Evaluation of a commercial enzyme-linked immunosorbent assay for detection of antibodies against the H5 subtype of Influenza A virus in waterfowl

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The ID Screen Influenza H5 Antibody Competition enzyme-linked immunosorbent assay was tested for the detection of antibodies to the H5 subtype of influenza A (IA) virus in waterfowl. Assays were conducted with sera obtained from Mallards (Anas platyrhynchos) and Pekin Ducks (Anas platyrhynchos domestica), experimentally infected with eight low pathogenic (LP) and nine highly pathogenic (HP) H5N1 IA viral strains. Three incubation periods (1, 4 and 18 hours) and two dilution factors (1:2 and 1:5) were evaluated. All serum samples from LP H5-infected birds tested positive; however, improved detection rates were observed for viruses belonging to the HP H5N1 clade 2.2.1 as compared with those belonging to clade 2.1.3.

Keywords Avian influenza, enzyme-linked immunosorbent assay, H5, serology, waterfowl.

Testing for antibodies to Influenza A (IA) virus is a common diagnostic tool used in poultry1 and also has recently been incorporated into wild bird surveillance efforts.2–8 These assays usually are based on the detection of IA virus nucleoprotein antibodies using agar gel immunodiffusion or enzyme-linked immunosorbent assays (ELISA), and hemagglutinin (HA) and neuraminidase antibodies using hemagglutination inhibition (HI) and neuraminidase inhibition tests, respectively. Several commercial ELISA's have recently been developed and evaluated for use in both poultry and wild birds.7,9,10

In the current study, the ID Screen Influenza H5 Antibody Competition ELISA (IDVET, Montpellier, France) was tested. We investigated the ability of the assay to detect H5 antibodies in sera obtained from Mallards (Anas platyrhynchos) and Pekin ducks (Anas platyrhynchos domestica) experimentally infected with eight low pathogenic (LP) and nine highly pathogenic (HP) virus strains, respectively.

For LP IA viruses, serum samples were obtained from 43 one-month-old Mallards experimentally infected with eight different virus subtypes (Table 1) as well as from eight sham-inoculated birds.10 Blood samples were collected at the end of the experiments (14 or 21 days post-infection) and sera stored at −20°C until tested. Virus isolation, PCR testing as well as NP ELISAs10 verified infections of inoculated birds (see references 11–14 for details related to the experimental infection trials).

For HP H5N1 viruses, serum samples were obtained from 38 Pekin ducks inoculated with nine different viral strains; five and four viruses being identified as belonging to the HP H5N1 clade 2.1.3 and 2.2.1, respectively (Table 2). Blood samples were collected at the end of the experiments (10 days post-virus inoculation), and sera stored at −20°C until tested. Virus isolation, PCR testing as well as HI assays verified infections of inoculated birds (Pantin-Jackwood et al. in preparation).

The H5 IA virus-specific ELISA was performed using slightly modified protocols from the manufacturer’s instructions: three incubation periods (1, 4 and 18 hours) and two dilution factors (1:2 and 1:5) were evaluated. Briefly, serum samples were diluted with sample diluent provided by the manufacturer, and 100 µl of the diluted samples were dispensed into the antigen-coated test plates. Samples were incubated at 36°C at 1, 4 or 18 hours and manually washed three times with approximately 300 µl of wash solution (provided in the kit), per well. Next, 50 µl of conjugate were
Table 1. Effect of the incubation period and sample dilution factor on the results obtained with the enzyme-linked immunosorbent assay for the detection of non-H5 Influenza A virus antibodies in experimentally infected Mallards

| Subtype               | Strain name                        | N  | 1 hour 1:5 Mean S/N | POS | 1 hour 1:2 Mean S/N | POS | 4 hours 1:5 Mean S/N | POS | 4 hours 1:2 Mean S/N | POS | 18 hours 1:5 Mean S/N | POS | 18 hours 1:2 Mean S/N | POS |
|-----------------------|------------------------------------|----|---------------------|-----|---------------------|-----|---------------------|-----|---------------------|-----|---------------------|-----|---------------------|-----|
| Sham-inoculated birds |                                    |    |                     |     |                     |     |                     |     |                     |     |                     |     |                     |     |
| H3N8                  | A/Mallard/Minnesota/5-0516/2007     | 5  | 0.94                | 0   | 0.89                | 0   | 0.89                | 0   | 0.89                | 0   | 1.00                | 0   | 0.85                | 0   |
| H4N6                  | A/Surface water/Minnesota/1-32/2006| 5  | 0.91                | 0   | 0.83                | 0   | 0.87                | 0   | 0.87                | 0   | 0.85                | 0   | 0.72                | 0   |
| H4N8                  | A/Mallard/Minnesota/5-0519/2007     | 5  | 0.96                | 0   | 0.79                | 0   | 0.89                | 0   | 0.78                | 0   | 0.84                | 0   | 0.70                | 0   |
| H6N1                  | A/Mallard/Minnesota/5-0517/2007     | 5  | 0.91                | 0   | 0.72                | 0   | 0.76                | 0   | 0.71                | 0   | 0.74                | 0   | 0.59                | 0   |
| H6N2                  | A/Duck/Minnesota/5-0107/2007        | 5  | 0.86                | 0   | 0.71                | 0   | 0.74                | 0   | 0.65                | 0   | 0.67                | 0   | 0.58                | 0   |
| H6N8                  | A/Green-winged teal/Minnesota/5-0197/2007 | 5  | 0.89                | 0   | 0.82                | 0   | 0.87                | 0   | 0.66                | 0   | 0.76                | 0   | 0.58                | 0   |
| H8N4                  | A/Mallard/Minnesota/5-0557/2008     | 4  | 0.89                | 0   | 0.80                | 0   | 0.81                | 0   | 0.73                | 0   | 0.83                | 0   | 0.58                | 0   |

N, Number of tested samples; S/N, sample-to-negative control ratio; POS, number of positive samples; Numbers in parenthesis represent samples considered doubtful (S/N: 0.35–0.40); 1:5 and 1:2, sample dilution factor.

