Evaluation of different serological tests for the detection of antibodies against highly pathogenic avian influenza in experimentally infected ostriches (*Struthio camelus*)

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In the present study we collected 177 serum samples from ostriches (*Struthio camelus*) infected experimentally with A/ostrich/South Africa/Middleton/2004 (H5N2) highly pathogenic avian influenza virus. We tested these samples using the haemagglutination inhibition (HI) test, the agar gel immunodiffusion test and three enzyme-linked immunosorbent assay kits. We considered the HI test, with homologous antigen and including pre-treatment of sera with 10% chicken red blood cells, as the gold standard. Detectable specific antibodies appeared on day 7 post-infection and persisted until the termination of the experiment. The relative sensitivity and specificity of the tests under evaluation and Cohen's *K* value were calculated. The results reported herein could be of assistance to decision-makers in drafting guidelines for the definition of the health status of ostriches and for trade purposes.

Introduction

Despite the inclusion of ostriches (*Struthio camelus*) in the World Organisation for Animal Health (OIE, 2008b) and European Union (EU, 1990) definitions of “poultry” (Terrestrial Animal Health Code, 2008 and 1990/539/EEC), they are phylogenetically—and thus anatomically and physiologically—very different from conventional poultry.

Ostriches are classified in the superorder *Paleognathae*, order *Ratites*, suborder *Struthioniformes*, family *Struthionidae* (Vigors, 1825)—distant from the *Galloanseriformes* that belong to a different superorder the *Neognathae* (Feduccia, 1999; Edward *et al.*, 2002). They are considered prehistoric birds (Cracraft, 1974) due to certain peculiarities, among which is the absence of a proper bursa of Fabricius (lymphoid tissue enclosed in the dorsal and lateral tissue of the proctodeum), and this is believed to influence the immune response of these birds to pathogens (Huchzermeyer, 1994).

It has been reported that ostriches have an atypical response to avian influenza (AI) infection. Both in natural and experimental infection it has been reported that both low pathogenicity AI and highly pathogenic (HP) AI viruses cause a very similar clinical condition in this species, with non-specific clinical signs and mortality occurring only in young birds despite extensive virus replication (Manvell *et al.*, 1998; Capua *et al.*, 2000; Mutinelli *et al.*, 2003; Olivier, 2006).

AI in its notifiable form must be reported to the OIE and to the veterinary authorities of most countries.

A suspicion must be followed by a laboratory confirmation, and, given the non-lethal nature of infection in ostriches, a serological analysis may be a tool to support diagnostic efforts and complement virus isolation or detection methods. To date there is a paucity of data on the reliability of serological assays performed on ostrich sera, and in general there is a lack of validation data for most infectious diseases of ostriches.

Scientific literature dealing with ostrich serology for the detection of AI antibodies is limited and most of it does not report data on the sensitivity or specificity of the tests used, due to the lack of availability of appropriate samples.

Zhou *et al.* (1998) tested 1261 ostrich sera collected from farms in the US and Canada by agar gel immunodiffusion (AGID), haemagglutination inhibition (HI) and competitive enzyme-linked immunosorbent assay (ELISA). Given the limited number of positive samples, it was not possible to evaluate the performances of the different tests. However, Zhou *et al.* demonstrated that AGID was not a sensitive test compared with HI and competitive ELISA because it was able to detect only one of the 29 samples that were positive by HI and competitive ELISA.

Ley *et al.* (2000) found only one positive AI sample by HI among 163 ostrich sera collected in a US slaughterhouse, and Sakai *et al.* (2006) failed in finding any positive samples among 181 ostrich sera collected in a Japanese slaughterhouse using AGID.

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Received 14 April 2009

ISSN 0307-9457 (print)/ISSN 1465-3338 (online)/10/010011-05 © 2010 Houghton Trust Ltd
DOI: 10.1080/03079450903431390

Clavjo *et al.* (2003) tested sera after an experimental challenge of 22 ostriches infected with $10^{6.6}$ median embryo infective dose/ml H5N2 HPAI. In this experiment, ostriches started to show low titred seroconversion by HI and competitive ELISA from day 7 post-infection until 14 day post-infection. The sensitivity of the AGID was shown to be lower than HI and ELISA.

