Running title: Commercial Competition ELISAs for AIV serology in wild birds

Serological Testing for Avian Influenza Viruses in Wild Birds: Comparison of Two Commercial Competition ELISAs

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Abstract

Serologic testing of wild birds for AIV surveillance poses problems due to species differences and non-specific inhibitors that may be present in sera of wild birds. Recently available competitive ELISA kits offer a new species independent approach. In this study we compare two commercial cELISAs, using a total of 184 serum and plasma samples from 23 species of wild birds belonging to 10 orders. Thirteen samples were from experimentally HPAI and LPAI infected red-legged partridges (*Alectoris rufa*), 77 samples were from a flock of sentinel hybrid ducks confirmed infected by AI by RTPCR and 94 samples were from wild birds admitted to a rehabilitation center. Both ELISAs detected AI antibodies in the experimentally infected partridges, while HI was negative. Concordance in results between the two ELISAS was 51.5%. When specific subtype H5/H7 HI positive samples were considered for comparison, ELISA 1 appeared to perform better on ducks while ELISA2 appeared to perform better in other wild bird species. Overall, 68.2% of H5/H7 positive samples tested positive by ELISA 1 and 36% by ELISA 2. Both ELISAs detected AIV antibody positive samples negative by specific HI against 9 of the 16 existing HA subtypes. Presumably this reflects either higher sensitivity of cELISA when compared to HI, presence of antibodies against HA subtypes not tested, or unspecific reactions. Performance of ELISA 1 on ducks appears to be comparable to in-house cELISA previously used by other authors in wild birds but requires a relatively large sample volume. Alternatively, while the ELISA 2 required a smaller sample volume, it was less effective at identifying HI positive samples. The results reflect the necessity of validation of cELISA tests for individual species or at least families as required by the OIE.

41 Key words: Avian influenza, cELISA, HI, serology, wild birds.

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43 Abbreviations: AGID = agar gel immunodiffusion; AIV = avian influenza virus; cELISA

44 = competitive enzyme-linked immunosorbent assay; HA = hemagglutinin; HI =

45 hemagglutination inhibition; HU = hemagglutinating units; iELISA = indirect enzyme-

46 linked immunosorbent assay; RTPCR = real time polymerase chain reaction.

Introduction

The continuing avian influenza epidemic due to subtype H5N1 has resulted in commitment of resources towards improving avian influenza virus detection tools, especially in wild birds as they are considered the natural reservoir of all hemagglutinin and neuraminidase subtypes of influenza A viruses (5).

Serologic testing is widely used for surveillance and disease control in domestic poultry, employing mainly AGID test and iELISA systems as pre-screening tools and HI test as confirmation assay since it is considered the serological standard method.

Nevertheless, some constraints exist in relation to serologic testing in wild birds. AGID tests can be useful when screening large numbers of birds, although it has not been thoroughly validated for use with other than gallinaceous species and has been shown to yield inconsistent results in some species, such as mallards, that do not always produce precipitating antibodies (6,8,13). HI provides information on the presence of antibodies directed against specific subtypes of AIV and can be applied to a variety of bird species. However, to avoid effect of non-specific inhibitors, the sera need to be pre-treated, potentially causing a decrease of antibody titers. Other constraints of the use of HI are related to steric inhibition from the antibodies to the NA protein that can affect the interaction of the HA protein and the antibodies directed to it, the fact that it is expensive, very labor intensive and not readily applicable to high-throughput testing (13).

An additional problem in serological testing in wild birds is the amount of serum or plasma needed in the test systems that frequently exceeds the maximum volume that can be extracted without harming lower body mass individuals, especially small songbirds.

For high-throughput screening of poultry, ELISAs have been developed to detect mainly anti-NP-antibodies directed against a cross-reactive antigen shared by all

influenza A viruses. However, most of the current commercial immunoenzymatic tests are based on indirect detection of specific antibodies using anti-species conjugates. These tests mostly employ anti-chicken IgG secondary antibodies and thus can generally not be employed to test wild bird samples (4). Some groups have tried approaches using peroxidase labeled anti-chicken IgA conjugate produced in rabbits with good results for the species tested, but the system has not been taken to a commercial format (1,2).

More recently, cELISA systems have been implemented to detect antibodies against AI NP in different avian species (12,14). This refined species independent approach has been used for wildlife surveillance (6,7), and is now commercially available.

