related to inherent deficiencies in duck immune responses with poor production of precipitating and hemagglutination-inhibiting antibodies (26,27) and different fractions of the primary serum antibody, immunoglobulin Y (IgY) (15). However, host responses may also vary depending on existing virus-host relationships and subtype adaptation (12) and life history, such as prior exposure to an AIV that can trigger anamnestic responses. Further study is needed to fully assess the range of immune responses to AIV infection in ducks.

Overall, the heterogeneity of wild-bird populations and dynamics of AIV infection presents interpretative difficulties for serologic testing, and this has detracted from the inclusion of these methods in wild-bird AIV surveillance. The type A RRT-PCR test, validated in domestic ducks (24) and poultry, has been found to be highly sensitive in wild birds (19) and is widely used for the detection of AIV in wild-bird surveillance. The standard World Organisation for Animal Health (OIE) procedure for test validation includes the use of defined positive and negative reference animals, and in wild birds this would require a multitude of experiments across many species and access to uninfected populations. Hence it is not surprising that AIV diagnostic methods have not been fully validated in wild birds, in that addressing the logistical, resource, and welfare concerns of this work would present significant impediments that far outweigh the likely benefits. In this study, we evaluated the diagnostic performance of NP c-ELISA, HI, Taqman type A RRT-PCR, and embryonating chicken egg virus isolation (ECE VI) methods from the results of a low-pathogenicity AIV (LPAIV) inoculation trial in three species of wild birds, and the serologic methods were further evaluated in wild-bird surveillance samples.

MATERIALS AND METHODS

A summary of the materials and methods for the challenge trial are described, with further detail provided in a separate publication (5).

Challenge trial and surveillance samples. Suitable species were selected for the challenge trial that were likely to adapt readily to captivity and could be caught locally. Three species, wandering whistling duck (*Dendrocygna arcuata*; $n = 7$; raised as orphans, aged 5 mo), silver gull (*Chroicocephalus novaehollandiae*; $n = 9$; one immature, wild-caught), and ruddy turnstone (*Arenaria interpres*; $n = 6$; adults, wild-caught), were inoculated with an LPAIV, A/Eurasian coot/WA/2727/79 (H6N2). Birds ($n = 19$) were inoculated with 0.5 ml by the oropharyngeal (OP) route, 0.15 ml in each naris and 0.1 ml in each conjunctiva, equivalent to an infectivity titer of $10^{7.95}$ 50% egg infectious dose (EID_{50}), as determined using the Spearman–Kärber method (28), with one of each species left as untreated in-contacts. Cloacal and OP swabs were collected from each bird every second day postinoculation (DPI) until day 16 and thereafter every 3 days in the ducks, with a final sample taken from all birds at 28 DPI. Cloacal sampling to 14 DPI included a duplicate swab (inserted simultaneously) from each bird. All swabs were ultimately stored at −170 C, with the duplicate swabs stored initially at 4 C for 48 hr to replicate conditions commonly found in field sampling. A blood sample was collected from each bird every 3 days from 7 DPI to 28 DPI. Serum samples from the surveillance for AIV in wild Charadriiformes and Anseriformes across northern Australia were analyzed.

NP c-ELISA. An Australian Animal Health Laboratory (AAHL) NP c-ELISA was used according to standard methods (4). Test results were expressed as percent inhibition relative to the optical density of the monoclonal antibody control. Sera with >60% inhibition are interpreted as positive, 40%–60% as equivocal, and <40% as negative. The diagnostic performance of the c-ELISA was evaluated at two levels: as a test for detection of NP antibody response to infection in trial birds, with infection status determined by sequential RRT-PCR testing (any bird with a cycle threshold [C_t] < 40 at 2 DPI or thereafter in either cloacal or OP swabs was considered infected), and as a screening test in the surveillance of wild-bird populations compared with HI test results. For the surveillance data, because the true AIV status of the test populations were unknown and virologic results were predominantly negative, the NP c-ELISA results were assessed at two cut-off thresholds. To assess the specificity parameter accurately in wild birds would have required larger sample numbers of AIV-naïve birds.

