Bulgarian Journal of Veterinary Medicine (2007), **10**, No 4, 257–266

COMPARATIVE EVALUATION OF THREE SEROLOGICAL DIAGNOSTIC TESTS IN A CHICKEN MODEL OF EXPERIMENTAL INTRATRACHEAL INFECTION WITH AVIAN INFLUENZA VIRUS A/DUCK/BULGARIA/05 H6N2

I. S. ZARKOV

Faculty of Veterinary Medicine, Trakia University, Stara Zagora, Bulgaria

Summary

Zarkov, I., 2007. Comparative evaluation of three serological diagnostic tests in a chicken model of experimental intratracheal infection with avian influenza virus A/DUCK/ BULGARIA/05 H6N2. *Bulg. J. Vet. Med.*, **10**, No 4, 257–266.

After experimental intratracheal infection of chickens with an avian influenza viral isolate A/duck/Bulgaria/05 H6N2 (0.2 mL), the potential of the haemagglutination inhibition test (HIT), the immunodiffusion test (IDT) and enzyme-linked immunosorbent assay (ELISA) for antibody detection was evaluated. The results evidenced that in birds, antibodies to haemagglutinin, precipitating antibodies and IgG antibodies, detectable with ELISA, were formed. Subtype-specific antibodies were detected in all infected chickens. Serum titres ranged between 1:4 and 1:128 with predomination of 1:64 titres (44.8 %). The mean arithmetic titre for the experiment was 1:56.1. Precipitins were found out in all infected chickens. The titres ranged from undiluted to 1:128 with prevailing of 1:2 titres (27.6%). By means of ELISA, 100% seropositive birds were detected by the 7 and 14th day whereas the percentage on days 21 was 85.7% and 28 was 80%. S/P values were the highest by the 14th day (up to 2.776). The sensitivity, specificity and test agreement was 100% for HIT and IDT and 93.1% for ELISA on the background of a specificity of 100 %. The comparison of the three tests showed a specificity of 100 % for IDT and HIT and 93.1% for ELISA, sensitivity of 100 % and test agreement of 97.4 % for ELISA. The data for detection of type-specific antigens showed 93.1 % sensitivity for IDT and ELISA, specificity of 100 % and test agreement of 97.4 %.

Key words: avian influenza viruses, chickens, ELISA, haemagglutination inhibition test, immunodiffusion test

INTRODUCTION

The analysis of blood sera is extensively used for detection of antibodies against avian influenza A viruses (AIV). It is utilized as a screening test for detection of infection (Arenas *et al.*, 1990; Allwright *et al.*, 1993; Otsuki *et al.*, 1995) in the course of experimental induction of a disease (Lu & Castro, 2004), for elucidation of sensitive bird species (Abraham *et al.*, 1986; Allwright *et al.*, 1993; Cadman *et al.*, 1994; Swayne & Slemons, 1995), for determination of the immune response depending on the avian species (Boer *et al.*, 1990; Allwright *et al.*, 1993; Cadman *et al.*, 1994; Swayne & Slemons, 1995; Zhou *et al.*, 1998), for determination of the relationship between the age of birds and the inoculation dose (Brugh, 1995; Beck & Swayne, 1997; Lu & Castro, 2004), the detection of the time of antibody formation (Meulemans *et al.*, 1987) and last but not least, as indicator for

evaluation of the efficacy of various vaccines (Stone, 1988; Abraham *et al.*, 1988; Donahoe, 1997; Garcia-Garcia *et al.*, 1997; Trani *et al.*, 2003).

The generally used serological tests detect type-specific antibodies raised against the nucleoprotein antigen and subtype-specific antibodies to the haemagglutinin and neuroaminidase. The most commonly used reactions with type-specific antigens are the immunodiffusion test (IDT) (Beard, 1970; Pearson & Senne, 1986; Meulemans *et al.*, 1987; Adair *et al.*, 1990; Otsuki *et al.*, 1995) and ELISA (Snyder *et al.*, 1985; Meulemans *et al.*, 1987; Fatumbi *et al.*, 1989; Adair *et al.*, 1990; Arenas *et al.*, 1990; Zhou *et al.*, 1997; Zhou *et al.*, 1998; Sala *et al.*, 2003), and with subtype-specific antigens – the haemagglutination inhibition test (HIT) (Salk, 1944; Beard, 1970; Meulemans *et al.*, 1987; Arenas *et al.*, 1990; Otsuki *et al.*, 1995; Sala *et al.*, 2003).