Generally, validation of these tests is carried out with influenza antibody positive samples obtained during experimental infections, as this way specific antibody titers and HA subtype against which they are directed are known (12,14). However, this means that only a reduced number of species is included in validation studies and in effect, the OIE standards (12) recommend these tests to be validated for each species tested. This has the inconvenience that in most cases only a limited number and amount of samples are available.

Recently, the first validation of a commercial bELISA (IDEXX) using 281 sera from experimentally infected and sham inoculated wild bird species has been reported. Results obtained from the bELISA as compared to AGID, revealed the usefulness of the kit as a preliminary screening tool and as a complement to agent identification methods (3). However, this kit was not evaluated using field samples in which antibody response is expected to be lower than in experimental infections.

The aim of this study is to compare results from two commercial cELISAs using firstly sera from experimentally infected red-legged partridges (*Alectoris rufa*), secondly plasma samples from a group of sentinel ducks confirmed infected by LPAI using

RTPCR and finally samples from different species of wild birds with the aim of assessing usefulness of this kind of assays in wild bird disease surveillance.

Material and Methods

In our study, we employed three different samples sets. A small number of sera (n=13) was available from a recently completed infection experiment using low and high pathogenic H7 subtype avian influenza virus in red legged partridges (Table 3). Briefly, the samples were from three control birds, five partridges infected with H7N9 LPAI on day 15 *p.i.*, just prior to euthanasia and five partridges infected with H7N1 HPAI on days 5, 6 and 8 *p.i.*

A total of 77 plasma samples were available from a flock of sentinel ducks (mallard hybrids, included in Tables 1 and 2 as *Anas sp.*) in which LPAI infection had been confirmed by RTPCR. The third set of samples originated from a plasma bank collected between 1995 to 2007 from birds of prey, white storks, and other waterbirds admitted to rehabilitation centers in Castilla –La Mancha in South central Spain. The sample type available for all individual birds from the field was plasma, as the extracting veterinarians used a single tube for blood extraction in order to maximize sample yield.

A total of 1400 plasma samples from the wild bird plasma bank, and from sentinel ducks were tested against H5 and H7 subtype AIV using the HI test with 4 HU as described previously (11; data not shown). The red-legged partridge sera were tested by HI against H7 subtype AIV only. Antigen for HI testing had been purchased from the influenza A virus reference laboratory of the OIE (Veterinary laboratories agency, VLA New Haw, Addlestone, UK). Antigens available for primary screening were H7N1 (A/African Starling/983/79) and H5N2 (A/Ostrich/Denmark/72420/96). Positive sera

were then confirmed using H5N1 (A/Chicken/Scotland/59) and H7N7 (A/Turkey/England/647/77) antigen. All antigens had been produced in specific pathogen free (spf) embryonated chicken eggs and were provided lyophilized. Prior to analysis all samples were inactivated 30 min at 56° C. Samples with a HI titer ≥ 1.16 were considered positive.

A subset of 94 plasma samples of wild birds was selected to include a considerable number of AIV antibody positive samples (as by H5/H7 subtype HI), and where possible, also a minimum of randomly selected HI (H5/H7) negative samples from individuals of the same species and families. Twenty three species, belonging to ten families were included in this study.

The samples were analyzed blindly using two different ELISAs (ELISA1 Ingezim Influenza A[®], Ingenasa, Spain; ELISA2 IDscreen, influenza A antibody competition[®], idvet, France). ELISA1 is based on a blocking protocol and ELISA2 is based on a competition protocol. ELISA kits were used according to manufacturer's instructions. HI test positive samples that tested negative with ELISA2 were re-tested including an overnight incubation period as described by the manufacturer for problem samples.

A sample was considered doubtful if the competition percentage was between 45 and 50% in the case of ELISA1 and between 40 and 45% in ELISA2, as defined by manufacturers. For subsequent comparisons, doubtful samples were considered positive. Finally, 49 plasma samples that tested positive in one or both of the ELISAs but negative in the HI test against H5 and H7 antigen, were submitted to HI tests against other HA subtypes, depending on the availability of sample. HA subtypes against which the problem samples were tested included H1, H2, H3, H6, H9, H10 and H12. HI tests for these additional HA antigens were carried out at the virology department of the Klinik für Vögel, Amphibien, Fische und Reptilien of the veterinary faculty in Giessen, Germany.