Hemagglutination inhibition. The HI assay was performed according to standard procedures (20) and assessed using the results from the challenge trial, since surveillance sera were sourced from birds with unknown infection history. For the NP c-ELISA evaluation, surveillance sera were tested at AAHL against a panel of inactivated H1–H16 antigens that prioritized exotic H5 and H9 subtypes and subtypes previously identified in Australia (H1, 3, 4, 5, 6, 7, 11, 12, and 15), subject to availability of sera and antigens. For the evaluation, a HI test result with a titer of ≥8 was considered positive. To assess the effect of receptor-destroying enzyme (II) Seiken (RDE; Denka Seiken Co., Tokyo, Japan) pretreatment of serum on nonspecific inhibition in the HI test for wild birds, a sub-sample of surveillance, and trial sera were tested with and without pretreatment of sera with RDE according to standard methods (29).

Taqman type A RRT-PCR and virus isolation testing. The cloacal and OP swab samples from the challenge trial were tested individually and similarly for the presence of influenza A RNA by Taqman RRT-PCR using the AAHL method of Heine and Trinidad (11). At the Department of Food and Agriculture Western Australia (DAFWA), minor variations included extraction of RNA from 100-µl samples using a Magmax 96 viral isolation kit (Ambion Inc., Austin, TX) on an Applied Biosystems Magmax Express magnetic particle processor (Life Technologies Corp., Melbourne, Australia) according to the manufacturer's instructions. At DAFWA, $C_t < 37$ is considered positive, with $C_t = 37$–40 an indeterminate reaction and $C_t \geq 40$ negative. The ECE method for VI follows the standard protocol of the OIE (20). A standard curve to provide estimates of viral titers (EID_{50}) that can include live and inactivated viral material was produced from RRT-PCR testing $\log_{10}$ titrations of extracted RNA from 100 µl of the H6N2 challenge inoculum.

Statistical methods. Median values with average deviation (AD), statistical differences, and assessment for Spearman's correlation coefficient (r_s) were all analyzed in either Microsoft Excel (2007) or

SPSS (version 17 for Windows; SPSS Inc., Chicago, IL). For HI results, the geometric mean titer (GMT) and standard deviation were calculated using the log $_2$-transformation method of reciprocal titers followed by calculation of the antilogarithm (28).

The NP c-ELISA and HI were evaluated by categorizing each trial bird as either infected or uninfected based on the detection of any $C_t <$ 40 RRT-PCR result postinoculation. Using recognized positive thresholds, the numbers in each category, including negative control data, were analyzed in EpiTools epidemiologic calculators (23) to assess diagnostic sensitivity (Se) and specificity (Sp) with 95% confidence interval (CI), likelihood ratio (LR), positive and negative predictive values (PPV and NPV), and Kappa statistic (K) for agreement (6). Using surveillance data, diagnostic values for the NP c-ELISA against HI results were further evaluated in EpiTools at various thresholds using a nonparametric two-graph receiver-operating characteristic (ROC) analysis with 95% accuracy based on the area under the curve (AUC) as described (10). The diagnostic performance of the RRT-PCR to detect AIV (by ECE VI) at two cut-offs, $C_t < 37$ and $C_t < 40$, was assessed in EpiTools, and C_t values were dichotomized into VI positive and negative groups to assess for correlations (r_s) in SPSS, with C_t values of ≥40 arbitrarily assigned a C_t value of 45. Paired RRT-PCR data were analyzed for differences using the paired samples t-test in SPSS.

RESULTS

For the LPAIV challenge trial, results relevant to this evaluation are outlined, with detailed results provided in a related publication (5). All birds appeared to be clinically healthy and were negative for AIV by RRT-PCR and VI before challenge. When the sera were tested by NP c-ELISA, only the turnstones (6/6; median of 57% inhibition) had evidence of past exposure to AIV. All sera tested negative for H6 antibodies by HI, and the six turnstone sera also tested negative for HI antibodies to other available HA subtypes at AAHL. After inoculation, 19 of 22 birds responded with serologic or virologic evidence of infection. The ducks responded with most uniformity, featuring high NP c-ELISA values (>80% median inhibition to 42 DPI), low HI titers (highest GMT of 16.1 at 19 DPI), moderate viral titers in cloacal excretions to 6 DPI (highest median titer of $10^{4.58}$ EID_{50}/0.1 ml at 4 DPI), and low viral titers in OP excretions to 4 DPI (highest median titer of $10^{1.69}$ EID_{50}/0.1 ml). In contrast, of the infected gulls and turnstones, only one turnstone had evidence of cloacal viral excretion ($10^{3.14}$ EID_{50}/0.1 ml at 10 DPI), and with the exception of one gull ($10^{4.26}$ EID_{50}/0.1 ml at 2 DPI), OP viral excretions were of low titer ($10^{1.43–2.09}$ EID_{50}/0.1 ml at 4 DPI). Although all infected turnstones and gulls responded with high NP c-ELISA values by 7 DPI, anti-HA antibody responses were inconsistent and, where detected, were characterized by moderately high HI titers (highest titer of 64 and 128, respectively, by species at 10–13 DPI).