The comparative investigations of sera from hens and chickens using IDT and ELISA showed a different sensitivity and specificity (Snyder *et al.*, 1985; Meulemans *et al.*, 1987; Zhou *et al.*, 1997; Zhou *et al.*, 1998; Fatumbi *et al.*, 1989; Adair *et al.*, 1990; Arenas *et al.*, 1990; Boer *et al.*, 1990). The comparison between ELISA and HIT also exhibited a variable sensitivity: equal (Lamichhane & Kirkegaard, 1997), higher for ELISA (Adair *et al.*, 1989; Adair *et al.*, 1990; Arenas *et al.*, 1990) or lower for ELISA (Zhou *et al.*, 1998). The route of penetration of the virus could be a probable cause for these discrepancies.

The predominant localization of AIV on respiratory or alimentary epithelial cells containing tripsin-like enzymes allows to assume that the intratracheal route of experimental infection is the closest to the natural one. It is used as a screning test for detection of infection (Otsuki *et al.*, 1982; Meulemans *et al.*, 1987; Brugh, 1995; Swayne & Slemons, 1995; Swayne, 1997; Beck & Swayne, 1997; Swayne & Beck, 2005).

For these reasons, we aimed to investigate the diagnostic potential of HIT, IDT and ELISA after experimental intratracheal infection of chickens with avian influenza isolate A/duck/Bulgaria/05 H6N2.

MATERIALS AND METHODS

Experimental chickens

The experiment was performed with 18 30-days old Dekalb chickens. Nine of them were intratracheally infected with 0.2 mL allantoic fluid of virus-infected chick embryos (CE) and the other nine remained uninfected (controls) and were intravenously treated with allantoic fluid of uninfected CE.

The chickens were housed in two isolated premises of 2 m^2 each, at daylight regimen of 13 hours, ambient temperature of 20 ^{0}C, air humidity 70%, a common feeding front and drinking front of 0.9 m. No vaccinations have been performed.

Virus

In this experiment, an AIV A/duck/ Bulgaria/05 H6N2 isolate from a wild duck (*Anas plathyrynchos*) was used (Zarkov *et al.*, 2006). The virus was titrated for detection of embryonic lethal dose (ELD_{50}) by the method of Reed & Muench (1938). A viral suspension from the 4th passage with a titre of $10^{5.0}$ ELD_{50}/0.1 mL was used.

Blood sampling

Blood samples were obtained at 7-day intervals: day 0 (prior to infection), and

days 7, 14, 21 and 28 after the infection. The total number of blood samples appropriate for testing was 77: 48 from healthy birds (39 control and 9 from birds subject to infection on day 0) and 29 from infected population of birds (obtained on post infection days 7, 14, 21 and 28).

Serological tests

Three tests were used, one of them (HIT) for detection of subtype-specific antibodies (antibodies to haemagglutinin) and two (IDT and ELISA) – for detection of type-specific antibodies – precipitating antibodies (precipitins) and IgG antibodies, respectively. The protocols of these serological assays are described in a previous work of ours (Zarkov, 2007).

Statistical analysis of data

The statistical analysis of data obtained from the different diagnostic tests was done only in chickens, challenged with a viral antigen. The Statistica v.6.0 software was used (StatSoft, Tulsa, OK, USA). The confidence interval (CI) was calculated at a probability of 0.95.

The relative sensitivity, relative specificity and test coverage were calculated according to Courtney *et al.* (1990) as described earlier (Zarkov, 2007).

RESULTS

The results from the HIT, IDT and ELISA are presented in Table 1. They show that 29 samples from infected chickens were positive in the HIT and in IDT (S = 100 %) and 27 – positive in ELISA (S = 93.1 %). False positive results in non-inoculated birds were not present (100% specificity for all tests).

All sera from the 7th to the 28th day were positive in HIT (S=100 %) (Table 2). There was a difference in individual titres. By days 7 and 28, the titres ranged between 1:8 and 1:128, by the 14th day – between 1:8 and 1:64 and by the 21st day: from 1:4 to 1:128. The mean arimethric titre (MAT) on the 7th day was 1:61.3, on the 14th day – 1:37, on the 21st day – 1:69.1 (the highest one in this experiment) and on the 28th day – 1:59.2.

