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Short Article

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

Camille Lebarbenchon,[a] Mary Pantin-Jackwood,[b] Whitney M. Kistler,[a] M. Page Luttrell,[a] Erica Spackman,[b] David E. Stallknecht,[a] Justin D. Brown[a]

[a]Southeastern Cooperative Wildlife Disease Study, Department of Population Health, College of Veterinary Medicine, The University of Georgia, Athens, GA, USA. [b]US Department of Agriculture, Southeast Poultry Research Laboratory, Agricultural Research Service, Athens, GA, USA.
Correspondence: Université de La Réunion, Avenue René Cassin, BP 7151, 97715 Saint-Denis Cedex, La Réunion. E-mail: camille.lebarbenchon@univ-reunion.fr

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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 dilutions (1:2 and 1:5) were tested. 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.

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Testing for antibodies to Influenza A (IA) virus is a common diagnostic tool used in poultry[1] 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 ELISAs[10] 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 dilutions 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

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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

			1 hour				4 hours				18 hours			
			1:5		1:2		1:5		1:2		1:5		1:2	
Subtype	Strain name	N	Mean S/N	POS	Mean S/N	POS	Mean S/N	POS	Mean S/N	POS	Mean S/N	POS	Mean S/N	POS
Sham-inoculated birds		8	0·94	0	0·89	0	0·89	0	0·89	0	1·00	0	0·85	0
H3N8	A/Mallard/MN/Sg-00169/2007	5	0·94	0	0·83	0	0·86	0	0·79	0	0·88	0	0·72	0
H4N6	A/Surface water/MN/NW1-T/2006	5	0·91	0	0·83	0	0·87	0	0·73	0	0·85	0	0·66	0
H4N8	A/Mallard/MN/Sg-00219/2007	5	0·96	0	0·79	0	0·89	0	0·78	0	0·84	0	0·70	0
H6N1	A/Mallard/MN/Sg-00170/2007	5	0·91	0	0·72	0	0·76	0	0·71	0	0·74	0	0·59	0
H6N2	A/Mallard duck/MN/Sg-00107/2007	5	0·86	0	0·71	0	0·74	0	0·65	0	0·67	0	0·58	0
H6N8	A/Green-winged teal/MN/Sg-00197/2007	5	0·89	0	0·82	0	0·87	0	0·66	0	0·76	0	0·58	0
H8N4	A/Mallard/MN/Sg-00570/2008	4	0·89	0	0·80	0	0·81	0	0·73	0	0·83	0	0·58	0 (+1)

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.

added to each well, and plates were incubated for 30 minutes at 23°C. Each well was washed again three times as described above. Finally, 50 μl of substrate solution was added to each well, and plates were incubated at 23°C in the dark for 10 minutes. The reactions were stopped by adding 50 μl of stop solution. Sample absorbance was measured at 450 nm with a BIO-RAD Benchmark microplate reader (Hercules, CA, USA). Serum samples with a sample-to-negative control (S/N) ratio values greater than or equal to 0·40 were considered negative. According to the manufacturer instructions, samples with S/N ratio values between 0·35 and 0·40 were considered to be suspect positives. Serum samples with S/N ratio values below 0·35 were considered positive for the presence of H5 antibodies. A single validation was performed for each sample.

Results obtained for non-H5 viruses are presented in Table 1. Although all sera tested positive for the presence of IA virus antibodies with two commercial ELISA,[10] H5-specific antibodies were not detected regardless of incubation time or sample dilution, suggesting that false-positive results were unlikely for the tested virus subtypes. A note of caution, however, is warranted as not all non-H5 subtypes were tested in this study (i.e. 16 different HA have been described in wild birds); in addition, one H8N4 serum sample was considered doubtful for the 18 hours incubation with a 1:2 dilution factor.

All low pathogenic H5N2 sera tested positive with the ELISA assay particularly when the duration of incubation was increased from 1 hour to 4 or 18 hours (Table 2). The same trend was observed for HP H5N1 sera, with slightly improved results obtained when the sample dilution was decreased to 1:2. Results obtained for the HP sera also were highly variable depending on the tested viral strain (Table 2), although all sera tested positive with a HI assay (Pantin-Jackwood *et al.* in preparation). Overall, an improved detection rate was obtained for viruses belonging to the H5N1 clade 2.2.1 as compared with those belonging to clade 2.1.3 (e.g. only 14% of tested samples yielded positive results for A/Chicken/West Java/SMI-PAT/2006). These results suggest that although the ELISA may be suitable for the detection of H5 IA virus-specific antibodies, the important antigenic diversity existing for viruses such as the HP H5N1 Asian lineage is likely to affect assay sensitivity and yield false-negative results.

Serology is an important diagnostic tool commonly used in poultry to detect anti-IA antibodies. When included in surveillance programmes, serology should be considered a flock-level test, as multiple variables relating to infection (unknown exposure date), host (antibody response), virus (virulence, antigenicity), or serologic assay (sensitivity) may influence results in an individual bird. Serology has historically been an underutilized diagnostic tool for IA in wild birds; however, the recent availability of accurate species-independent commercially available assays has initiated new research and surveillance efforts in a variety of wild

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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/MN/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.

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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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