Virus detection evaluation. Of the 28 ECE VI–positive swabs, virus was detected in 25 of these on first passage. The RRT-PCR detected virus in 16 of 18 (89%) cloacal swabs positive by VI to 10 DPI, and 9 of 10 (90%) OP swabs positive by VI to 4 DPI. The three RRT-PCR–negative, VI-positive swabs were from ducks that had a C_t-positive result in prior or subsequent samples. Prevalence of OP swabs with target nucleic acid was higher compared with cloacal swabs from 2–4 DPI; however, the median C_t value was significantly lower ($P < 0.001$) at all sample times in the cloacal swabs (Fig. 1). When RRT-PCR C_t and VI results were compared to 10 DPI for cloacal swabs, there was substantial agreement and significant correlation ($K = 0.8$; $r_s = 0.82$, $n = 83$, $P < 0.001$), and fair agreement for OP swabs to 4 DPI ($K = 0.23$; $r_s = 0.37$, $n = 44$, $P = 0.013$).

The RRT-PCR evaluation at each sampling DPI in EpiTools showed high diagnostic Se (mostly 83.3%–100%), and high diagnostic Sp (77.8%–100%) in cloacal swabs, with comparable Se (85.7%–100%) but lower Sp (40%–52.6%) in OP swabs (Table 1). In comparison, from parallel testing of test-negative surveillance swab samples from 306 Charadriiformes, RRT-PCR and VI agreed 100%, suggesting high RRT-PCR specificity. In the cloacal swabs, sensitivity at the positive and indeterminate C_t thresholds were not different because all VI-positive samples were $C_t < 37$. When test performance in OP swabs at the $C_t < 37$ threshold were compared with that in $C_t < 40$ swabs, Se values were lower (43%) at 2 DPI, equivalent (100%) at 4 DPI, whereas Sp values were higher (66.7%, 79%) both times. The values are related to differences in median C_t values between VI-positive and -negative results in cloacal swabs (C_t = 28.2 and 36.5, respectively; $P < 0.001$) and OP swabs (C_t = 36 and 37, respectively; $P = 0.22$).

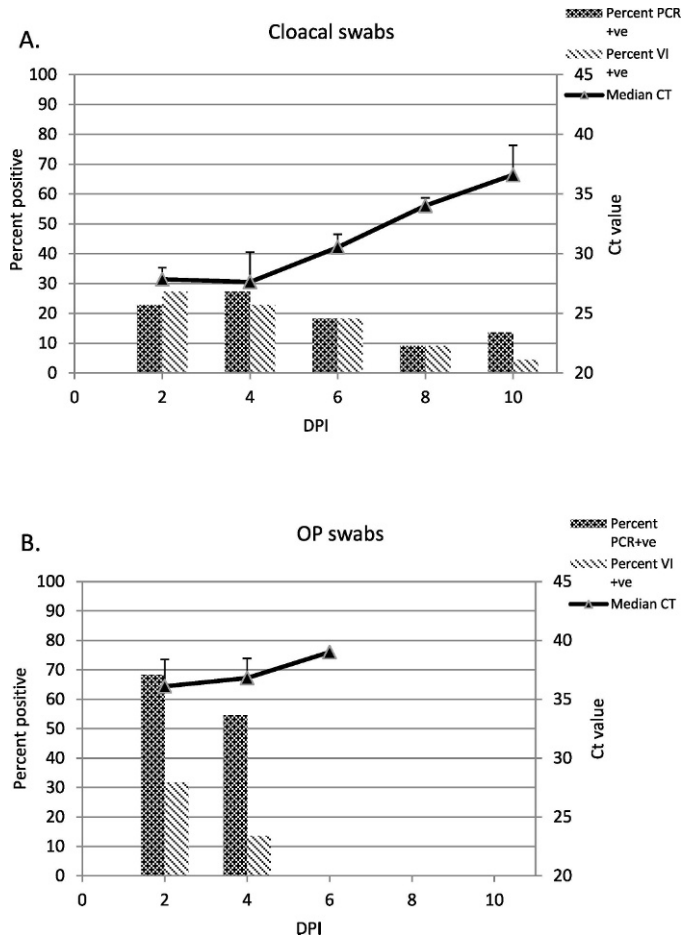

Fig. 1. Percentage of LPAIV challenge birds positive by RRT-PCR ($C_t < 40$) and positive by VI, with the median C_t value of positive results with error bars (AD) in (A) cloacal swabs to 10 DPI and (B) OP swabs to 4 DPI.