Other experimental infections reported by Manvell *et al.* (1998, 2003) showed detectable seroconversion by HI in a group of 10 birds infected with both HPAI and low pathogenicity AI H5 and H7 viruses, using HI with ostrich red blood cells (RBCs).

The data currently available are essentially insufficient to make any statement on recommended diagnostic tests for serology of AI in ostriches. The aim of the present experiment was to test a significant number of ostrich serum samples collected during an experimental infection by means of different serological assays in order to establish which test(s) may be recommended for trade or diagnostic purposes.

Materials and Methods

Experimental protocol and collection of samples. Thirty-three, 3-month-old to 4-month-old ostriches were infected with 3 ml solution containing 10^9 median embryo infective dose/0.1 ml A/ostrich/South Africa/Middleton/2004 (H5N2) HPAI virus. One millilitre of the inoculum was placed in one eye, 1 ml in one nostril and 1 ml in the trachea. The challenge virus was supplied by the Virology Division, Onderstepoort Veterinary Institute and the animal challenge experiment was carried out in Onderstepoort Veterinary Institute—Exotic Diseases Division.

At 0, 3 or 5 days post-infection (pi), 7 or 10 pi, 13 or 15 or 18 pi and 21 days post-infection, ostriches were bled to obtain serum samples in sufficient amount to be tested by different methods. In total 177 serum samples were collected. Serum samples were heat inactivated (56°C for 30 min) and stored at -20°C until testing.

Serological tests. Each serum sample was tested by the following serological tests.

Haemagglutination inhibition. HI tests were performed according to the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (OIE, 2008a). Briefly serum samples were tested in “V”-bottom plates using 4 haemagglutinating units of two different antigens. Namely, we used the challenge strain A/ostrich/South Africa/Middleton/2004 (H5N2) as a homologous antigen and a heterologous genetically distant H5N2 virus (A/turkey/Italy/80).

Each serum sample was tested by HI with and without pre-treatment with 10% chicken RBCs. The pre-treatment of the sera with RBC solution was performed as follows (OIE, 2008a): 50 μl phosphate-buffered saline (PBS) were dispensed into wells in the first column of a microplate (wells A1 to E1), and the second row (A2 to E2) was left empty. Then 25 μl PBS were subsequently dispensed into all other wells of the microtitre plate. A 50 μl sample of test sera was added to the first wells of the microplate (column 1) and then 50 μl of a 10% RBC suspension was added to the first wells (column 1). The plates were incubated for 30 to 40 min at room temperature ($+20$ to 24°C), to allow to the 10% RBC suspension to settle. Subsequently, 25 μl supernatant of the samples in the first column was transferred to the wells of the second column. An additional 25 μl supernatant from the wells of the first column was transferred to the wells of the third column. Two-fold serial dilutions of the samples in the third column were performed, and the last 25 μl were discarded. From this point onwards the sera were processed as chicken sera. The first column was excluded from the test.

Agar gel immunodiffusion test. The AGID test was performed as described by Beard (1970) and according to the OIE diagnostic manual (OIE, 2008a).

Competitive ELISA test. A competitive IZSve ELISA detecting antibodies against AI nucleoprotein developed and validated at the Istituto Zooprofilattico Sperimentale delle Venezie was used. Briefly, ELISA plates (Maxisorp, Nunc) were coated overnight with an antigen containing the whole purified virus (H5N2 A/turkey/Italy/80) with coating buffer (pH 9.6). Sera were diluted 1:10 in a solution of sterile PBS containing 1% yeast extract and 4% foetal calf serum. Then 50 μl of each serum were distributed into wells and incubated for 1 h at 37°C . After washing the plates three times, an anti-nucleoprotein monoclonal antibody developed in the framework of the FLUAID EC funded project and conjugated with horseradish peroxidase was added and incubated for 1 h at 37°C . The plate was washed three times and the substrate solution added. The reaction was stopped after 10 min incubation in the dark and the optical density was read. Samples obtaining a percentage of inhibition $\geq 40\%$ of the negative controls were considered positive.