Throughout the entire experiment, samples with titres 1:64 predominated (44.83 %). The MAT values of the individual chickens titres in this trial were from 1:7 to 1:96 whereas MAT values of

Table 1. Sensitivity and specificity of the haemagglutination inhibition test (HIT), the immunodiffusion test (IDT) and ELISA in 77 sera from chickens – healthy and intratracheally infected with 0.2 mL avian influenza isolate A/duck/Bulgaria/05 H6N2, tested at 1-week intervals after the infection for a period of 4 weeks

Test		Healthy population (n=48)	Infected population (n=29)	Relative sensitivity (S); false negative (FN)	Relative specificity (Sp); false positive (FP)
HIT	+	0	29	S: 100 %	Sp: 100 %
	–	48	0	FN: 0 %	FP: 0 %
ELISA	+	0	27	S: 93.1 %	Sp: 100 %
	–	48	2	FN: 6.9 %	FP: 0 %
IDT	+	0	29	S: 100 %	Sp: 100 %
	–	48	0	FN: 0 %	FP: 0 %

Table 2. Time- and titre-dependent distribution of antibodies to the haemagglutinin (HIT-positive sera) in chickens, intratracheally infected with 0.2 mL avian influenza isolate A/duck/Bulgaria/05 H6N2

Antibody titres	Days after the infection				
	0	7	14	21	28
1:4	0 %	0 %	0 %	14.3 %	0 %
1:8	0 %	11.1 %	12.5 %	0 %	20 %
1:16	0 %	0%	0 %	0 %	0 %
1:32	0 %	11.1 %	62.5 %	14.3 %	20 %
1:64	0 %	66.7 %	25 %	42.8 %	40 %
1:128	0 %	11.1 %	0 %	28.6 %	20 %

Table 3. Time- and titre-dependent distribution of precipitins (IDT-positive sera) in chickens, intratracheally infected with 0.2 mL avian influenza isolate A/duck/Bulgaria/05 H6N2

Items	Days after the infection				
	0	7	14	21	28
Total number of seroreagents	0	(100 %)	(100 %)	(100 %)	(100 %)
Antibody titres					
Undiluted sera	0 %	0 %	0 %	14.3 %	20 %
Diluted sera with titre 1:2	0 %	0%	37.5 %	28.5 %	60 %
Diluted sera with titre 1:4	0 %	22.2 %	0 %	42.9 %	20 %
Diluted sera with titre 1:8	0 %	0%	12.5 %	14.3 %	0 %
Diluted sera with titre 1:16	0 %	22.2 %	50 %	0 %	0 %
Diluted sera with titre 1:32	0 %	0%	0 %	0%	0 %
Diluted sera with titre 1:64	0 %	33.4 %	0 %	0%	0 %
Diluted sera with titre 1:128	0 %	22.2 %	0 %	0%	0 %

all infected birds were 1:56.14.

IDT-detected precipitating titres (IDT-positive sera) in infected chickens (Table 3) in undiluted and diluted samples were up to 1:128, and all were positive (S = 100 %).

By the 7th day, the titres of precipitins in positive samples were from 1:4 to 1:128. On the 14th day from 1:2 to 1:16, on the 21st day from undiluted sera to 1:8. At the last testing (day 28) from undiluted sera to 1:4. The mean titres for the experiment were 1:20.76 and prevailing samples had titres 1:2 (27.6 %).

By means of ELISA (Table 4) the samples from all chickens were positive on the 7th and the 14th days (S=100 %). By days 21 and 28, one bird (No 6) exhibited a negative result. The sensitivity at these time intervals was 85.71 % (day 21) and 80 % (day 28).

The values of the ratio of tested samples to positive control (S/P) (considered negative when S/P $\leq$ 0.5 and positive when S.P $\geq$ 0.5), ranged between 0.701

and 2.701 on the 7th day after the infection,. On the 14th day they were from 0.539 to 2.776, on the 21 day from 0.942 to 1.950 and the 28 day from 0.892 to 1.929. S/P values over 2.000 were observed in 55.6 % of samples on the 7th day and in 62.5 % on the 14th day. At subsequent intervals (days 21 and 28) there were no birds with S/P values higher than 2.000. In birds, positive throughout the entire experimental period (No No 1, 3, 5 and 9) there was a tendency towards lower values on the 7th day (No No 1, 5), increasing on day 14 and decreasing again in subsequent periods. In the other chickens (No No 3, 9) S/P values gradually decreased from the 7th to the 28th day.