Swab storage conditions. There were slightly more (Pearson's chi-square, $P = 0.65$) virus isolations ($n = 15$) from the duck swabs stored at −170 C soon after collection compared to the duplicate duck swabs stored initially at 4 C for 48 hours ($n = 13$). Virus was isolated from two of the 4 C swabs and one of the swabs stored entirely at −170 C that were not detected in the alternate paired swab. Paired C_t values were significantly correlated ($r_s = 0.86$, $n = 19$, $P < 0.001$), with substantial agreement between RRT-PCR and VI (4 C: $K = 0.62$; −170 C: $K = 0.73$).

NP c-ELISA evaluation in trial birds. When the NP c-ELISA was assessed against infection status, the results showed that on all

Table 1. Assessment of RRT-PCR performance ($C_t < 40$ positivity threshold) against ECE virus isolation in cloacal and OP swabs from the AIV challenge trial.

	% Sensitivity (95% CI)	% Specificity (95% CI)	Likelihood ratio[A]	Kappa statistic
Cloacal swabs				
2 DPI	83.3 (35.9–99.6)	100 (79.4–100)	NC	0.88
4 DPI	100 (47.8–100)	94.1 (71.3–99.9)	17	0.88
6 DPI	100 (39.8–100)	100 (81.5–100)	NC	1.00
8 DPI	50 (1.3–98.7)	80 (28.4–99.5)	2.5	0.30
10 DPI	100 (2.5–100)	77.8 (40–97.2)	4.5	0.41
OP swabs				
2 DPI	85.7 (42.1–99.6)	40 (16.3–67.7)	1.4	0.20
4 DPI	100 (29.2–100)	52.6 (28.9–75.6)	2.1	0.23

[A]Positive likelihood ratio shown; NC = not calculated since estimated specificity at this point was 100%.

sampling days (7–28 DPI), the percentage of infected birds positive by c-ELISA (range, 72.2%–100%) was higher than the percent positive by HI (range, 33.3%–83.3%). The numbers pre- and postchallenge were dichotomized into uninfected ($n = 22$) and infected groups ($n = 18$) based on RTT-PCR results for assessment, excluding one gull that had serologic evidence of infection without evidence of viral excretion (94% c-ELISA inhibition, HI titer of 16). This analysis showed that the NP c-ELISA (>60% inhibition threshold) had a diagnostic Se of 100% (95% CI: 81.5%–100%) at 10 DPI and a diagnostic Sp of 91% (95% CI: 70.8%–98.9%) in negative controls. Two turnstones had positive c-ELISA values before inoculation, and if these results were excluded, the diagnostic Sp of the c-ELISA would have been 100% (95% CI: 83.2%–100%).

The median NP c-ELISA percent inhibition value for the infected group was 89.5% (AD = 4.9%), which was significantly higher ($P < 0.0001$) than for the uninfected (negative control) group at 36% (AD = 11.2%). The percent positive by c-ELISA declined gradually after 10 DPI (from 100% to 72.2%), which was directly attributable to the gull and turnstone results, because 100% of the ducks remained positive to 28 DPI. The NP c-ELISA had a high positive LR of 11, and the diagnostic Se and Sp values indicate that this test would have a high precision rate when used in high–AIV prevalence settings (e.g., a PPV/NPV of 0.92/1.0 at 50% prevalence). Assessment for correlations between the results from 7 to 28 DPI, including negative controls, showed significant positive correlation between NP c-ELISA values and infection status ($r_s = 0.70$, $P < 0.001$, $n = 189$) and outstanding test agreement between NP c-ELISA and RRT-PCR ($K = 0.9$, $n = 40$).