Results

In the red legged partridge samples included in the study, H7 AIV subtype infection was confirmed by RTPCR on feathers and swabs on consecutive days. AIV antibodies were detected by both cELISAs but not by the HI test (Table 3). Concordance between the two ELISAS was 53.8%.

Among the sentinel ducks, 62 samples (80.5%) tested positive by ELISA 1, while only 16 (20.8%) samples were identified as positive by ELISA2 (Table 1). HI confirmed 14 (22.2%) of the samples positive by ELISA 1 and 5 (31.3%) of the samples that had tested positive by ELISA2. Two of the total of 17 samples that tested positive against H5 or H7 subtypes in HI (Table 1) were not detected by any of the two cELISAS. Concordance between the two ELISAS for the sentinel duck samples was 36.4%.

With ELISA1, 14 (14.89%) of the 94 wild birds tested positive, while 38 (40.4%) of the birds tested positive by ELISA2 (Table 2). Both ELISAs agreed on 60 (63.82%) of the samples. Five of these birds had H5 or H7 subtype specific antibodies as demonstrated by HI.

If only H5/H7 HI positive samples are selected and compared to the results of the two ELISAs, concordance of ELISA1 and HI was 82.4 % (14 ELISA positive of 17 HI positives) in the sentinel duck samples and 20% (one ELISA positive of 5 HI positives) for samples of the other bird species included. ELISA2 showed 29.4% concordance with H5/H7 HI positive results on sentinel duck samples (5 ELISA positives of 17 HI positives) and 60% of the wild bird samples (three ELISA positives of 5 HI positives). Thus, overall (integrating sentinel duck and wild bird samples), considering the H5/H7 HI test results and the ELISA positive results with the cut-off defined by the

manufacturer, concordance of the HI test and the cELISAs was 68.2% and 36% respectively for ELISA1 and ELISA2. Both ELISAs agreed on 51.5% of the samples.

Concordance of AIV antibody detection between the HI test and cELISAs was not related to the titer in the HI test. Overnight incubation of negative samples with ELISA 2 did not modify the results.

Among the samples negative by H5/H7 subtype HI, 19 were identified as positive by both ELISAs. In total, 60 samples were identified as negative by all three tests.

None of the ELISA-positive/HI (H5/H7)-negative samples that could be tested against additional HA subtypes (n = 49) gave a positive result. Due to the reduced amount of sample volume it was not possible to test the samples against all HA subtypes. Thus HA AIV subtypes not tested in this study include: H4, H8, H11, H13, H14, H15 and H16. Also, AGID testing could not be carried out due to the lack of sample.

H5/H7 HI negative but ELISA positive results were encountered in eight of the ten families included in the study.

Discussion

Serologic testing of multiple different avian species for AIV antibodies is a challenge that the use of cELISA technology tries to solve (3,15).

In our study we compared the usefulness of recently available commercial multispecies ELISA kits for assessment of AIV seroprevalence in multiple wild bird species. ELISAs have been previously used in wild bird surveillance in Italy (6,7), but in this case an in-house double antibody sandwich blocking ELISA was employed that is not commercially available. Studies carried out in the 90s in southern Spain employed an in-house iELISA based on anti-chicken IgA conjugate (1,2). A cELISA developed in the frame of the German AIV surveillance program was thoroughly tested and gave

promising results but again, is not available commercially (14). All these in-house ELISAs require extensive reagent production and manipulation, and thus may not be feasible in many laboratories (15), a situation that a commercially available kit using blocking ELISA technology could solve.

In this study, samples from 23 species of wild birds are included, most of which are related to wetland environments that is considered the reservoir for AIV. Other predominantly terrestrial species included might become exposed to AIV through consumption of susceptible birds.

In our sample set, correlation both between the cELISAs and HI and between the two cELISAs was variable. As the HI test and the blocking ELISAs investigated detect antibodies directed against different antigens, direct comparison is not appropriate. Therefore, we used proportion of concordance between the different tests for comparison.