Results obtained for non-H5 viruses are presented in Table 1. Although all sera tested positive for the presence of non-H5 antibodies with two commercial ELISA (H5-specific antigens, CA, USA), serum samples with a sample-to-negative control (S/N) ratio values greater than or equal to 0.40 were considered negative. Serum samples with S/N ratio values below 0.35 were considered positive for the presence of non-H5 antibodies. A single validation was performed for the presence of non-H15 viruses are presented in Table 1. Although all sera tested positive for the presence of H15 antibodies with two commercial ELISA (H5-specific antigens, CA, USA), serum samples with a sample-to-negative control (S/N) ratio values greater than or equal to 0.40 were considered negative. Serum samples with S/N ratio values below 0.35 were considered positive for the presence of non-H5 antibodies.
Table 2. Effect of the incubation duration and sample dilution factor on the results obtained with the enzyme-linked immunosorbent assay for the detection of low- and highly pathogenic H5-specific Influenza A virus antibodies

| Subtype          | Strain name                        | Species | N 1 hour 1:5 Mean S/N | POS | 1:2 Mean S/N | POS | 4 hours 1:5 Mean S/N | POS | 1:2 Mean S/N | POS | 18 hours 1:5 Mean S/N | POS | 1:2 Mean S/N | POS |
|------------------|------------------------------------|---------|------------------------|-----|--------------|-----|----------------------|-----|--------------|-----|-----------------------|-----|--------------|-----|
| H5N2 (LP)        | A/Mallard/Mallard/355779/2000       | Mallard | 9                      | 0.36| 4 (+1)       | 0.20| 8 (+1)              | 0.21| 9             | 0.09| 9                      | 0.14| 9             | 0.06| 9             |
| H5N1 (HP) Clade 2.1.3 | A/Chicken/Garut/BBVW-223/2007          | Pekin duck | 3                        | 0.71| 0            | 0.61| 0                   | 0.50| 1             | 0.48| 1                      | 0.43| 1             | 0.39| 1             |
|                  | A/Chicken/Pekalongan/BBVW/2007       | Pekin duck | 2                        | 0.51| 0            | 0.33| 2                   | 0.44| 1             | 0.29| 2                      | 0.27| 1 (+1)        | 0.15| 2             |
|                  | A/Chicken/West Java/29/2007          | Pekin duck | 3                        | 0.55| 1            | 0.51| 1                   | 0.44| 1             | 0.41| 1                      | 0.43| 1             | 0.41| 1             |
|                  | A/Chicken/West Java/SMI-PAT/2006     | Pekin duck | 7                        | 0.76| 0            | 0.60| 1                   | 0.64| 0             | 0.60| 0 (+1)                 | 0.63| 0             | 0.53| 1 (+1)        |
|                  | A/Chicken/West Java/TASIKO B/2006    | Pekin duck | 5                        | 0.55| 0 (+1)      | 0.72| 0                   | 0.36| 2 (+2)       | 0.26| 4                      | 0.30| 4 (+1)       | 0.22| 4 (+1)        |
| H5N1 (HP) Clade 2.2.1 | A/Chicken/Egypt/06207-NLQP/2006        | Pekin duck | 4                        | 0.63| 1            | 0.36| 2 (+2)              | 0.56| 3             | 0.28| 4                      | 0.44| 2 (+1)       | 0.25| 4             |
|                  | A/Chicken/Egypt/07118-NLQP/2006      | Pekin duck | 6                        | 0.42| 2 (+1)      | 0.34| 4                   | 0.29| 4             | 0.22| 5                      | 0.21| 5             | 0.17| 6             |
|                  | A/Chicken/Egypt/0813-NLQP/2008       | Pekin duck | 6                        | 0.47| 1            | 0.34| 4                   | 0.27| 5             | 0.22| 6                      | 0.28| 5 (+1)       | 0.22| 6             |
|                  | A/Duck/Egypt/0923-NLQP/2009          | Pekin duck | 2                        | 0.71| 0            | 0.54| 0                   | 0.44| 0             | 0.35| 1 (+1)                 | 0.30| 1 (+1)       | 0.22| 2             |

N, Number of tested samples; S/N, sample-to-negative control ratio; POS, number of positive samples; Numbers in parenthesis represent samples considered doubtful (S/N: 0.35–0.40); 1:5 and 1:2, sample dilution factor; LP, low pathogenic; HP, high pathogenic.
avian species throughout the world. As with poultry, these serosurveys have been performed on a population level and have complimented traditional isolation or molecular approaches to expand our understanding on IA natural history and epidemiology.

Rapid serological tools for subtype-specific IA antibody testing can greatly enhance our ability to evaluate both exposure and potential reservoir status of diverse wild bird populations. These tests also provide a means for detecting species involvement in IA virus epidemiology in situations where virus detection is difficult, for instance because of limited shedding. Although the results presented in this study present limitation as they were not compared with a reference test (e.g. H5 HI), our findings suggest that with slight and reasonable modifications to the manufacturer’s protocols, the commercial ELISA may perform adequately enough to provide valuable LP H5 exposure data in waterfowl. We, however, warrant that the important antigenic diversity existing for viruses such as the Asian lineage of HP H5N1 IA virus is likely to affect the sensitivity of the assay and yield to false-negative results.

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