NP c-ELISA evaluation in surveillance samples. From the challenge study, HI titers ≥8 were associated with infection status, and this was further quantified by examining the NP c-ELISA results from 82 surveillance and 68 challenge trial samples that had HI titers ≥8. Of these, 122 (81.3%; 95% CI: 74.2%–87.2%) were NP c-ELISA positive (81% mean inhibition). Given this result and the outstanding agreement ($K = 0.9$) with RRT-PCR and 100% Se value of NP c-ELISA in the challenge trial, it was decided to use a HI titer of ≥8 as a positive test result for comparing sensitivity evaluations of the NP c-ELISA. The surveillance sera could not be tested with all subtypes because of insufficient sera; therefore, arbitrarily, the assessment was restricted to those samples ($n = 1194$) that were tested against any 10 or more of the HA subtypes prioritized for testing at AAHL. Using this criterion, the number of sera that tested HI positive to at least one HA subtype were analyzed at two c-ELISA thresholds. At the ≥40% c-ELISA inhibition threshold, diagnostic Se was 99.6% (95% CI: 97.8%–100%), diagnostic Sp was 18.7% (95% CI: 16.3%–21.3%), and positive LR was 1.5, whereas at the >60% c-ELISA inhibition threshold, diagnostic Se was 90.5% (95% CI: 86.2%–93.8%), diagnostic Sp was 41.2% (95% CI: 38.1%–44.5%), and positive LR was 1.2. In low–AIV prevalence surveillance testing, these values for NP c-ELISA suggest the test would have less precision (e.g., a PPV/NPV of 0.07/0.99 at 5% prevalence).

To further evaluate performance parameters using surveillance data, the NP c-ELISA results for ''infected'' and ''uninfected'' groups based on HI testing were further analyzed using a two-graph ROC curve in EpiTools. The AUC was calculated as 75.6% (95% CI: 72.5%–78.7%), indicating that the NP c-ELISA has moderate accuracy. When compared, the Se and Sp values at the 40% and 60% c-ELISA inhibition thresholds by ROC (Fig. 2) were comparable to the estimated values for surveillance samples calculated in EpiTools.

Evaluation of the HI assay. The HI test was evaluated against RRT-PCR using trial data and negative control data and showed that, by 13 DPI, HI titers (≥8) were found in 100% of infected ducks and gulls and 40% of infected turnstones. Overall, the results at 13 DPI show that the HI test (titer ≥8) had a high diagnostic Se (83.3%) for detection of prior AIV infection in wild birds (Table 2). The lower test Se value of 40% in the turnstones was because only two of the five birds that gave weak OP C_t values ($C_t = 36$ and 38) at 2 DPI had HI antibody responses ≥8.

Correlation between test results. When the results from the challenge trial 7–28 DPI were analyzed for correlations using HI titer ≥8 and NP c-ELISA ≥40% threshold, 104 of 176 (59.1%; 95% CI 51.4%–66.4%) were positive to both tests, 47 (26.7%; 95% CI: 20.3%–33.9%) were positive to the c-ELISA only, and 1 (0.6%;

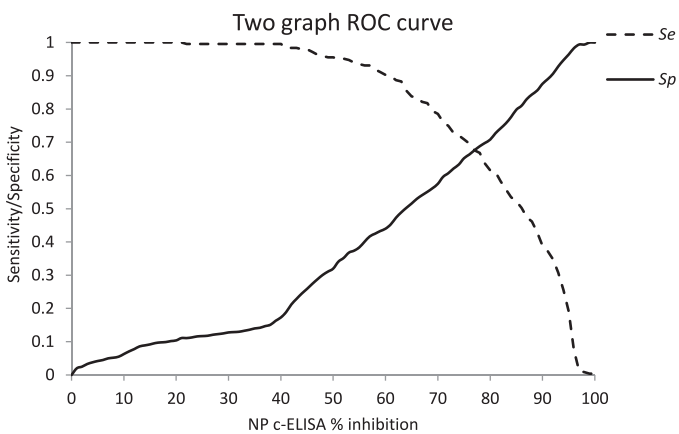

Fig. 2. Two-graph nonparametric ROC analysis of the NP c-ELISA performance in wild-bird surveillance samples, with birds dichotomized into ''infected'' or ''uninfected'' groups based on their HI results.

Table 2. Evaluation of the HI test performance against RRT-PCR results using the challenge trial data (95% CI) with sensitivity calculated in birds categorized as infected at 13 DPI and specificity calculated in prechallenge negative control birds.