While a high concordance was observed between ELISA1 and HI in duck samples, this test appeared to perform less well with other species. Correlation between HI and ELISA 2 was relatively poor for duck samples but average in other bird species. However, the sample set available for comparative testing was limited and contained only five H5/H7 subtype HI positive samples. In general, testing the samples by AGID also could have made interpretation of the results easier. Nevertheless, the small amount of sample available and the possibility that only part of the species might produce precipitating antibodies (13) led to the choice of HI as a comparative test.

In the case of ELISA1 the manufacturers recommend the use of a volume of 50µl for each sample. Given that this quantity exceeds in many cases the maximum volume that can be extracted without harming the individuals, this test is not very practical when working with small birds. ELISA2, trying to improve this aspect, is configured for use with 10µl of sample. This drastic volume reduction is of advantage for the analysis of

wild bird samples, especially in songbirds. However, it might have negative effects on performance, especially in animals with low antibody titers. Despite the absence of a direct relation between specific HI titers and detection by either of the two ELISAs, sample dilution may be an important factor.

On the other hand, numerous samples tested positive by one or both of the ELISAs but were negative by HI against nine of the 16 existing HA subtypes. Potentially, these samples may contain antibodies against any of the subtypes that were not tested, although some of these subtypes are fairly rare in the species and geographic sample location (8). Also, non-specific reactions or inhibition of the samples in the HI test system are possible, especially as in this case the material available was plasma. Nevertheless, authors that have developed and used cELISAs previously have encountered similar situations with field samples (7,14). Part of the samples employed by Starick *et al.* (2006) in which this problem was observed were from experimentally infected ducks. HI positivity of consecutive samples, titration and confirmation using an iELISA with a multispecies conjugate led these authors to conclude that in part of the cases the cELISA was more sensitive than HI. In field samples of ducks and geese tested thereafter by the same authors, this assumption was confirmed by titration curves of cELISA positive/HI negative samples, while in some other cases incongruent results between HI and ELISA were not resolved. However, the overall conclusion was that the test was very sensitive for flock screening as well as for the screening of wild bird samples (14).

Unfortunately in our case, no further samples were available from experimentally infected red-legged partridges, because all partridges infected with HPAI had died by day 8 *p.i.*, while all LPAI infected partridges were euthanized on day 15 *p.i.* Thus we suppose that the HPAI infected partridges may not have had time to produce subtype

specific agglutinating antibodies. The performance of ELISA1 and ELISA2 on these samples was similar to the set of general wild bird samples.

In their study, Arenas *et al.* (1990) detected an AIV seroprevalence of 40% using an in-house iELISA based on anti-chicken IgA conjugate, in comparison to a 10% AIV seroprevalence in HI using HA subtypes H1, H3, H5 and H7. In a following study by researchers of the same group, using only the iELISA, overall AIV seroprevalence was much lower, namely 6.2% (2).

It has to be bared in mind that although the class “birds” is generally treated as a whole entity, it comprises a great variety of species, and therefore also a considerable variation in the immune response (3,10,14). As an example, antibodies may be induced in ducks, as well as other anatid species, that cannot be detected in conventional HI test performed with intact virus (10,14). In their surveillance studies, De Marco *et al.* (2004) detected an AIV seroprevalence of 52.2% in mallard ducks and 7.1% in coots using an in-house cELISA, however only 16.6% of the ELISA positive duck sera and 33.3% of the positive coot sera were positive against one or several HA subtypes in the HI test. In our case, overall species, 19.48% of the ELISA 1 and 14.81% of the ELISA 2 positive samples were consistent with positive (H5/H7 subtype specific) HI. More specifically in ducks, HI results agreed with 21.21% of the ELISA 1 and 19.23% of the ELISA 2 positives. Thus performance of both ELISAs appears to be comparable to the tests used by De Marco *et al.* (2004) and Starick *et al.* (2006). However, as said before, we were not able to test the incongruent samples against all existing HA subtypes, due to the lack of sample.

Recently, a bELISA using commercially available recombinant nucleoprotein antigen, was tested successfully using samples of a variety of experimentally and naturally infected bird and mammal species (15), but comparative data of performance between the three tests is not yet available.

In conclusion, the cELISA system appears to be useful for the general screening of wild birds for AIV antibodies if it is kept in mind that results may vary with the species/family of bird tested and that specific validation as recommended by OIE may be necessary. In our case, ELISA1 performed better on duck samples while ELISA2 did so in other bird species. Also, it could be interesting to amplify results by testing the cELISA positive samples against specific HA and NA subtypes using HI tests.