Two commercially available competitive ELISA kits (here called COM. ELISA 1 and COM. ELISA 2) were also used following the manufacturer's instructions.

Statistical analysis. We considered the threshold of positivity recommended by OIE and EU guidelines; that is, sera with an HI titre $\geq 1:16$ are considered positive. The HI performed with the homologous antigen following pre-treatment with a 10% chicken RBC suspension was considered the gold standard method and was the reference test to which other assays were compared. All sera under examination were divided into two categories: positive samples (for ELISA this included sera testing doubtful) and negative samples.

We calculated the sensitivity of each test from day 7 post infection onwards, which previous tests have indicated as the date by which antibodies are detectable.

The relative sensitivity (Se), relative specificity (Sp) and the relative confidence interval (CI) were also calculated between each test (HI, AGID and competitive ELISAs) and the gold standard chosen (Everitt, 1989).

The McNemar test and Cohen's agreement value (K) were then calculated (Cohen, 1960).

Results

Following challenge, almost all birds exhibited respiratory signs, such as conjunctivitis, ocular discharge, nasal discharge, tracheal foam, pharyngitis and coughing. A few birds showed diarrhoea. The average duration of clinical signs was 5 days with a range from 1 to 11 days. Eight birds showed clinical signs for a single day only. In general, the birds recovered rapidly and, despite the high dose of infection, clinical signs were mild (A. Olivier, personal observation).

The sensitivity of each different test/methods is presented in Table 1.

The relative Se and Sp, the results of Cohen's and McNemar's tests of each method compared with the gold standard (HI with RBCs 10% pre-treatment) and the sensitivity of each method at different days post-infection are presented in Tables 2 and 3, respectively.

We arbitrarily considered as false positive results the ELISA readings at days 0 and 3. All tests under examination were able to detect a variable percentage of serologically positive birds from day 7 onwards. With the exception of AGID, all tests exhibited a sensitivity value over 70% by day 9 post-infection. Detailed results on the onset and duration of antibody response are shown in Table 4 and Figure 1.

Table 1. Sensitivity of each test/method starting from day 7 post infection

	Positive (confidence interval)	Negative (confidence interval)
HI with homologous antigen without pre-treatment	67.57 (58.02; 76.15)	100 (94.48; 1*)
HI with homologous antigen and pre-treatment with 10% RBC solution	80.37 (71.58; 87.42)	100 (94.48; 1*)
HI with heterologous antigen without pre-treatment	24.11 (16.53; 33.10)	100 (94.48; 1*)
HI with heterologous antigen and pre-treatment with 10% RBCs solution	45.00 (23.06; 68.47)	100 (73.54; 1*)
COM. ELISA 1	78.18 (69.30; 85.49)	92.19 (82.70; 97.41)
COM. ELISA 2	83.64 (75.38; 90.00)	100 (94.48; 1*)
IZSVe ELISA	89.29 (93.70; 99.78)	96.92 (89.32; 99.63)
AGID	75.89 (66.90; 83.47)	100 (94.48; 1*)

*One-sided, 97.5% confidence interval.

Table 2. Relative sensitivity and specificity, agreement (K) and P value of McNemar test of the different tests/methods compared with the gold standard