The comparison of data obtained in the HIT, IDT and ELISA is done in Table 5. At a specificity of 100 % for all tests, the sensitivity was different in ELISA – 93.1% vs 100 % for both IDT and HIT. The test agreement percentage of ELISA was 97.4 % compared to 100 % in IDT and HIT.

There was a correlation between IDT/ELISA and HIT/ELISA at $P<0.001$ and $r=0.9453$. The correlation between HIT and IDT was at $P<0.001$ with $r=1.0000$.

Table 4. Time-dependent distribution of ELISA-positive chickens, intratracheally infected with 0.2 mL avian influenza isolate A/duck/Bulgaria/05 H6N2 with regard to the result of the tested samples vs the positive control (S/P)

Chicken	Days after the infection and S/P values				
	0	7	14	21	28
No 1	0.207	1.855	2.025	1.469	1.199
No 2	0.295	2.701	2.776	n.t.	n.t.
No 3	0.176	1.880	1.577	0.942	0.892
No 4	0.265	2.444	2.564	1.855	n.t.
No 5	0.291	1.432	1.768	1.498	1.104
No 6	0.212	0.701	0.539	0.295	0.281
No 7	0.243	2.299	n.t.	n.t.	n.t.
No 8	0.288	2.564	2.282	1.635	n.t.
No 9	0.231	2.510	2.278	1.950	1.929

Legend: The maximum value of S/P in the test with negative result was 0.295; n.t.= not tested.

Table 5. Potential of ELISA and IDT compared to HIT (accepted for 100%) for detection of antibodies in chickens, intratrachealli infected with 0.2 mL avian influenza isolate A/duck/Bulgaria/05 H6N2: relative sensitivity (S); relative specificity (Sp) and between-test agreement

		HIT-negative (n =48)	HIT-positive (n =29)	Relative sensitivity (S); false negative (FN)	Relative specificity (Sp); false positive (FP)	Between-test agreement
ELISA	+	0	27	S: 93.1 %	Sp: 100 %	97.4 %
	–	48	2	FN: 6.9 %	FP: 0 %	
IDT	+	0	29	S: 100 %	Sp: 100 %	100 %
	–	48	0	FN: 0 %	FP: 0 %	

Table 6. Potential of ELISA and IDT (accepted for 100%) for detection of type-specific antibodies in chickens, intratracheally infected with 0.2 mL avian influenza isolate A/duck/Bulgaria/05 H6N2: relative sensitivity (S); relative specificity (Sp) and between-test agreement

		IDT-negative (n =48)	IDT-positive (n =29)	Relative sensitivity (S); false negative (FN)	Relative specificity (Sp); false positive (FP)	Test agreement
ELISA	+	0	27	S: 93.1 %	Sp: 100 %	97.4 %
	–	48	2	FN: 6.9 %	FP: 0 %	

The results of detection of type-specific antibodies by means of ELISA and IDT showed that 27 sera (out of 29 positive ones) matched in both tests. The relative sensitivity of ELISA and IDT was 93.1 % (Table 6). False negative results in ELISA tests were obtained in 6.9 % of sera. The relative specificity was 100 %. The agreement of both tests was 97.4 %.

DISCUSSION

The data obtained in the present experiments showed that after intratracheal infection with the AIV isolate A/duck/ Bulgaria/05 H6N2, the chickens reacted with formation of antibodies to haemagglutinin, precipitins and IgG antibodies. A common feature of all three tests was the maximum percentage of seroreagents in infected birds as early as the 7th day. The seroreagents' percentage persisted to the end of the experiment in two tests (HIT and IDT), whereas in ELISA, some of infected birds showed false negative results on the 21st and the 28th days (14.29 % and 20.00 %, respectively).

The data of our experiment and those from other investigators showed a similarity or differences in used tests depending on the antigen entry site in the organism and the avian species. They are related to the period of emergence of antibodies, the percentage of positive samples and the antibody titres.

The results in the HIT test confirmed the early emergence of antibodies to the haemagglutinin – as early as the 7th day. Similar data (presence of antibodies from the 4th day) are reported by Meulemans *et al.* (1987), whereas Beck & Swayne (1997) detected antibodies to the haemagglutinin from the 10th day onward and Otsuki *et al.* (1982) – after the 14th day. The objectivity of our results is substantiated by the fact that the haemagglutinin is located on the surface of the viral particle and is the first to provoke an antigenic challenge. At the same time, our data showed for the first time that the adaptation of the viral strain to the avian species was important for antibody titres against haemagglutinin, tested in HIT. After infection with a virus, adapted to chickens (chicken isolate), resulting antibody titres are high – up to 1:2048 (Meulemans *et al.*, 1987; Brugh, 1995; Lu & Castro, 2004), whereas in chickens infected with our isolate obtained from a wild duck, antibody titres were 16-fold lower (up to 1:128).

