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Original Contribution

PERIOD OF SHEDDING OF THE AVIAN INFLUENZA A H6N2 SUBTYPE VIRUS ISOLATE IN YOUNG DOMESTIC FOWL

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ABSTRACT

The aim of the present study was to compare the susceptibility of 4 bird species (ducklings, goslings, turkeys and chickens) to a low pathogenic avian influenza A virus (H6N2 subtype) and to evaluate the corresponding virus shedding periods. For that purpose, 9 turkey poults, 9 chickens, 7 ducklings and 6 goslings, were experimentally infected by the H6N2 virus (10^5 ELD_{50}) by intravenous route whereas another 16 birds (2 ducklings, 2 goslings, 6 turkey poults and 6 chickens) were not infected and served as negative controls. The virus re-isolation method was performed on cloacal and oropharyngeal samples collected before inoculation and 3, 5, 7, 10, 14, 21 and 28 days after inoculation. The virus was re-isolated from all ducklings (100%), 67 % of goslings, 56% of turkey poults and from 33% of chickens essentially during the first 10 days after virus inoculation. The proportion of positive cloacal samples was higher compared to that of oropharyngeal samples (positive cloacal samples were 72%, 83%, 80 % and 87% from all positive samples in ducklings, turkey poults, chickens and goslings respectively). The mean virus shedding periods determined from cloacal samples were 10.6, 6.6, 5.4 and 4.3 days in ducklings, turkeys, goslings and chickens respectively. The longest periods observed in this study were 21 (ducklings), 10 (turkeys and goslings) and 5 days (chickens). The oropharyngeal virus shedding periods were shorter in the 4 species. Consequently, cloacal samples may be more relevant to exploring spontaneous avian influenza cases.

Key words: Avian influenza virus, H6N2 subtype, shedding, duckling, goslings, turkey poults, chickens.

INTRODUCTION

Wild waterfowl, in particular mallard ducks (*Anas plathyrynchos)* and geese, are natural hosts and constitute a reservoir of the influenza A virus which can be transmitted to domestic fowl, and mainly to poultry (*Gallus domesticus)* and turkeys (*Meleagridis gallopavo)* (1-5). Infection with the low-pathogenic avian influenza A virus (LPAIV) depends on the avian species. The durations of shedding and virus re-isolation from the oropharynx and the cloaca vary widely (6-9). The Influenza A virus isolation rates were from 8.3% (10) to 45.7% (7) in ducklings, 51.4% in turkey poults (7), 30% in goslings and 0% (7), 4% - 43% (8) and 95.2% (11) in chickens. It was generally stated that the virus shedding lasted 0-8 days in geese (9), 3-7 days in turkey poults (7,8), 5-7 days in chickens (6,8,12,13,14) and 4-7 days in ducklings (7, 15) but some studies have reported longer shedding periods: until 14 days in chickens (16) and 30 days in ducklings (17). Furthermore, oropharyngeal shedding is considered to last longer than cloacal one (8, 9) albeit some authors have reported a same duration of shedding in both sites (11, 14) or a shorter duration in the oropharynx (6). Virus isolation and duration of shedding depend also on the type of the strain. In experiments with the H13N2 strain LAUDERT and others (1993) have re-isolated the virus from 51.4% of the turkeys and from 45.7% of the ducklings whereas no chicken was infected. Tumpey and others (2004) obtained more isolates from turkey poults than from chickens experimentally infected with the LPAIV H7N2 for a different period of virus shedding in both species according to the site (5 days from the

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cloaca and 7 days from the oropharynx). These authors also pointed out that efficient infection of chickens required high virus dosages (100-250 fold the dose necessary for infecting turkey poults).

The aim of the present study was to determine the specific number of isolates, the duration and the site of virus shedding in different avian species (ducklings, goslings, turkey poults and chickens) experimentally infected with the LPAIV H6N2 subtype.

MATERIAL AND METHODS

VIRUS AND INOCULUM PREPARATION

The low-pathogenic avian influenza A virus (LPAIV) of the H6N2 subtype obtained from a mallard duck (*Anas plathyrynchos)* was used at a titre of 10^5 ELD_{50} /0.1 mL (ELD_{50} mean embryo lethal doses causing a 50% death rate in inoculated chicken embryos) (19). Allantoic fluid was collected after inoculation of LPAIV (H6N2 subtype) into the allantoic sac (100 μL) of 5 to 9-day old chicken embryos (CE). Embryos were observed daily for 120 hours (when all were dead). Allantoic fluid derived from them was explored by haemagglutination assay (HA) (20). Samples with haemagglutin titres of 1:128 were stored at -84°C until used in the experiment.