	Ducks ($n^A = 14$)	Gulls ($n^A = 15$)	Turnstones ($n^A = 11$)	All birds ($n^A = 40$)
Sensitivity (%)	100 (59–100)	100 (54.1–100)	40 (5.3–85.3)	83.3 (58.6–96.4)
Specificity (%)	100 (59–100)	100 (66.4–100)	100 (54–100)	100 (84.6–100)
Kappa statistic	1.0	1.0	0.42	0.85

[A]The number includes prechallenge negative control and infected birds as follows: seven prechallenge and seven infected ducks, nine prechallenge and six infected gulls, six prechallenge and five infected turnstones; total of 22 prechallenge and 18 infected birds.

95% CI: 0%–3.1%) was positive by HI only, demonstrating moderate agreement ($K = 0.43$). When the highest HI titer, highest NP c-ELISA value, lowest C_t value, and duration of shedding for each bird were compared, correlations were only significant between the C_t value and HI titer ($r_s = -0.43$, $P = 0.044$, $n = 22$) and the c-ELISA and HI titer ($r_s = 0.61$, $P = 0.002$, $n = 22$).

Assessment of RDE treatment method for HI testing. A subsample of surveillance and challenge sera with HI titers (≥8) were treated with RDE, and antibody titers were compared with untreated sera by HI testing. Agreement was positive for a HI result ≥8 in 73 of 85 sera (86%; 95% CI: 78.5%–93.3%), positive correlations were significant ($r_s = 0.72$, $P < 0.0001$), and differences between RDE treated and untreated sera were not significant (paired samples *t*-test, $P = 0.470$).

DISCUSSION

This is first time AIV diagnostic methods, including an NP c-ELISA, HI, and Taqman RRT-PCR, have been evaluated at the same time in a captive species of the three bird groups primarily sampled in AIV surveillance studies: wild duck, gull, and wader. Characterization of the performance of diagnostic tests is influenced by many biological factors, such as host immune function and stage of infection (9). In AIV infection, measures of test performance are likely to vary considerably when testing biologically and genetically diverse populations of wild birds where there is complex ecological interplay between virus and host. Moreover, when wild-bird populations are tested, it is unlikely that all infected birds will show a homogeneous response. This could manifest as inconsistent antibody production, variability in the level of viral replication and shedding from different sites, and variable virus isolation rates, depending on the wild-bird species, location, time of the year, and adaptation of the virus in the host.

Evaluation of the ECE method with other VI methods was beyond the scope of this study. Moreover, ECE VI is widely accepted as the gold standard test with high Se and Sp values in chickens (7) and is considered to be the most efficient system for growth of wild-bird LPAIV isolates compared with various other embryo types and cell lines (18,19). In this study, ECE VI performed well, with 89% (95% CI: 72%–98%) of viruses isolated on first passage, which is comparable to the 87% isolation rate reported by others (19).

The Taqman type A RRT-PCR targets highly conserved sequences of the matrix gene common to all type A AIVs and has wide acceptance as an effective and highly efficient tool for AIV surveillance in poultry and wild birds. In this study, the RRT-PCR performed with high accuracy to detect 25 of 28 (89%) virus-positive swabs, with only two cloacal and one OP virus-positive swab negative by RRT-PCR. Given that the ducks provided 24 of the 28 virus isolations, the results of the assessment are more specific to this species. Overall, at 4 DPI, the RRT-PCR had high diagnostic Se (100%) and Sp (94.1%); however, values varied postinoculation (Se of 83.3%–100% and Sp of 94.1%–100% from 2 to 6 DPI; Table 1). These performance values are comparable to those reported in domestic poultry (7) and reflect marked improvement in sensitivity compared with earlier methods (25). Other statistical analyses between RRT-PCR and VI results in cloacal swabs from 2 to 6 DPI showed substantial to outstanding agreement ($K = 0.88$–1.0), and to 10 DPI, showed significant correlations ($r_s = 0.82$). From 2 to 4 DPI in the OP swabs, RRT-PCR had high diagnostic Se (85.7%–100%) but lower Sp (40%–52.6%), with only fair agreement ($K = 0.2$–0.23) and poor correlations ($r_s = 0.37$). The lower specificity was most likely due to limited viral replication and neutralization of infectious virus, as is evident in significantly higher ($P < 0.001$) median C_t values in OP swabs compared with cloacal swabs (Fig. 1). Moreover, although respiratory viral replication was prevalent, the results show that titers were probably less than the detection limit by VI or that the detections by PCR were of inactivated viral material. Because RRT-PCR detects both viable and nonviable virus, test specificity will be affected by the stage of infection and various host-virus factors that influence the site and level of replication.