References

- (1) Arenas, A., J. Carranza, A. Perea, A. Miranda, A. Maldonado, and M. Hermoso. Type A influenza viruses in birds in southern Spain: Serological survey by enzyme-linked immunosorbent assay and haemagglutination inhibition tests. *Avian Path.* 19(3):539-546. 1990.
- (2) Astorga, R. J., L. Leon, M. J. Cubero, A. Arenas, A. Maldonado, M. C. Tarradas, and A. Perea. Avian influenza in wild waterfowl and shorebirds in the Doñana National Park: Serological survey using the enzyme-linked immunosorbent assay. *Avian Path.* 23(2): 339-344. 1994.
- (3) Brown, J.D., D.E. Stallknecht, R.D. Berghaus, M.P. Luttrell, K. Velek, W. Kistler, T. Costa, M. J. Yabsley, and D. Swayne. Evaluation of a commercial blocking enzyme-linked immunosorbent assay to detect avian influenza virus antibodies in multiple experimentally infected avian species. *Clin. and Vaccine Immunol.* 16(6):824-829. 2009.

(4) Cattoli G., and I. Capua. Diagnosing avian influenza in the framework of wildlife surveillance efforts and environmental samples. *J. Wildlife Dis.* 43(3):S35-S39. 2007.

(5) Charlton, B., B. Crossley, and S. Hietala. Conventional and future diagnostics for avian influenza. *Comp. Immunol. Microb. and Infect. Dis.* 32:341-350. 2009.

(6) De Marco, M.A., G.E. Foni, L. Campitelli, E. Raffini, L. Di Trani, M. Delongu, G. V. Guberti, G. Barigazzi, and W. Di Donatelli. Circulation of influenza viruses in wild waterfowl wintering in Italy during the 1993-99 period: Evidence of virus shedding and seroconversion in wild ducks. *Av. Dis.* 47:861-866. 2003.

(7) De Marco, M.A., L. Campitelli, E. Foni, E. Raffini, G. Barigazzi, M. Delogu, V. Guberti, L. Di Trani, M. Trollis, I. Donatelli. Influenza surveillance in birds in Italian wetlands (1992-1998): is there a host restricted circulation of influenza viruses in sympatric ducks and coots? *Vet. Microb.* 98:197-208. 2004.

(8) Higgins, D.A. Precipitating antibodies of the duck (*Anas platyrhynchos*). *Comp. Biochem. and Physiol.* 94:135-144. 1989.

(9) Kaleta, E. F., G. Hergarten, and A. Yilmaz. Avian influenza A viruses in birds – an ecological, ornithological and virological view. *Dtsch. Tierärztl. Wschr.* 112:448-456. 2005.

(10) Kida, H., R. Yanagawa, Y. Matsuoka. Duck influenza lacking evidence of disease signs and immune response. *Infect. and Immun.* 30:547-553. 1980.

(11) OIE. 2008. Manual of diagnostic tests and vaccines for terrestrial animals.
http://www.oie.int/eng/normes/mmanual/2008/pdf/2.03.04_AI.pdf.

(12) Shafer, A.L., J.B. Jatz, and K.A. Eernisse. Development and validation of a competitive enzyme-linked immunoabsorbent assay for detection of type A influenza antibodies in avian sera. *Av. Dis.* 42:28-34. 1998.

(13) Spackman, E., D.L. Suarez, and D.A. Senne. Avian influenza diagnostics and surveillance methods. In: *Avian Influenza*. D.E.Swayne Ed. Blackwell Publishing, Oxford, UK. pp 299-308. 2008.

(14) Starick, E., O. Werner, H. Schirrmeyer, B. Kollner, R. Riebe, and E. Mundt. Establishment of a competitive ELISA (cELISA) system for the detection of influenza A virus nucleoprotein antibodies and its application to field sera from different species. *J. Vet. Med. B* 53:370-375. 2006.

(15) Sullivan, H.J., B.J. Blitvich, K. VanDalen, K.T. Bentler, A.B. Franklin, and J.J. Root. Evaluation of an epitope-blocking enzyme-linked immunosorbent assay for the detection of antibodies to influenza A virus in domestic and wild avian and mammalian species. *J. Virol. Methods* 161:141-146. 2009.