BIRDS AND PROTOCOL DESIGN

Forty-seven 30–day-old birds (15 Turkey poults of the Beltsville White breed, 15 chickens of the Dekalb breed, 9 mallard ducklings and 8 goslings of the White Bulgarian/Benkovska breed) were used in this experiment. Thirty-one birds (9 turkey poults, 9 chickens, 7 ducklings and 6 goslings) were intravenously infected with 100 μL allantoic fluid from infected chicken embryos (CE) while 100 μL allantoic fluid from intact CE was intravenously injected to the other birds (uninfected control group, n = 16). The 2 groups of infected and uninfected birds were kept separately in 4 x 4 m rooms at 1.8 m feeding and watering front, 20°C and 70% humidity. No vaccine and antibiotic were administered to the birds.

Cloacal and oropharyngeal swabs from all infected and uninfected birds were collected on day 0 (before infection) and on days 3, 5, 7, 10, 14, 21 and 28 post infection (P.I.). Consequently, 434 samples were obtained from infected birds and 318 - from uninfected birds (from controls and prior to infection – day 0).

VIRUS RE-ISOLATION METHOD

A 10% suspension of the samples (w/v) was prepared in MEM (pH: 7.2-7.4) supplemented with Penicillin G (2.10^6U/L), Streptomycin (200 mg/L), Polymyxin B (2.10^6 U/L), Gentamicin sulfate (250 ml/L), Nystatin dehydrate ($0.5.10^6$ U/L), Sulphamethoxazole (0.2 g/L) and foetal bovine serum (0.5%). After homogenization and centrifugation (800 g, 4°C for 10 min), the supernatant (200 μL) was inoculated into the allantoic sac of three 9-day old CE. The infected embryos were incubated at 36°C for 120 hours, then the dead and living CE were cooled at 4°C for another 2 hours and the allantoic fluid was collected. The presence of the haemagglutinating virus were determined by the haemagglutination assay (HA) and the viral haemagglutinins by the haemagglutination inhibition (HI) test. Serial dilutions (50 μL) of the allantoic fluids (1:2 – 1:4096) were prepared in a micro plaque with buffered saline and 50 μL of 1% hen erythrocyte suspension were added. HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The highest dilution of the allantoic fluid preventing the spot-like agglutination of erythrocytes corresponded to the haemagglutinating viral titre. The haemagglutinins from the H6 isolates were identified by the HI test using a chicken monospecific hyperimmune serum diluted to 1:256. The micro plaque remained at room temperature for 30 min before the results were read. Positive HI (presence of agglutination) evidenced the subtype of the viral haemagglutinins.

RESULTS

Control birds and birds prior to infection (day 0) gave always negative results for H6N2 subtype virus re-isolation from cloacal and oropharyngeal samples for the whole experimental period.

The H6N2 subtype of avian influenza A virus was re-isolated from all ducklings (100%) between the 3 to the 5 P.I. days **(Table 1).** The highest proportions of positive birds were recorded on the first days (Days 3 and 5) and thereafter these percentages decreased with time (Day 7: 50% - Day 21: 29%). Virus re-isolation was no more possible on day 28. The overall positive reisolation rate (cloacal and oropharyngeal samples) was 30%: the frequency of positive samples gradually decreased with time from 71% on Day 3 to

14% on Day 21 (where only positive cloacal samples were still obtained). Positive reisolation from cloacal samples prevailed (43% of cloacal samples were positive, i.e. 72% of positive re-isolations originated from cloacal samples) whereas the proportion of positive oropharyngeal samples were 2.5 times less (16%). Besides, no positive oropharyngeal samples were obtained after the 14th P.I. day.

***Table 1**. Number of positive cloacal and oropharynx samples and number of infected ducklings as evidencing by the virus re-isolation method after intravenous injection of allantoic fluid from infected chicken embryos (100 μL) with the LPAIV H6N2 virus subtype (10^5 ELD_{50}) on day 0 on 30-day-old ducklings (n = 7).*

Positive samples (number)				**Infected ducklings**	
Days	**Total**	**Cloacae**	**Oropharynx**	**Identification number**	**Total number**
3	10/14	5/7	5/7	N° 1, 2, 3, 4, 7	5/7
5	6/14	4/7	2/7	N° 1, 4, 5, 6, 7*	7/7
7	4/14	4/7	0/7	N° 4, 5, 6, 7	
10	4/14	3/7	1/7	N° 5, 6, 7	
14	3/14	3/7	0/7	N° 5, 6, 7	
21	2/14	2/7	0/7	N° 5, 6	
28	0/14	0/7	0/7	0	
Overall	29/98	21/49	8/49	7/7	7/7

* 3 birds with positive cloacal samples, 1 bird with positive cloacal and oropharyngeal samples and 1 bird with only oropharyngeal sample positive.

