

Survival of avian influenza viruses in filtered and natural surface waters of different physical and chemical parameters

I. STOYANOV ZARKOV

Department of Microbiology, Infectious and Parasitic Disease, Veterinary Faculty, Thracian University, 6000 Stara Zagora, Bulgaria
E-mail : ivanzarkov@yahoo.com

SUMMARY

Avian influenza viruses (AIV) survival was studied in five basins of different physical and chemical parameters of water, including pH, temperature and salinity. Places are known for the considerable aggregation of wild waterfowl. After 1:1000 dilution H6N2 isolate with titer $10^{1.63}$ EID₅₀/0.1 ml water and A/duck/England/56 H11N6 with titer $10^{3.83}$ EID₅₀/0.1 ml water were used. Survival did not exceed 24 h at 9.34 pH, reached one week at the highest salinity and more than 2 weeks at close to tap water physical and chemical characteristics.

Presence of living microorganisms in some waters further reduced AIV survival (with 12.5 % in H6N2 and with 14.29 % in A/duck/England/56 H11N6 in the waters with microorganism count as small as 8,9. 10^4 cells/ml). When microorganism count was high ($6,64.10^6$ cells/ml and $5,2.10^6$ cells/ml) the period of survival was with 40 % and 35.71 % shorter. Titer values in waters containing microorganisms were with 0.17 \log_{10} EID₅₀/0,1 ml to 2.16 \log_{10} EID₅₀/0,1 ml lower.

Keywords: Avian influenza virus, natural waters, persistence, titration

RESUME :

Persistence du virus de la grippe aviaire dans les eaux filtrées et naturelles de surface en fonction des paramètres physiques et chimiques.

Cette étude a pour objectif de caractériser la persistance du virus de la grippe aviaire dans 5 points d'eau présentant une composition physico-chimique différente et une forte concentration d'oiseaux sauvages. On a utilisé après dilution au 1 :1000 un isolat H6N2 avec ($10^{1.63}$ EID₅₀/0.1 ml) et A / duck/England/56 H11N2 ($10^{3.83}$ EID₅₀/0.1ml). La survie des isolats n'a pas dépassé 24h à pH 9,34, a atteint 1 semaine pour les conditions de salinité les plus élevées et plus de 2 semaine dans des conditions proches de celles rencontrées dans l'eau potable.

La présence d'une flore microbienne vivante réduit encore la période de persistance dans les eaux de 12.5 % pour l'isolat H6N2 et de 14.29 % pour A / duck/England/56 H11N6 dans des eaux avec le moindre taux de microorganismes ($8,9.10^4$ de cellules/ml) . Dans les eaux ayant un taux élevé de microorganismes ($6,64.10^6$ de cellules/ml et $5,2.10^6$ de cellules/ml) la période de persistance est raccourcie respectivement de 40% et 35,71 %. Les valeurs des titres des virus dans les eaux avec des microorganismes sont inférieures de 0.17 \log_{10} EID₅₀/0.1 ml à 2.16 \log_{10} EID₅₀/0.1 ml.

Mots-clés : AIV, grippe aviaire, eaux naturelles, persistance, titrage

Introduction

Bulgaria is situated on the main migratory route of Via Pontica used by numbers of migrating waterfowl. In most cases avian influenza virus (AIV) have been isolated from waterfowl [13, 14]. During 2004/5 migratory season for the first time in Bulgaria AIV subtype H6N2 has been isolated from a mallard [19]. AIV replication occurs mainly in the intestinal tract of *Anseriformes* order [12] with high concentration of viruses in feces [5, 17]. Feces are shed mainly in water where birds reside longer than on the coast [10, 11]. Hence aquatic habitats are important reservoir for propagation of AIV among wild waterfowl and are potentially hazardous for domestic birds and swine that share the same water sources.