Test/method	Se (confidence interval)	Sp (confidence interval)	Cohen's K (P value)	McNemar's test P value
HI with homologous antigen without pre-treatment	81.39 (71.55; 88.98)	98.84 (93.69; 99.97)	80.23 (<0.05)	<0.05
HI with heterologous antigen without pre-treatment	27.91 (18.77; 38.62)	100 (95.80; 100*)	27.91 (<0.05)	<0.05
HI with heterologous antigen and pre-treatment with 10% RBC solution	50 (23.04; 76.96)	93.75 (69.77; 99.84)	44.95 (<0.05)	<0.10
COM. ELISA 1	91.86 (83.95; 96.66)	89.41 (80.85; 95.04)	82.28 (<0.05)	NS
IZSVe ELISA	98.84 (93.69; 99.97)	86.05 (76.89; 92.58)	84.88 (<0.05)	<0.05
COM. ELISA 2	97.64 (91.76; 99.71)	93.02 (85.43; 97.40)	90.65 (<0.05)	NS
AGID	91.86 (86.95; 96.66)	97.67 (91.85; 99.71)	89.53 (<0.05)	<0.10

Gold standard is HI with homologous antigen and pre-treatment with 10% RBC solution. *One-sided, 97.5% confidence interval. NS, not significant.

Table 3. Sensitivity (confidence interval) of the different tests at different days post-infection

Day post-infection	COM. ELISA 1	COM. ELISA 2	IZSVe ELISA	HI (gold standard)	AGID
7	19.05 (5.45; 41.91)	33.33 (14.59; 56.97)	47.62 (25.71; 70.22)	19.05 (5.45; 41.91)	9.52 (1.17; 30.37)
9	90.90 (58.72; 99.77)	90.90 (58.72; 99.77)	90.90 (58.72; 97.77)	72.72 (39.03; 93.98)	54.54 (23.38; 83.25)
12	95.00 (75.13; 99.87)	95.00 (75.13; 99.87)	100 (83.16; 100*)	100 (83.16; 100*)	90.00 (68.30; 98.77)
15	100 (73.54; 100*)	100 (73.54; 100*)	100 (73.54; 100*)	100 (73.54; 100*)	100 (73.54; 100*)
18	100 (78.20; 100*)	93.75 (69.77; 99.84)	100 (79.41; 100*)	100 (76.84; 100*)	100 (79.41; 100*)
21	88.46 (69.85; 97.55)	96.00 (79.65; 99.90)	100 (87.23; 100*)	100 (85.75; 100*)	96.30 (81.03; 99.90)
22	60.00 (14.66; 94.73)	100 (47.82; 100*)	100 (47.82; 100*)	100 (47.82; 100*)	100 (47.82; 100*)

*One-sided, 97.5% confidence interval.

Table 4. Percentage of positive birds after infection during the study period obtained by different tests

Day post-infection	COM. ELISA 1	COM. ELISA 2	IZSVe ELISA	HI (gold standard)	AGID	Expected result
0	12.50	0.00	0.00	0.00	0.00	0.00
3	5.00	0.00	10.00	0.00	0.00	0.00
5	0.00	0.00	0.00	0.00	0.00	0.00
7	19.05	33.33	47.62	19.05	9.52	100.00
9	90.91	90.91	90.91	72.73	54.55	100.00
12	95.00	95.00	100.00	100.00	90.00	100.00
15	100.00	100.00	100.00	100.00	100.00	100.00
18	100.00	93.75	100.00	100.00	100.00	100.00
21	88.46	96.00	100.00	100.00	96.30	100.00
22	60.00	100.00	100.00	100.00	100.00	100.00

Figure 1. Percentage of positive birds after infection during the study period compared with the expected result.

The results of Cohen's *K* test, measuring the agreement between the test results at different days post infection and the expected results, are reported in Table 5.

Discussion

The present study has enabled us to compare the ability of different serological tests to detect antibodies against AI virus in serum samples collected from experimentally infected ostriches. A total of 177 serum samples from 33 birds were tested. To our knowledge this is the first study on ostrich serology supported by a statistically significant number of samples.

All serum samples have been analysed with three different test systems, namely HI, AGID and competitive ELISA. To gather as much information as possible, we applied different methods for HI (with and without 10% chicken erythrocyte pre-treatment and with two different antigens), and for ELISA (three different competitive kits).