The H6N2 subtype of avian influenza A virus was re-isolated from 67% of the goslings between the 3rd and the 10th P. I. days **(Table 2)**. The highest proportions of positive birds were recorded between the 3rd and the 7th days and percentages decreased to 17 % by the 10th day. Virus re-isolation was no more possible on days 14, 21 and 28. A total of 15/48 (31.2%) samples were positive only between the P.I. days 3 (41.7% of positive samples) and 10 (8.3% of positive samples). Positive reisolations from cloacal samples prevailed (86.7 % positive cloacal samples) whereas positive oropharyngeal samples were 6.5 times less (13.3%).

A total of 5/9 (56%) experimentally inoculated turkey poults gave positive H6N2 subtype virus re-isolation from samples collected from the 3rd to the 7th days P. I. **(Table 3).** No positive samples were obtained after this time. Only 12/126 samples (10%) were positive between the days 3 (22% of positive samples) and 10 (11% of positive samples) and again, the proportion of positive cloacal samples among all positive samples (10/12, i.e. 83%) was considerably greater than that of oropharyngeal samples (2/12, i.e. 17%).

Positive re-isolations were achieved in 33% of the infected chickens only in the first days (days 3 and 5) after experimental inoculation **(Table 4)**. Re-isolates constituted 4% of all samples tested: 4 were from cloacal origin and only one was an oropharyngeal sample.

Table 2. *Number of positive cloacal and oropharynx samples and number of infected goslings as evidencing by the virus re-isolation method after intravenous injection of allantoic fluid from infected chicken embryos (100 μL) with the LPAIV H6N2 virus subtype (10^5 ELD_{50}) on day 0 on 30 day old goslings (n = 6).*

Positive samples (number)				**Infected goslings**	
Days	**Total**	**Cloacae**	**Oropharynx**	**Identification number**	**Total number**
3	5/12	4/6	1/6	N° 2, 3, 4, 6*	4/6
5	5/12	4/6	1/6	N° 2, 3, 4, 6	
7	4/12	4/6	0/6	N° 2, 3, 4, 6	
10	1/12	1/6	0/6	N° 3	
14	0/12	0/6	0/6	0	
21	0/12	0/6	0/6	0	
28	0/12	0/6	0/6	0	
Overall	15/84	13/42	2/42	4/6	4/6

* 2 birds with positive cloacal samples, 2 birds with positive cloacal and oropharyngeal samples

Table 3. *Number of positive cloacal and oropharynx samples and number of infected turkey poults as evidencing by the virus re-isolation method after intravenous injection of allantoic fluid from infected chicken embryos (100 μL) with the LPAIV H6N2 virus subtype (10^5 ELD_{50}) on day 0 on 30-day- old turkey poults (n = 9).*

Positive samples (number)				**Infected turkey poults**	
Days	**Total**	**Cloacae**	**Oropharynx**	**Identification number**	**Total number**
3	4/18	3/9	1/9	N° 1, 4, 8*	3/9
5	3/18	3/9	0/9	N° 1, 4, 6	4/9
7	3/18	2/9	1/9	N° 1, 6, 9	5/9
10	2/18	2/9	0/9	N° 6, 9	5/9
14	0/18	0/9	0/9	0	
21	0/18	0/9	0/9	0	
28	0/18	0/9	0/9	0	
Overall	12/126	10/63	2/63	5/9	5/9

* 2 birds with positive cloacal samples, 1 bird with positive cloacal and oropharyngeal samples.

Table 4. *Number of positive cloacal and oropharynx samples and number of infected chicken as evidencing by the virus re-isolation method after intravenous injection of allantoic fluid from infected chicken embryos (100 µL) with the LPAIV H6N2 virus subtype (10^5 ELD_{50}) on day 0 on 30-day-old chickens (n = 9).*

Positive samples (number)				**Infected chicken**	
Days	**Total**	**Cloacae**	**Oropharynx**	**Identification number**	**Total number**
3	3/18	2/9	1/9	N° 3, 7*	2/9
5	2/18	2/9	0/9	N° 3, 9	3/9
7	0/18	0/9	0/9	0	
10	0/18	0/9	0/9	0	
14	0/18	0/9	0/9	0	
21	0/18	0/9	0/9	0	
28	0/18	0/9	0/9	0	
Overall	5/126	4/63	1/63	3/9	3/9

* 1 birds with positive cloacal samples, 1 bird with positive cloacal and oropharyngeal samples.