Persistence of viral infectivity in water is important from epidemiological point of view. STALLNECHT and al. (1990) have studied AIV survival in distilled water and proved the role of water temperature and virus subtype. Its survival has been shorter at higher temperature (30 – 102 days at 28° C

increasing to 126 – 146 days at 17° C). Survival has varied from 30 % - 76 % in relation to the subtype. Same authors have revealed importance of pH, temperature and salinity as principal parameters influencing persistence of AIV infectivity in water [16]. Their tests have included 12 combinations at 17° C and 28° C; 6.2, 7.2 and 8.2 pH and 0 ppt and 20 ppt salinity and shown that all three parameters influence virus survival. AIV infectivity has persisted for nine days only at 8.2 pH, 20 ppt salinity and 28° C. If any of the parameters has been reduced, survival increased. For example, at 6.2 pH or at 17° C survival has increased three times and at 0 ppt – 5 times.

The aim of the present experimental design was to determine AIV survival in natural surface waters of different physical and chemical parameters in the presence and in the absence of microorganisms. AIV survival was estimated by the minimum AIV dose, in agreement with the natural one, that could be detected after infection of chicken embryos (CE).

Material and methods

VIRUSES

Two AIV subtypes were used, H6N2 isolated by us in Bulgaria and A/duck/England/56 H11N6 supplied by the Bulgarian National Bank of Industrial Microorganisms and Cell Cultures. Their infectious titers were $10^{5.0}$ EID₅₀/0.1 ml and $10^{7.75}$ EID₅₀/0.1 ml, respectively.

WATERS

Surface water specimens were obtained from the Black Sea, the Burgas Lake Vaya region, Mandra dam Poda region, Ovcharitsa dam and Koprinka dam. Specimens were collected during December – February of 2004/2005 and again in 2005/2006, 10 m inside the shore and 0,5 m in depth. Samples in sterile glass jars were transported cooled for determination of pH, electric conductivity on a C833 consort conductometer, sulfate content by gravimetric analysis, chlorides, calcium and magnesium contents and total hardness by titrimetry, nitrate and phosphate contents by colorimetry on a specol 11 spectrophotometer. Water temperature was measured at collecting the specimens.

The total count of microorganisms was determined after staining with methylene blue in Burker's chamber [8]. The microorganisms were cultured in blood agar and in brain-heart infusion agar at 20°C and at 4°C and the colony forming units (CFU/ml) in the isolates were determined [7].

EXPERIMENTAL DESIGN

A part of each water specimen was filtered through millipore filters (GE polypropylene Syringe filters, filter size 17 mm, pore size of 0.45 micron – Osmonics,) and another part remained intact (natural). Virus suspension of 0.1 ml was added to 100 ml of each water specimen. The initial dilutions of viruses were $10^{1.63}$ EID₅₀/0.1 ml for H6N2 isolate and $10^{3.83}$ EID₅₀/0.1 ml for A/duck/England/56 H11N6. The virus containing water samples were put at temperature corresponding to that in the basin of origin. Attempts for reisolation of viruses and titration of reisolates were performed on days 0, 1, 3, 5, 7, 9, 12, 14, 16 and 18. Each time 2 ml from the natural water and virus suspension were filtered and used for re-isolation and titration.

Re-isolation of viruses was carried out by inoculation of 0.1 ml suspension into the allantoic sac of six 9-day-old CE. The samples from which viruses were re-isolated were titrated. Ten-fold serial dilutions were done to 10^5 in MEM containing antibiotics (Penicillin G – 2×10^6 U/l, Streptomycin – 200 mg/l, Gentamicin sulfate – 250 mg/l). Six 9-day-old CE were inoculated with 0.1 ml of each viral dilution.

All inoculated CE were observed daily, those dead after the 24th h and those that survived up to the 144th h being placed at 4°C for 2 h. Hemagglutination test was performed with 1% chick erythrocytes (micro variant) on allantoic

fluid [1]. Virus titers were calculated by the method of REED & MUENCH (1938).