The results from the IDT, detecting type-specific antibodies (precipitins) raised against the nucleoprotein antigen, are also interesting. Regardless of its location in the core of the viral particle, antibodies in our experiments were detected

as early as the 7th day and in those of Swayne & Beck (2005) on the 5th day. These time intervals could be explained with phagocytosis of the virion, most likely followed by its destruction and the early occurring antigen challenge of immunocompetent cells. The difference between our data and those of Beck & Swayne (1997) for this early period consists in the fact that the latter detected seroreagents only with precipitins (positive IDT), whereas in the present study, the chickens are positive to both HIT and IgG antibodies (detected in ELISA).

Another specific feature observed by us in the IDT were the high (maximum) titres by the 7th day (precipitin titres up to 1:128), that decreased later (up to 1:4 by the 28th day). The lack of respective literature data about the level of precipitins does not allow us to make any comparisons in this connection.

The summarized data from the ELISA in this experiment showed that it was able to detect 93.1 % of seroreagents after intratracheal introduction of AIV. The probable reasons for the high percentage are the route of infection and the amount of the used antigen. This is further supported by the experiments of Beck & Swayne (1997) and Lu & Castro (2004).

The first antibodies, detected in ELISA in the experiment of Meulemans *et al.* (1987) appeared on the 6th day. These results are close to ours, showing positive samples from the 7th day onward. In another trial (Beck & Swayne, 1997) the emergence of antibodies was at a later period – after the 14th day. In the beginning however, the authors observed false negative results (8 %), absent in our study at that time. The occurrence of false negative results according to Beck & Swayne (1997) is attributed to the used viral titres. In high-titre strains (10^5 $\log_{10}$ ELD_{50}), the percentage of falsely negative results reaches 0 %.

The S/P values on the 14th day in our experiments (S/P = 2.776) are three times higher than those reported in the experiments of Beck & Swayne (1997) in birds at the same age, infected with a virus with equal titre (maximum S/P of 0.941 by the 14th day). Despite the fact that reported S/P values increased later, they did not reach the rate from our trial.

With regard to the potential of the three tests (HIT, IDT and ELISA) for detection of the time of emergence of antibodies, our studies support the data of Lamichhane & Kirkegaard (1997) for equal potential and differ from these of Meulemans *et al.* (1987) and Beck & Swayne (1997) in favour of IDT and HIT compared to ELISA.

Data comparing the results from HIT, IDT and ELISA with regard to their sensitivity, specificity and test agreement are reported from Zhou *et al.* (1997) and Zhou *et al.* (1998). They are about investigations on field chicken samples without stating the route of viral penetration (oral or intratracheal). The authors demonstrated better values for all HIT parameters, and IDT and ELISA potentials were found to be equal (Zhou *et al.*, 1997). Later studies with IDT and ELISA, determined the superiority of ELISA (Zhou *et al.*, 1998). Provided that the route of penetration of the virus in chickens is known (experimental intratracheal infection), we observed equal parameters in HIT and IDT (100% sensitivity and specificity). We did not confirm the reported equal or superior characteristics of ELISA vs IDT. In our experiment ELISA showed a lower sensitivity (93.10 %) and test agreement (97.40 %) compared to IDT. The differences with the experiments of Zhou *et al.* (1997) and Zhou *et al.* (1998),

in our opinion, are probably related to the absence of information about the route of infection of birds, the amount of viral inoculum and the lack of standardized methods for preparations of tests for antibody diagnostics that makes the comparative assessment not always objective.

REFERENCES

Abraham, A., V. Sivanandan, D. A. Halvorson & J. A. Newman, 1986. Standardization of enzyme-linked immune sorbent assay for avian influenza virus antibodies in turkeys. *American Jurnal of Veterinary Resarch*, **47**, 561–566.

Abraham, A., V. Sivanandan, D. Karunakaran, D. A. Halvorson & J. A. Newman, 1988. Comparative serological evaluation of avian influenza vaccine in turkeys. *Avian Disease*, **32**, 4, 659–662.

