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**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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## Abstract

In this study we collected 177 serum samples from ostriches (*Struthio camelus*) infected experimentally with A/ostrich/South Africa/Middleton/2004 (H5N2) HPAI virus. We tested these samples using the haemagglutination inhibition (HI) test, the agar-gel immunodiffusion (AGID) test and three ELISA kits. We considered **the HI test, with homologous antigen and including pre-treatment of sera with 10% chicken red blood cells, as 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) and European Union (EU) 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 et al., 1999; Edward et al., 2002).**

They are considered as prehistoric birds (Cracraft, 1974) and this is due to certain peculiarities among which the absence of a proper bursa of Fabricius (lymphoid tissue enclosed in the dorsal and lateral tissue of 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

(LP) 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; Olivier, 2006; Capua et al., 2000, Mutinelli et al., 2003).

Avian influenza in its notifiable form (NAI) 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 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 avian influenza 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 1,261 ostriches sera collected from farms in the US and Canada by agar gel immunodiffusion (AGID), haemagglutination inhibition (HI) and competitive ELISA (c-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 to HI and competitive ELISA because it was able to detect only one of the 29 samples that were positive by HI and c-ELISA.

Ley et al., (2000) found only 1 positive sample by HI among 163 ostrich sera collected in US slaughterhouse and Sakai et al., (2006) failed in finding any positive samples among 181 ostrich sera collected in a Japanese slaughterhouse using AGID.

Clavjo et al., (2002) tested sera coming from an experimental challenge of 22 ostriches infected with  $10^{6.6}$  EID<sub>50</sub>/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., in 1998 and 2003 showed detectable seroconversion by HI in a group of 10 birds infected with both HPAI and LPAI H5 and H7 viruses by using HI with ostrich red blood cells (RBCs).

The data that is currently available is essentially insufficient to make any statement on recommended diagnostic tests for serology of AI in ostriches. The aim of this 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- to 4-month-old ostriches were infected with 3 ml of solution containing  $10^9$  EID<sub>50</sub>/0.1ml of A/ostrich/South Africa/Middleton/2004 (H5N2) HPAI virus. One ml 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 (OVI) and the animal challenge experiment was carried out in Onderstepoort Veterinary Institute–Exotic Diseases Division.

At 0, 3 or 5, 7 or 10, 12 or 15 or 18 and 21 days post infection ostriches were bled to obtain serum samples in sufficient amount to be tested with different methods. A total of 177 serum samples were collected. Serum samples were heat inactivated (56°C for 30 minutes) and stored at -20°C until testing.

### *Serological tests*

Each serum sample was tested by the following serological tests:

- Haemagglutination Inhibition (HI)

Haemagglutination inhibition (HI) tests were performed according to the manual of diagnostic tests and vaccines for terrestrial animals (OIE, 2008). 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 viruses (A/turkey/Italy/80). Each serum sample was tested by HI with and without pre-treatment with 10% chicken red blood cells (RBCs). The pre-treatment of the sera with RBCs solution was performed as follows (OIE, 2008): 0.050 ml of PBS were dispensed into wells in the first column of a microplate (wells A1–E1), the second row (A2–E2) was left empty. 0.025 ml of PBS were subsequently dispensed into all other wells of the microtitre plate. 0.050 ml of test sera were added to the first wells of the microplate (column 1) and then 0.050 ml of a 10% RBCs suspension was added to the first wells (column 1). The plates were incubated for 30-40 min at room temperature (+20–24°C), to allow to the 10% RBC suspension to settle. Subsequently, 0.025 ml of the supernatant of the samples in the first column was transferred to the wells of the second column. An additional 0.025 ml of the 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 0.025 ml 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 (AGID)

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

- Competitive ELISA test

A competitive ELISA detecting antibodies against avian influenza nucleoprotein (NP) 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. 50  $\mu$ l of each serum were then distributed into wells and incubated for 1 hour at 37 °C. After washing the plates three times an anti-NP monoclonal antibody developed in the framework of the FLUAID EC funded project and conjugated with horseradish peroxidase was added and incubated for 1 hour at 37 °C. The plate was washed three times and the substrate solution added. The reaction was stopped after 10 minutes of incubation in the dark and the optical density (OD) read. Samples obtaining a percentage of inhibition (PI) more or equal than 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, i.e. sera with an HI titre higher or equal to 1:16 are considered positive. The HI performed with the homologous antigen following pre-treatment with a 10% chicken red blood cell 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 c-ELISAs) and the gold standard chosen (Everitt, 1989).