Results

From a physical and chemical point of view water from the different basins differs mainly in temperature, pH and electroconductivity/salinity (Table 1). Ovcharitsa water has pH of 9.34 and temperature of 10°C – 12°C during the migratory season. Black Sea water has the highest salinity (2480 mg/l chlorides, 431.88 mg/l magnesium ions, 854.4 mg/l sulfates and electroconductivity 17300 µS/cm). Poda water had 10 times lower values compared to Black Sea water (193 mg/l chlorides and conductivity 1840 µS/cm). Vaya water has about 30 times less salts than Black Sea water (77 mg/l chlorides and 651 µS/cm electroconductivity). Koprinka water is used as drinking water source and has pH of 7.61 and the lowest salinity (7 mg/l chlorides; 26.05 mg/l magnesium ions; 0.03 mg/l phosphates; 25.09 mg/l sulfates).

Low microorganism counts were determined in Koprinka water ($8.9 \cdot 10^4$ cells/ml), followed by Ovcharitsa ($1.11 \cdot 10^5$ cells/ml) and Black Sea ($1.21 \cdot 10^5$ cells/ml) and higher in Vaya ($5.2 \cdot 10^6$ cells/ml) and Poda ($6.64 \cdot 10^6$ cells/ml). We isolated less microorganisms than we had determined (0,002 % from Koprinka and 0,056 % from Vaya) confirming the results of BUSSMAN and al. (2001).

Data of virus survival in water are presented in Tables 2 and 3. Infective viruses disappeared from Ovcharitsa water early and by day 1 no infective viruses were found. In filtered Black Sea and Poda waters infective viruses were present in the isolate until day 5; in Vaya water until day 7 and in Koprinka water until day 14. Survival of subtype A/duck/England/56 H11N6 in filtered water lasted from day 7 (Black Sea), to day 12 (Poda), day 14 (Vaya) and day 16 (Koprinka).

Survival in natural waters was shorter. In Ovcharitsa water virus was found only on day 0. In Black Sea and Poda waters survival lasted 3 days, in Vaya 5 days and in Koprinka 12 days. Survival of A/duck/England/56 H11N6 differed with 2 days only in natural waters from Black Sea (5 days) and Koprinka (14 days). Difference of 3 days was observed in Poda water and 5 days in Vaya water (until day 9 in both cases).

Reduction of titers varied in relation to the viral strains, type of water and presence of other microorganisms. Lower titers were observed with the H6N2 subtype on the 3rd day dropping to less than $1.0 \log_{10}$ EID₅₀/0.1 ml in the filtered Black Sea and Poda waters (Figure 1). In Vaya water these values were attained by day 5. Titers in Koprinka water decreased gradually and values lower than $1.0 \log_{10}$ EID₅₀/0.1 ml were observed by day 7.

Meanwhile lower intermediate titer values were found out in all natural waters. On the 3rd day in Black Sea and Vaya; 1st, 3rd days in Poda and 5th, 7th, 9th days in Koprinka waters their value was lower with $0.17 \log_{10}$ EID₅₀/0.1 ml than in the filtered waters (Figure 2). In natural Vaya (3rd day) and Koprinka (5th day) waters the intermediate titers were $0.33 \log_{10}$ EID₅₀/0.1 ml. This trend persisted for

Parameter	Units	Water basins				
		Black Sea	Poda zone, Mandra dam	Vaya zone, Burgas lake	Ovcharitsa dam	Koprinka dam
pH	pH units	7.76	7.34	7.78	9.34	7.61
Nitrates	mg/l	4.6	17.0	2.0	2.6	2.8
Chlorides	mg/l Eqv/l	2480	193	77.0	58.0	7.0
Total hardness	mg/l	41.8	7.3	4.0	5.2	2.3
Calcium	mg/l	128.25	30.06	34.06	54.11	26.05
Magnesium ions	mg/l	431.88	70.76	28.06	30.50	12.20
Phosphates	mg/l	0.18	0.34	0.27	0.05	0.03
Sulfates	mg/l	854.48	188.0	71.17	192.95	25.09
Electroconductivity	μ S/cm	17300	1840	651	802	244
Temperature (December 2004 -February 2005)	$^{\circ}$ C	5-6 ⁰	5-6 ⁰	5-6 ⁰	10-12 ⁰	5-6 ⁰
Microorganism/ml		1,21.10 ⁵	6,64.10 ⁶	5,2.10 ⁶	1,11. 10 ⁵	8,9. 10 ⁴
CFU/ml, % isolation		12,17 0,01	2600 0,039	2910 0,056	3,2 0,003	1,6 0,002