Adair, B. M., D. Todd, E. R. McKillop & M. S. McNulty, 1989. Detection of influenza A type-specific antibodies in chicken and turkey sera by enzyme linked immunosorbent assay. *Avian Pathology*, **18**, 455–463.

Adair, B. M., K. Burns, M. S. McNulty & D. Todd, 1990. A study of ELISA systems incorporating pooled viral and *Mycoplasma* antigen preparations for antibody screening of chicken sera. *Avian Pathology*, **19**, 263–278.

Allwright, D. M., W. P. Burger, A. Geyer & A. W. Terblanche, 1993. Isolation of influenza A virus from ostriches (*Struthio camelus*). *Avian Pathology*, **22**, 59–65.

Arenas, A., J. Carranza, A. Perea, A. Miranda, A. Maldonado & M. Hermoso, 1990. Type A influenza viruses in birds in southern Spain: Serological survey by enzyme linked immunosorbent assay and haemagglutination inhibition tests. *Avian Pathology*, **19**, 539–546.

Beard, C. W., 1970. Demonstration of type-specific influenza antibody in mammalian and avian sera by immunodiffusion. *Bulletin WHO*, **42**, 779–785.

Beck, J. R. & D. E. Swayne, 1997. Evaluation of ELISA for avian influenza serologic and diagnostic programs: Comparison with agar gel precipitin and hemagglutination inhibition tests. In: *Proceedings of the Fourth International Symposium on Avian Influenza*, May 28–31, The University of Georgia, Athens, GA, USA, American Association of Avian Pathologists, Lib. Cong. Cat. Card No. 97-051243, 297–304.

Boer, G. F., W. Back & A. D. M. E. Osterhaus, 1990. An ELISA for detection of antibodies against influenza nucleoprotein in human and various animal species. *Archives of Virology*, **115**, 47–61.

Brugh, M., 1995. Pathogenicity of three avian influenza viruses for Leghorn hens of different ages. *Avian Diseases*, **40**, 725–728.

Cadman, H. F., P. J. Kelly, R. Zhou, F. Dovelaar & P. R. Mason, 1994. A serosurvey using enzyme-linked immunosorbent assay for antibodies against poultry pathogens in ostriches (*Struthio camelus*) from Zimbabwe. *Avian Diseases*, **38**, 621–625.

Courtney, C. H. & J. A. Cornell, 1990. Evaluation of heartworm immunodiagnostic tests. *Journal of the American Veterinary Medical Association*, **197**, No 6, 724–729.

Donahoe, J. P., 1997. Inactivated avian influenza whole virus vaccines. In: *Proceedings of the Fourth International Symposium on Avian Influenza*, May 28–31, The University of Georgia, Athens, GA, USA, American Association of Avian Pathologists, Lib. Cong. Cat. Card No. 97-051243, 223–227.

Fatumbi, O. O., J. A. Newman, V. Sivanandan & D. A. Halvorson, 1989. A broad-spectrum avian influenza subtype antigen for indirect enzyme-linked immunosorbent assay. *Avian Diseases*, **33**, 264–269.

Garcia-Garcia, J., H. Rodriguez, A. Hernandez, T. R. Mickle, R. Fernandez, E. Mon-

tiel & H. Tinoco-Garcia, 1997. Experimental studies and field trails with recombinant fowlpox vaccine in broilers in Mexico. In: *Proceedings of the Fourth International Symposium on Avian Influenza*, May 28–31, The University of Georgia, Athens, GA, USA, American Association of Avian Pathologists, Lib. Cong. Cat. Card No. 97-051243, 245–252.

Lamichhane, C. M. & L. Kirkegaard, 1997. Comparison of serological methods for the detection of antibodies to avian influenza virus in chickens sera. In: *Proceedings of the Fourth International Symposium on Avian Influenza*, May 28–31, The University of Georgia, Athens, GA, USA, American Association of Avian Pathologists, Lib. Cong. Cat. Card No. 97-051243, 366–372.

Lu, H. & E. Castro, 2004. Evaluation of the infectivity, length of infection, and immune response of a low-pathogenicity H7N2 avian influenza virus in specific-pathogen-free-chickens. *Avian Diseases*, **48**, 263–270.

Meulemans, G., M. S. Carlier, M. Gonze & P. Petit, 1987. Comparison of hemagglutination–inhibition, agar gel precipitin, and enzyme-linked immunosorbent assay for measuring antibodies against influenza viruses in chickens. *Avian Diseases*, **31**, 560–563.