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



## 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 the different test/procedures are shown in table 1.

The relative Se and Sp, the results of Cohen's and McNemars' tests of each method compared to 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 p.i.. Detailed results on the onset and duration of antibody response are shown in table 4 and graphic 1.

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

This study has enabled us to compare the ability of different serological tests to detect antibodies against avian influenza 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 3 different test systems, namely HI, AGID and c-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 ELISA (three different competitive kits).

Previous work (Zhou et al., 1998) indicated that AGID is not satisfactory test because of its low sensitivity. In our experiment the sensitivity was lower compared to that of the other tests but after day 12 p.i. 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; Sakai et al., 2007; Manvell et al., 1998) that ostrich sera are highly cross-reactive when tested by HI for the presence of non-specific inhibitors of haemagglutination with the consequence of 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 ostriches sera may cause non-specific agglutination of chickens red blood cells. Some authors (Manvell et al., 1998) tested ostrich sera by 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 red blood cell 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 an HA antigen were not satisfactory, thus we 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 p.i., 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 p.i.. 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 this study may give indications as to which tests can be used for serological diagnosis of AIV infections in ostriches. We conclude that validated ELISA tests for the detection of AI antibodies in ostriches sera can be considered suitable test 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 antigen remains a suitable test to monitor the circulation of AI virus of known subtype.

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**Table 1:** *Sensitivity of each test/method starting form day 7 post infection*

|          | HI with<br>homologous<br>ag without<br>pre-<br>treatment | HI with<br>homologous<br>ag and pre-<br>treatment<br>with 10%<br>RBCs sol. | HI with<br>hetelogenous<br>ag without<br>pre-<br>treatment | HI with<br>hetelogenous<br>ag and pre-<br>treatment<br>with 10%<br>RBCs sol. | Com.<br>ELISA 1   | Com.<br>ELISA 2   | IZSVe<br>ELISA    | AGID              |
|----------|--|--|--|--|-------------------|-------------------|-------------------|-------------------|
| Positive | 67.57  | 80.37  | 24.11  | 45.00  | 78.18             | 83.64             | 89.29             | 75.89             |
| [CI]     | [58.02;<br>76.15]  | [71.58;<br>87.42]  | [16.53;<br>33.10]  | [23.06;<br>68.47]  | [69.30;<br>85.49] | [75.38;<br>90.00] | [93.70;<br>99.78] | [66.90;<br>83.47] |
| Negative | 100  | 100  | 100  | 100  | 92.19             | 100               | 96.92             | 100               |
| [CI]     | [94.48; 1*]  | [94.48; 1*]  | [94.48; 1*]  | [73.54; 1*]  | [82.70;<br>97.41] | [94.48; 1*]       | [89.32;<br>99.63] | [94.48; 1*]       |

(\*) one-sided, 97.5% confidence interval

Legend:

HI= Inhibition of haemagglutination test

CI= confidence interval

RBCs= red blood cells

**Table 2** *Relative sensitivity and specificity, agreement (K) and p-value of McNemar test of the different tests/methods compared with the gold standard (HI with homologous ag and pre-treatment*

| Test/method                                      | Se and [CI]             | Sp and [CI]             | Cohen's K and p-value | p-value of McNemar test |
|--|-------------------------|-------------------------|-----------------------|-------------------------|
| HI with homologous ag<br>without pre-treatment   | 81.39<br>[71.55; 88.98] | 98.84<br>[93.69; 99.97] | K=80.23 p<0.05        | p<0.05                  |
| HI with hetelogenous ag<br>without pre-treatment | 27.91<br>[18.77; 38.62] | 100<br>[95.80; 100*]    | K= 27.91 p<0.05       | p<0.05                  |

*with 10% RBCs solution)*



|  |                         |                         |                |        |
|--|-------------------------|-------------------------|----------------|--------|
| HI with hetelogenous ag and<br>pre-treatment with 10%<br>RBCs solution | 50<br>[23.04; 76.96]    | 93.75<br>[69.77; 99.84] | K=44.95 p<0.05 | p<0.10 |
| Com. ELISA 1   | 91.86<br>[83.95; 96.66] | 89.41<br>[80.85; 95.04] | K=82.28 p<0.05 | p=NS   |
| IZSVe ELISA  | 98.84<br>[93.69; 99.97] | 86.05<br>[76.89; 92.58] | K=84.88 p<0.05 | p<0.05 |
| Com. ELISA 2   | 97.64<br>[91.76; 99.71] | 93.02<br>[85.43; 97.40] | K=90.65 p<0.05 | p=NS   |
| AGID   | 91.86<br>[86.95; 96.66] | 97.67<br>[91.85; 99.71] | K=89.53 p<0.05 | p<0.10 |

(\*) one-sided, 97.5% confidence interval

Legend:

Se= Sensitivity

Sp= Specificity

CI= confidence interval

NS= not significant

p=p-value

RBCs= red blood cells

**Table 3:** Sensitivity of the different tests at different days post-infection. The confidence intervals are in brackets.