TABLE 1. Physico-chemical parameters in surface waters.

Day	Presence of virus									
	Black Sea		Poda		Vaya		Ovcharitsa		Koprinka	
	H6N2	H11N6	H6N2	H11N6	H6N2	H11N6	H6N2	H11N6	H6N2	H11N6
0	+*	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	-	-	+	+
3	+	+	+	+	+	+	-	-	+	+
5	+	+	+	+	+	+	-	-	+	+
7	-**	+	-	+	+	+	-	-	+	+
9	-	-	-	+	-	+	-	-	+	+
12	-	-	-	+	-	+	-	-	+	+
14	-	-	-	-	-	+	-	-	+	+
16	-	-	-	-	-	-	-	-	-	+
18	-	-	-	-	-	-	-	-	-	-

Legend: * - presence of infective virus ** - absence of infective virus

TABLE 2. Persistence of infective viruses in surface waters from the different water basins.

longer periods in Vaya water (1st – 5th day) and in Koprinka water (5th – 12th day).

Subtype A/duck/England/56 H11N6 titers in filtered water are presented on Figure 3. More rapid reduction in titers occurred by day 7 in Black Sea water and by day 9 in Poda and Vaya water. Titers lower than 1.0 log₁₀EID₅₀/0.1 ml were established in Koprinka water by day 14.

In natural water titers of that subtype differed related to the basin of water origin during the whole period. Difference smaller than 1.0 log₁₀EID₅₀/0.1 ml (0.17 log₁₀EID₅₀/0.1 ml – 0.67 log₁₀EID₅₀/0.1 ml) was determined in Poda water on the 3 - 7 days, in Black Sea water on the 1st day, in Vaya water from 1st – 5th and on the 9th day and in Koprinka water

from 3rd – 5th and from 12th – 14th day (Figure 4). Differences in titers from 1.0 log₁₀EID₅₀/0.1 ml – 2.16 log₁₀EID₅₀/0.1 ml were estimated during two consecutive studies on Black Sea water (on 3rd and on 5th day), on Koprinka water (on 7th and 9th day) and on Vaya water (only on the 7th day).

Discussion

Survival of AIV subtypes in waters with different physical and chemical parameters depends on their infectious titers. In 4 out of 5 waters we studied (except Ovcharitsa) the subtype of higher infectious titer survived longer (with 4 – 6 days).

Day	Presence of virus									
	Black Sea		Poda		Vaya		Ovcharitsa		Koprinka	
	H6N2	H11N6	H6N2	H11N6	H6N2	H11N6	H6N2	H11N6	H6N2	H11N6
0	+*	+	+	+	+	+	+	+	+	+
1	+	+	+	+	+	+	-	-	+	+
3	+	+	+	+	+	+	-	-	+	+
5	-**	+	-	+	+	+	-	-	+	+
7	-	-	-	+	-	+	-	-	+	+
9	-	-	-	+	-	+	-	-	+	+
12	-	-	-	-	-	-	-	-	+	+
14	-	-	-	-	-	-	-	-	-	+
16	-	-	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-	-	-

Legend: * - presence of infective virus ** - absence of infective virus

TABLE 3. Persistence of infective viruses in surface waters from the different water basins with microorganisms.