Otsuki, K., Y. Kawaoka, T. Nakamura & M. Tsubokura, 1982. Pathogenicity for chickens of avian influenza viruses inoculated from whistling swans and a black-tailed gull in Japan. *Avian Diseases*, **26**, No 1, 314–320.

Otsuki, K., H. Yoneda & Y. Iritani, 1995. Distribution of antibodies to influenza A virus in chickens in Japan. *Journal of Veterinary Medical Science*, **56**, No 6, 1063–1066.

Pearson, J. E. & D. A. Senne, 1986. Diagnostic procedures for avian influenza. *In: Proceedings of the 2nd International Symposium on Avian Influenza*, September 3–5, Athens, GA, Richmond, VA: U.S., Animal Health Association, Lib. Cong. Cat. Card No. 86-051243, 222–227.

Reed, L. J. & H. Muench, 1938. A simple method of estimating fifty percent endpoints. *Journal of Hygiene*, **27**, 493–497.

Sala, G., P. Cordioli, A. Moreno-Martin, M. Tolles, E. Brocchi, A. Piccirillo & A. Lavazza, 2003. ELISA test for the detection of influenza H7 antibodies in avian sera. *Avian Diseases*, **47**, 1057–1059.

Salk, J. E., 1944. A simplified procedure for titrating hemagglutinating capacity of influenza virus and the corresponding antibody. *Journal of Immunology*, **49**, 87–98.

Snyder, D. B., W. W. Marqardt, F. S.Yancey & P. K.Savage, 1985. An enzyme-linked immunosorbent assay for the detection of antibody against avian influenza virus. *Avian Diseases*, **29**, 136–144.

Stone, H. D., 1988. Efficacy of avian influenza oil-emulsion vaccines in chickens of various ages. *Avian Diseases*, **31**, No 3, 483–490.

Swayne, D. E. & R. D. Slemons, 1995. Comparative pathology of intravenously inoculated wild duck- and turkey-origin type A influenza viruses in chickens. *Avian Diseases*, **39**, No 1, 74–84.

Swayne, D. E., 1997. Pathobiology of H5N2 Mexican avian influenza virus infections of chickens. *Veterinary Pathology*, **34**, No 6, 557–567.

Swayne, D. E. & J. R. Beck, 2005. Experimental study to determine if low-pathogenicity and high-pathogenicity avian influenza viruses can be present in chicken breast and thigh meat following intranasal virus inoculation. *Avian Diseases*, **49**, 81–85.

Trani, L. D., P. Cordioli, E. Falcone, G. Lombardi, A. Moreno, G. Sala & M. Tollis, 2003. Standardization of an inactivated H7N1 avian influenza vaccine and efficacy

against A/chicken/Italy/13474/99 high-pathogenicity virus infection. *Avian Diseases*, **47,** 1042–1046.

Zarkov, Iv., R. Manvell, W.Shell & Il. Bochev, 2006. Isolation of avian influenza virus in Bulgaria. *The Veterinary Record*, **158**, No 3, 106–107.

Zarkov, I., 2007. Diagnostic potential of the haemagglutination inhibition test, the immunodiffusion test and ELISA for detection of antibodies in chickens, intravenously infected with A/duck/Bulgaria/05 H6N2 avian influenza virus isolate. *Bulgarian Journal of Veterinary Medicine*, **10**, No 3, 169–178.

Zhou, E.-M., M. Chan, R. A. Heckert, J. Riva & M.-F. Cantin, 1997. A competitive enzyme linked immunosorbent assay for avian influenza serological surveillance. In: *Proceedings of the Fourth International Symposium on Avian Influenza*, May 28–31, The University of Georgia, Athens, GA, USA, American Association of Avian Pathologists, Lib. Cong. Cat. Card No. 97-051243, 305–312.

Zhou, E.-M., M. Chan, R. A. Heckert, J. Riva & M.-F. Cantin, 1998. Evaluation of a competitive ELISA for detection of antibodies against avian influenza virus nucleoprotein. *Avian Diseases*, **42**, 517–522.

Paper received 03.07.2006; accepted for publication 12.11.2007

Correspondence:

Assoc. Prof. Ivan Zarkov,
Department of Veterinary Microbiology,
Infectious and Parasitic Diseases,
Faculty of Veterinary Medicine,
Trakia University, Student's Campus,
6000 Stara Zagora, Bulgaria