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

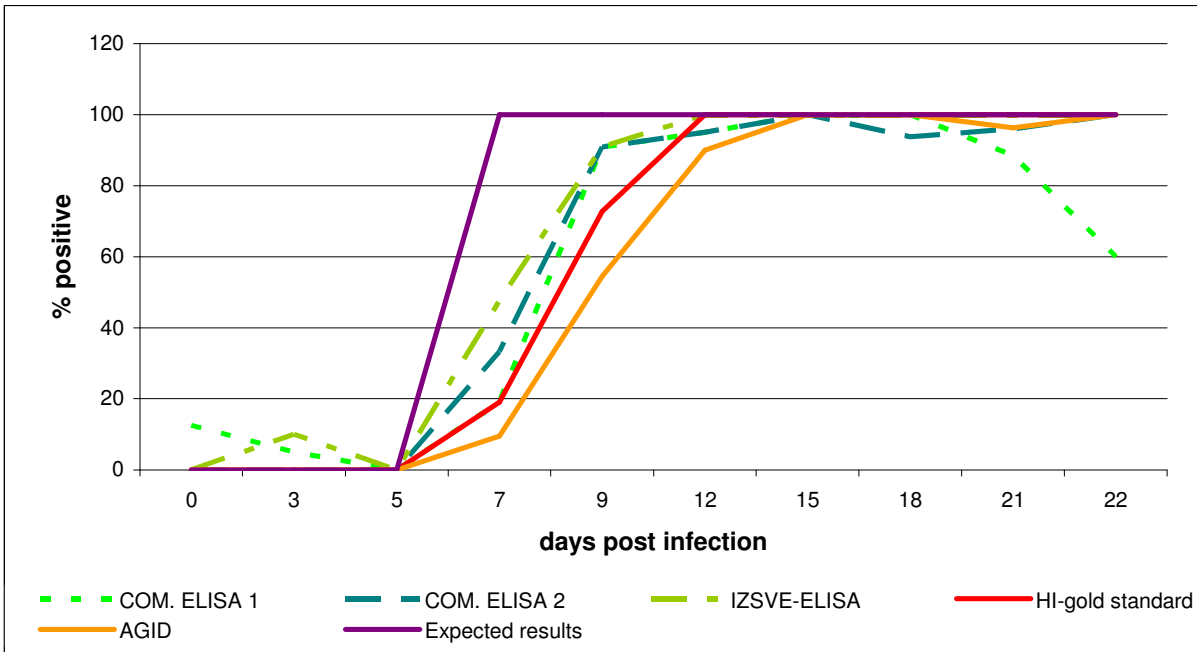
**Table 5:** Cohen's agreement (K) of different tests with the expected result

| Days | Com. ELISA<br>1     | Com. ELISA 2 | IZSVE ELISA | AGID     | HI (gold<br>standard) |
|------|---------------------|--------------|-------------|----------|-----------------------|
| 0-7  | 0.1390 <sup>+</sup> | 0.4305**     | 0.5210**    | 0.1373** | 0.2624**              |
| 0-9  | 0.7222**            | 0.9448**     | 0.8464**    | 0.6724** | 0.8202**              |
| 0-12 | 0.8158**            | 0.9667**     | 0.9368**    | 0.9323** | 1**                   |
| 0-15 | 0.7884**            | 1**          | 0.9076**    | 1**      | 1**                   |
| 0-18 | 0.8176**            | 0.9601**     | 0.9256**    | 1**      | 1**                   |
| 0-21 | 0.7885**            | 0.972**      | 0.9487**    | 0.9735** | 1**                   |
| 0-22 | 0.4088**            | 1**          | 0.8182**    | 1**      | 1**                   |

\*\*p<0.01; \*p<0.05; <sup>+</sup> p<0.10

**Note:** To better understand the results use the *Landis & Koch* scale: ≤ 0.01 = Poor agreement; 0.02-0.20 = Slight agreement; 0.21-0.40 = Fair agreement; 0.41-0.60 = Moderate agreement; 0.61-0.80 = Substantial agreement; 0.81-1.00 = Almost perfect agreement

Graphic 1: Percentage of positive birds after infection during the study period compared with the expected result



er Review Only