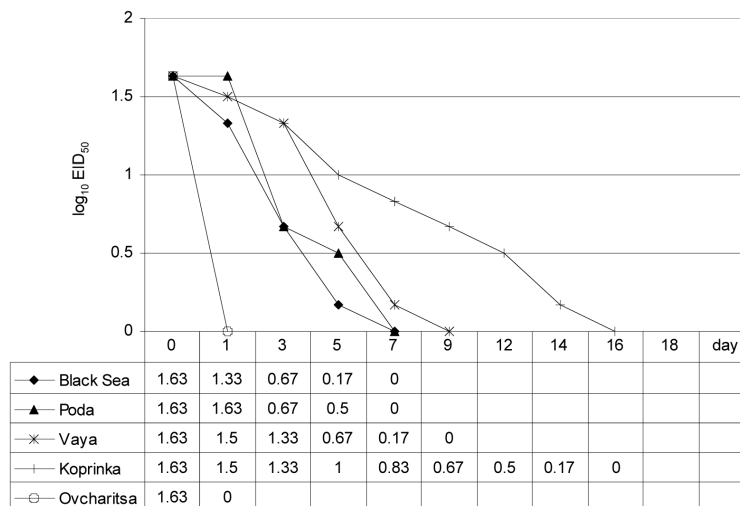


FIGURE 1. Titres of the H6N2 isolate in surface waters of various physico-chemical parameters.

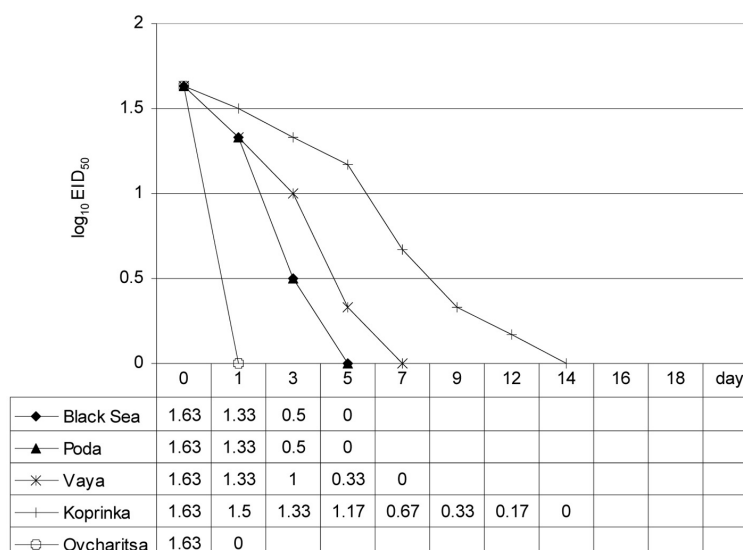


FIGURE 2. Titres of the H6N2 isolate in surface waters of various physico-chemical parameters and with microorganisms.

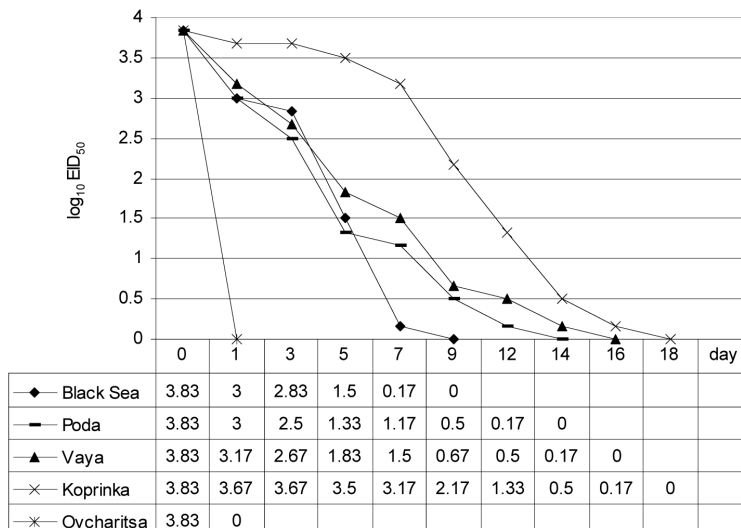


FIGURE 3. Titres of the A/duck/England/56 – H1N6 strain in surface waters of various physico-chemical parameters.