When the RRT-PCR was assessed using the $C_t < 37$ threshold relative to VI in cloacal samples to 10 DPI, there were no differences in the diagnostic sensitivity and specificity, except for higher specificity at 10 DPI. When OP samples ($C_t < 37$) were assessed, Se was lower (43%) at 2 DPI and equivalent (100%) at 4 DPI relative to the values for the $C_t < 40$ threshold. In this study, excluding OP samples with C_t values in the indeterminate range ($C_t = 37$–40) from confirmatory virus isolation testing would have missed the isolation of a virus in three swabs in the early stages of infection.

These findings confirm that the Taqman type A RRT-PCR using the method described was highly accurate and efficient for the detection of AIV in the three wild-bird species included in this study using in-house standardized threshold cut-off values. Although the detection of virus in OP swabs were interesting results, the rate of VI was much higher from cloacal swabs compared with OP swabs, a finding in agreement with other studies of LPAIV in wild birds (13,19). In this study, most ducks that had viral material detected in OP swabs had concomitant detections in cloacal swabs.

The comparison of test parameters by DPI in the three species of the LPAIV trial also provides some insight into the expected performance of RRT-PCR when testing wild-bird populations under natural conditions with affected birds at different stages of infection. The RRT-PCR performed well in the cloacal samples since positive results were mostly from ducks infected with an adapted subtype that readily replicated in the gastrointestinal tract with prominent virus shedding. In cloacal samples from 6 to 10 DPI, RRT-PCR Sp values decreased from 100% to 77.8%, which was likely related to the end of the excretion phase of the infection. After 10 DPI, viral replication and excretion was virtually not detected, indicating that the surveillance for AIV in wild birds affords only a very narrow window of time for LPAIV detection. The assessment of two protocols found that swab storage at −170 C had higher success for VI compared with storage at 4 C for 48 hr before storage at −170 C,

although the differences were not significant. In one study, higher rates of success with VI were found in RRT-PCR–positive samples stored at −80 C compared with samples stored at either −4 C or −20 C (19), and another study found that degradation of AIV RNA in viral transport media under constant storage conditions of 4 C or 20 C was minimal over a 2-wk period (8).

In the evaluation of the serologic tests, performance estimates need careful interpretation given the design of this study. Moreover, in the challenge trial, NP c-ELISA and HI were assessed against infection status using RRT-PCR results, rather than by virus isolation as used in other studies (2). This approach may affect performance estimates in wild-bird studies, given the variation in virologic results between species and individuals and the different sampling times in this study. For instance, results from one gull excluded from the analysis had detectable AI antibodies with no evidence of viral excretion. Furthermore, NP c-ELISA antibodies were more consistently found in infected birds from 7 DPI than was detection of viral material by RRT-PCR to 10 DPI. In poultry, HI has been used to assess c-ELISA (26); however, HI may not be as useful for wild-bird studies given the variation in HI results found between the three trial species. For the surveillance data, c-ELISA was compared with HI, although the sera could not be tested against all available serotypes.

The HI test is considered the reference serologic test for confirming antibody against an HA subtype in poultry with high diagnostic Se and Sp values, particularly when field sera are tested against reference antigens that have high HA antigenic and genetic relatedness to the viruses in the field. When the HI test was done using the homologous antigen and evaluated against infection status in the LPAIV challenge trial using an antibody titer of ≥8, overall, HI had high diagnostic Se (83.3%) for AIV infection at 13 DPI and high diagnostic Sp (100%) in negative controls. The sensitivity of the HI differed between species (100% in the ducks and gulls and 40% in the turnstones). In the ducks, a peak reciprocal GMT of 16.1 at 19 DPI is consistent with the understanding that ducks have poor anti-HA responses and reduced ability to produce hemagglutination-inhibiting antibodies (27). In three turnstones, the lack of anti-HA antibody response was probably due to low-level virus replication and weak immune response rather than poor test sensitivity in this species. Although by OIE guidelines, a HI reciprocal titer of ≥16 is considered positive for AIV infection (20), the findings from this study suggest that a reciprocal titer of ≥8 is a positive result in wild birds, providing that test internal quality control standards are meticulously followed. This finding should be further investigated. Under field conditions, HI test performance in wild birds is likely to be affected by variations in biological host response, antibody longevity, infection history, and the strain of virus used as the antigen, rather than intrinsic test performance. This could result in: false-negatives when the reference antigen strain is unrelated, or distantly related to the test serum antibody; or interpretative challenges due to the presence of multiple subtype antibodies from prior exposure or concomitant active infections. More accurate assessment of HI test performance would require challenge trials in other wild birds using multiple subtypes and extensive testing with a panel of antigens. Although RDE pretreatment of serum for the HI test is routinely used to remove nonspecific inhibitors in some species, the sera from the majority of avian species may only have low or undetectable levels of nonspecific inhibitors of agglutination (21). The findings from this limited study do not support the RDE pretreatment of avian sera.