Previous work (Zhou *et al.*, 1998) indicated that AGID is not a satisfactory test because of its low sensitivity. In our experiment the sensitivity was lower compared with that of the other tests but after day 12 post-infection the sensitivity of the test was surprisingly high. However, it was very difficult to read the plates because the precipitation lines were often extremely thin and at times incomplete.

It has been often reported (Williams *et al.*, 1997; Manvell *et al.*, 1998; Sakai *et al.*, 2006) that ostrich sera are highly cross-reactive when tested by HI for the presence of non-specific inhibitors of haemagglutination with the consequence of a high number of false positives.

On the other hand, false negatives could also occur with ostrich sera. This could be due to the fact that ostrich sera may cause non-specific agglutination of chicken RBCs. Some authors (Manvell *et al.*, 1998) tested ostrich sera using ostrich RBCs, but this method it is not generally recommended as it has not been validated.

Sera tested in this study did not exhibit any non-specific haemagglutination inhibition artefact in negative (pre-challenge) samples, regardless of pre-treatment.

The HI test, when performed with homologous antigen (challenge virus), was found to be a sensitive and specific test. Pre-treatment with 10% chicken RBC suspension increased the sensitivity of the test and aided the interpretation of results by eliminating non-specific agglutination of chicken RBCs that was observed in certain samples. Interestingly, this was seen only in samples collected from the same animals (2/33 birds), thus suggesting that it could be related to the characteristics of the individual.

Notwithstanding the results obtained using the challenge virus as an antigen, the results generated using another virus (of the same H and N subtype) as a haemagglutinating antigen (HA) were not satisfactory. We therefore recommend that if the field virus is not available, a selection of viruses should be used in order to identify the most suitable antigen.

Generally speaking the ELISA tests used all yielded comparable results with high Se and Sp and substantial agreement with the gold standard. Using ELISA tests, antibodies could be detected in the sera of experimentally infected ostriches from day 7 post infection, exhibiting sensitivity values greater than or equal to the HI (Table 4). Two of the ELISA kits used produced apparently false positive results within day 5 post-infection. It also appeared that one of the commercial kits was unable to maintain high sensitivity values towards the termination of the experiment.

In conclusion, we believe that the results of the present study may give indications as to which tests can be used for serological diagnosis of AI virus infections in ostriches. We conclude that validated ELISA tests for the detection of AI antibodies in ostrich sera can be considered suitable tests for screening purposes and should be preferred to AGID. The HI test with pre-treatment with 10% chicken RBC suspension, and using the virus to which the birds have been exposed (or genetically and antigenically closely related with it) as the HA, remains a suitable test to monitor the circulation of AI virus of known subtype.

Table 5. Cohen's agreement (*K*) of different tests with the expected result

Days	COM. ELISA 1	COM. ELISA 2	IZSVE ELISA	AGID	HI (gold standard)
0 to 7	0.1390 [†]	0.4305**	0.5210**	0.1373**	0.2624**
0 to 9	0.7222**	0.9448**	0.8464**	0.6724**	0.8202**
0 to 12	0.8158**	0.9667**	0.9368**	0.9323**	1**
0 to 15	0.7884**	1**	0.9076**	1**	1**
0 to 18	0.8176**	0.9601**	0.9256**	1**	1**
0 to 22	0.4088**	1**	0.8182**	1**	1**

***P* < 0.01, **P* < 0.05, [†]*P* < 0.10. To better understand the results, use the Landis & Koch scale: ≤0.01 = poor agreement; 0.02 to 0.20 = slight agreement; 0.21 to 0.40 = fair agreement; 0.41 to 0.60 = moderate agreement; 0.61 to 0.80 = substantial agreement; 0.81 to 1.00 = almost perfect agreement.

Acknowledgements

The present work was supported financially by the EU-funded project FLUAID (SSPE-CT-2005-022417). The authors gratefully acknowledge the South African Ostrich Business Chamber for funding the serum collection trial and Dr G.H. Gerdes and all the staff of Onderstepoort Veterinary Institute for serum preparation and submission to IZSVe.

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