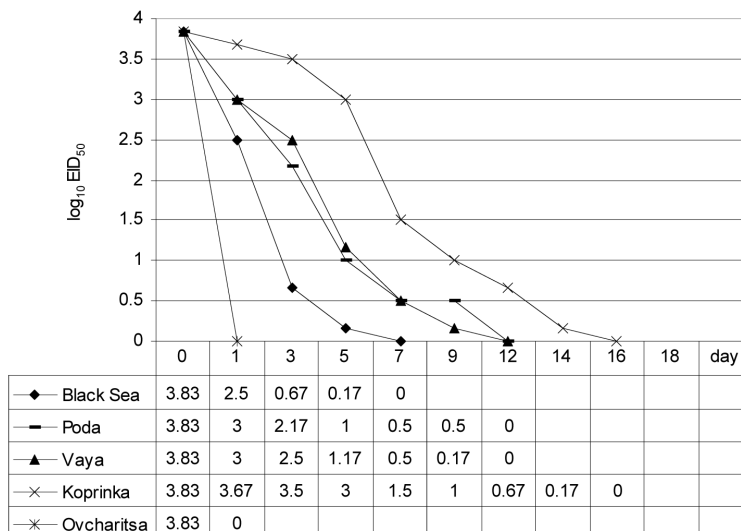


FIGURE 4. Titres of the A/duck/England/56 – H1N6 strain in surface waters of various physico-chemical parameters and with microorganisms.

Results from this study show that the biggest reduction in infectivity in both subtypes occurred in Ovcharitsa water with 9.34 pH, a value out of the range used by STALLKNECHT and al. [16]. Infectivity of viruses persisted less than 24 h. AIV is stable when pH ranges from neutral to 8.5, its infectivity decreasing rapidly under and over this range [4, 6, 7].

STALLKNECHT and al. [16] observe that salinity affects AIV survival. This was confirmed throughout our experiment with natural surface waters. In waters of high salinity (Black Sea) viruses survived 5 and 7 days in relation to the subtype, in medium salinity waters survival was longer (5 and 12 days in Poda water and 7 and 14 days in Vaya water). In drinking water from Koprinka survival was the longest of 14 and 16 days, respectively.

The mechanisms by which water pH, salinity and temperature, independently or in combination, affect AIV survival is not clear. However, pH below 5.0 causes changes in the hemagglutinin glycoprotein which is responsible for the contact and fusion of the virus into the host cells [3, 18]. This process is enhanced by increasing the temperature [16].

There is no evidence about the effect on AIV of the other water parameters, such as magnesium salts, calcium salts and sulfates with the exception of NaCl. Their content in water might also be of certain importance.

Microorganisms in natural water also influence AIV survival through their metabolism and synthesis of various products including toxins. During the whole experimental period strains had lower titers in natural waters in comparison to those in filtered waters which fact was the reason for the difference in the survival period. Differences were

smaller in waters with less microorganisms (12,5 % and 14,29 %, respectively, in Koprinka water). Differences as high as 40% were determined in the isolates from Black Sea and Poda water and 35,71 % in A/duck/England/56 H11N6 from Vaya. The last two waters contained large number of microorganisms.

Of an epidemiological merit is the length of persistence of AIV infectivity in the water basins as well as the period when they could be source of infection. Moreover, both factors depend on the specific physical and chemical properties of water.