The NP c-ELISA performed well in sera from the LPAIV challenge trial, with 100% diagnostic Se for AIV antibody at 10 DPI and 91% diagnostic Sp in negative controls, values that are in relative agreement with the evaluation in chickens at AAHL. At all times in the trial, the NP c-ELISA was a more reliable and sensitive diagnostic indicator of exposure to AIV than HI antibody, which is in agreement with comparative evaluations in other avian species (16,17,22,26). Other performance values at 10 DPI included high positive LR and predictive values in high-prevalence situations, significant positive correlations ($r_s = 0.7$) and outstanding agreement ($K = 0.9$) between NP c-ELISA values and infection status in control and challenge data to 28 DPI. More detailed assessment of NP c-ELISA specificity that would require greater numbers of naïve wild birds was beyond the scope of this study. The selection of an appropriate diagnostic threshold for the NP c-ELISA is likely to be more relevant in low-prevalence settings, depending on the epidemiologic situation. In surveillance sera at the ≥40% c-ELISA inhibition threshold relative to the HI test, Se was higher (99.6%) and Sp was lower (18.7%) compared with the >60% inhibition threshold (Se = 90.5% Sp = 41.2%), with comparable results from ROC analysis. The NP c-ELISA > 60% inhibition threshold for positivity relative to the HI test is a good compromise in low-prevalence populations, providing better specificity without significant loss of sensitivity. The higher threshold also reduces the amount of costly, labor-intensive HI testing required. In low–AIV prevalence flocks, the low Sp value of the c-ELISA relative to the HI test is likely to result in false positives. However, this is more likely related to differences in the longevity of the NP and HA antibody and not a reflection of the test's analytical specificity.

In the challenge trial, the AAHL NP c-ELISA performed with high accuracy for detection of AIV antibody in infected birds and should be used as a complementary test in diagnostic investigations. In low–AIV prevalence wild-bird surveillance, the test has high validity as a screening test to detect exposure to AIV and has the potential to fill information gaps on the ecology of AIV when virology results are mostly negative. Moreover, the c-ELISA is suitable for profiling at-risk wild-bird species that could be targeted to optimize early warning surveillance strategies to make efficient use of limited resources. These performance findings are in agreement with others that conclude that the NP ELISA (both blocking and competitive protocols) is a highly sensitive and specific sero-diagnostic screening test for AIV across a number of wild-bird species (2,22). The NP c-ELISA is more practical for large-scale screening, is semiautomated, and performs better in detecting exposure to AIV than the labor-intensive HI test, which requires testing against a multitude of HA subtypes.

These diagnostic tests have previously been used routinely in wild-bird surveillance without proper evaluation and with the assumption that the tests have equivalent performance to that found with poultry. In this study, assessment of the AIV diagnostic methods was limited to using wild-bird data from experimental inoculation of an AIV in three species and from low–AIV prevalence flocks. These findings should be assessed further in other wild birds and with different AIV subtypes and expanded to include emerging diagnostic methods. Overall, this evaluation endorses the suitability of the serologic and virologic test methods outlined, and provides performance parameters that should provide greater confidence to the diagnostic interpretation of test results in wild birds. The inclusion of these diagnostic methods in wild-bird surveillance programs would depend on the purpose (early warning, outbreak, or ecological study), scope, and cost of the activity and, in developing countries, access to suitable sample-handling systems and regional laboratory support.

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ACKNOWLEDGMENTS

Funding for this study was provided by the Australian Biosecurity Cooperative Research Centre for Emerging Infectious Disease. Laboratory testing of wild birds was undertaken with the technical support of the DAFWA Virology Laboratory and AAHL.