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383 Table 1. Wild bird species and families included in this study and results of HI and
 384 cELISAs.

FAMILY	SPECIES	N	HI H5/H7+	ELISA1 +	ELISA2 +
Accipitridae N=45	Spanish Imperial Eagle (<i>Aquila adalberti</i>)	10	1	4	5
	Golden eagle (<i>Aquila chrysaetos</i>)	7	0	0	2
	Marsh harrier (<i>Circus aeruginosus</i>)	6	0	0	1
	Eurasian black vulture (<i>Aegypius monachus</i>)	5	0	0	0
	Black kite (<i>Milvus migrans</i>)	5	0	0	0
	Red Kite (<i>Milvus milvus</i>)	4	0	0	0
	Common buzzard (<i>Buteo buteo</i>)	8	1	0	3
Anatidae N=88	Gadwall (<i>Anas strepera</i>)	1	1	0	0
	Northern Pintail (<i>Anas acuta</i>)	1	0	0	1
	Mallard (<i>Anas platyhrynchos</i>)	6	0	3	6
	Sentinel duck (<i>Anas sp.</i>)	77	17	63	16
	Greylag geese (<i>Anser anser</i>)	1	0	0	1
	<i>Anas sp</i>	1	0	0	1
	Red crested pochard (<i>Netta rufina</i>)	1	0	0	1
Ardeidae N=2	Purple heron (<i>Ardea purpurea</i>)	1	0	1	1
	Grey Heron (<i>Ardea cinerea</i>)	1	0	0	0
Ciconidae N=16	White stork (<i>Ciconia ciconia</i>)	16	0	4	8
Corvidae N=4	Magpie (<i>Pica pica</i>)	4	0	0	0
Laridae N=3	<i>Larus sp</i>	3	0	1	3
Phalacrocoracidae N=2	Great cormorant (<i>Phalacrocorax carbo</i>)	2	0	1	1
Phasianidae N=2	Red-legged partridge (<i>Alectoris rufa</i>)	2	1	0	0
Phoenicopteridae N=1	Greater flamingo (<i>Phoenicopterus ruber</i>)	1	0	0	1
Strigidae N=8	Eurasian Eagle-owl (<i>Bubo bubo</i>)	8	1	0	3
Total		171	22	77	54

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387 Table 2. Comparative results of H5/H7 HI test, ELISA1 and ELISA2

Family	N	ELISA 1				ELISA 2			
		+		-		+		-	
		HI H5/H7+	HI H5/H7-	HI H5/H7+	HI H5/H7-	HI H5/H7+	HI H5/H7-	HI H5/H7+	HI H5/H7-
<i>Accipitridae</i>	45	1	3	1	40	2	9	0	34
<i>Anatidae (sentinels)</i>	77	14	49	3	11	5	11	12	49
<i>Anatidae</i>	11	0	3	1	8	0	10	1	0
<i>Ardeidae</i>	2	0	1	0	1	0	1	0	1
<i>Ciconidae</i>	16	0	4	0	12	0	8	0	8
<i>Corvidae</i>	4	-	-	0	4	-	-	0	4
<i>Laridae</i>	3	0	1	0	2	0	3	-	-
<i>Phalacrocoracidae</i>	2	0	1	0	1	0	1	0	1
<i>Phasianidae</i>	2	-	-	1	1	-	-	1	1
<i>Phoenicopteridae</i>	1	-	-	0	1	0	1	-	-
<i>Strigidae</i>	8	-	-	1	7	1	2	0	5
TOTAL	171	15	62	7	87	8	46	14	103
		77		94		54		117	

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395 *Table 3.* Comparative results of HI test, ELISA1 and ELISA2 from experimental
 396 samples.

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Reference	Group	HI H7	ELISA1	ELISA2
1	HPAI H7N1	-	+	+
2	HPAI H7N1	-	-	+
3	HPAI H7N1	-	-	+
4	HPAI H7N1	-	+	+
5	HPAI H7N1	-	-	+
6	LPAI H7N9	-	-	+
7	LPAI H7N9	-	-	+
8	LPAI H7N9	-	-	+
9	LPAI H7N9	-	-	-
10	LPAI H7N9	-	-	-
11	Control	-	-	-
12	Control	-	-	-
13	Control	-	-	-