References

- 1 — ANONYMOUS. Office International des Epizooties (OIE): *Manual of diagnostic tests and vaccines for terrestrial animals. Highly pathogenic avian influenza*. 2004, chapt. 2.1.14, 1-12.
- 2 — BUSSMANN I., PHILLIPP B., SCHINK B.: Factors influencing the cultivability of lake water bacteria. *Journal Microbiology Methods*, 2001, **47** (1), 41-50.
- 3 — DOMS R.W., HELENIUS A., WHITE J.: Membrane fusion activity of influenza virus hemagglutinin. *Journal of Biological Chemistry*, 1985, **260**, 2973-2981.
- 4 — GRATHE H., STRITTMATTER H.U., KUNRL M., SINNECKER H.: Uber den Einfluss niedriger pH-Werte and die Infektiositat und Neuraminidaseaktivitat menschlicher und tierischer Stamme des Influenzavirus Typ A. *Acta Biologica et Medica Germanica*, 1982, **41**, 1075-1078.
- 5 — HINSHAW V. S., WEBSTER R. G.: The natural history of influenza A viruses. In *Basic and Applied Influenza Research*, Ed A. S. Beare, CRC. Press Inc., Boca Raton, Fl., 1982, 79-104.
- 6 — LANG G., ROUSE B. T., NARAYAN O., FERGUSON A. E., CONNELLI M. C.: A new influenza virus infection in turkeys. Isolation and characterization of virus 6213. *Canadian Veterinary Journal*, 1968, **9**, 22-29.
- 7 — PUJALTE M., ORTIGOSA M., GARAY E.: Aerobic and facultative anaerobic heterotrophic bacteria associated to Mediterranean oysters and seawater. *Intrnational Microbiology*, 1999, **2**, 259-266.
- 8 — RADJASA O., URAKAVA H., KITA-TSUKAMOTO K., OHWADA K.: Characterization of psychrotrophic bacteria in the surfac and deep-sea waters from the Northwestern pacific ocean based on 16s ribosomal DNA analysis. 2001, *Marinas Biotechnology*, **3**, 454-462.
- 9 — REED L. J., MUENCH H.: A simple method of estimating fifty per cent endpoints. *American Journal of Hygiene*, 1938, **27**, 493-497.
- 10 — SANDU T., HINSHAW V. S.: Influenza A virus infection in domestic ducks. *Proceedings 1st Internationale Symposium on Avian Influenza*. Ed. R. A. Bankowski, Carter Composition Corp., Richmond, VA, 1981, 93-99.
- 11 — SINNECKER R., SINNECKER H., ZILSKE E., KOHLER D.: Surveillance of pelagic birds for influenza A viruses. *Acta Virologica*, 1983, **27**, 75-79.
- 12 — SLEMONS R. D., EASTERDAY B. C.: Type A influenza viruses in the feces of migratory waterfowl. *Journal of the American Veterinary Medical Association*, 1977, **171**, 947-948.
- 13 — STALLKNES D. E.: Ecology and epidemiology of avian influenza virus in wild bird population: Waterfowl, shorebirds, pelecans, cormorans. *IVth Internationale Symposium of Avian Influenza*, USA, 1997, 61-69.
- 14 — STALLKNES D. E., SHANE S. M.: Host range of avian influenza virus in free-living birds. *Veterinary Research Communications*, 1988, **12**, 125-141.
- 15 — STALLKNES D. E., SHANE S. M., KEARNEY M. T., ZWANK P. J.: Persistence of avian influenza viruses in water. *Avian Diseases*, 1990, **34**, 406-411.
- 16 — STALLKNES D. E., KEARNEY M. T., SHANE S. M., ZWANK P. J.: Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water. *Avian Diseases*, 1990, **34**, 412-418.
- 17 — WEBSTER R. G., YAKHON M., HINSHAW V. S., BEAN JR., W. J., MURTI K. G.: Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology*, 1978, **84**, 268-278.
- 18 — WEBSTER R. G., KAVAOKA Y.: Avian influenza. *CRC Critical Reviews in Poultry Biology*, 1988, **1**, 211-246.
- 19 — ZARKOV I., BOCHEV IL., MANVELL R., SHELL W.: Isolation of avian influenza in Bulgaria. *Veterinary Record*, 2006, **158**, 3, 106-107.