The period of virus shedding varied according to the bird species and to the sample type **(Table 5).** In infected ducklings, the mean duration of this period at the cloacal site was 10.6 days and virus shedding period ranged from 3 to 21 days. By contrast, this period was shorter at the oropharynx site: only one duckling (bird N° 7) exhibited a virus shedding until the 10th day and the calculated mean was 4.0 days. In goslings, the shedding period lasted 3 to 10 days on the average from the cloaca and 3 to 5 days from the oropharynx. The mean duration of this period at the cloacal site was 5.4 days and at the oropharynx 4.0 days. In turkey poults, the shedding period lasted 7 days on the average, from 3 days in one bird (bird N° 8) to 10 days in 2 birds (birds N° 6 and 9). The mean virus shedding period was 7.0 days from the cloaca and 5 days from the oropharynx. The chickens shed the virus until the 5th day P. I. through the cloaca and only on day 3 through the oropharynx (the mean virus shedding period was 4.3 days from the cloaca and 3 days from the oropharynx).

DISCUSSION

In the present study, the LPAIV H6N2 subtype virus was successfully re-isolated from previously intravenously infected birds of the all four species: in 100% of *Anas plathyrynchos* ducklings, 67 % of goslings, 56% of turkey poults and 33% of chickens. These data show that ducklings (the natural host) are much more sensitive to the virus than the 3 other avian species whereas chickens are much more resistant. Moreover, the efficiency of the infectious challenge in the present experiment was superior to the previous results of Laudert and others (1993) which recorded influenza A virus prevalence of 45.7%, 51.4% and 0% in ducklings, turkey poults and chickens respectively, with H13N2 isolated from spontaneous cases of local respiratory infection in waterfowl and turkeys.

The present results also show that when the virus is obtained from a given species and experimentally inoculated into other species, the course of infection changed in agreement with previous studies (7, 8, 18). The maximal length of the virus re-isolation period was shorter in the recipient species (5 days in chickens and 10 days in goslings and turkey poults respectively) than in the original host species (until 21 days in ducklings) and the mean virus shedding periods were 10.6, 7.0, 5.4 and 4.3 days in ducklings, turkey poults, goslings and chickens respectively. These

results are similar to those obtained by most authors for chickens: 5 – 7 days (6, 8, 12, 13, 14), they are closer to those obtained by Webster and others (1978) for ducklings (30 days); to those of Tumpey and others 2004, Slemons and others (1990) – 7 days for turkey poults and to those of Brown and others (2008) in geese: up to 8 days. The probable reasons for observed differences are the used of viral inocula titres (8, 16) and the bird age (8). The viral persistence was longer when viral inocula with high titres (10^5 - 10^7 ELD_{50} / 100 μL) were used or when birds were older (4 and 8 week old).

Table 5.*Viral shedding period in the cloaca and oropharynx according to the species after intravenous injection of allantoic fluid from infected chicken embryos (100 μL) with the LPAIV H6N2 virus subtype (10^5 ELD_{50}) on day 0 in 25 birds (7 ducklings, 9 turkey poults, 9 chickens and 6 goslings). Seven ducklings, 5 turkey poults, 4 goslings and 3 chickens were considered as really infected by the virus re-isolation method.*

Virus shedding period (in days)																
Birds	**In cloaca**								**In oropharynx**							
	3	5	7	10	14	21	28	mean	3	5	7	10	14	21	28	mean
Ducklings	2	1	1	0	1	2	0	10.6	3	1	0	1	0	0	0	4.0
Turkey poults	1	1	1	2	0	0	0	7.0	1	0	1	0	0	0	0	5.0
Goslings	4	4	4	1	0	0	0	5.4	1	1	0	0	0	0	0	4.0
Chickens	1	2	0	0	0	0	0	4.3	1	0	0	0	0	0	0	3.0

Intense viral replication occurred in the digestive and respiratory tracts from where the virus was spread and isolated. This H6N2 subtype and other LPAIV strains are found out to locate in specific sites (the respiratory and digestive tracts) characterized by the presence of trypsin-like enzymes. In the present experiment, the number of cloacal samples positive for virus re-isolation was superior to the number of positive oropharynx samples in the 4 avian species and, in parallel, the mean virus shedding period was greater in the cloaca than in the oropharynx (mean virus shedding periods in cloacal samples of 10.6, 7.0, 5.4 and 4.3 days and 4.0, 5.0, 4.0, 3.0 days in oropharyngeal samples, for ducklings, turkey poults, goslings and chickens respectively).

These results were in accordance with previous studies (6,14), although some researchers observed a longer persistence of the virus (7 days) and a higher titre in oropharyngeal samples (8). As the intravenous inoculation induced a rapid and wide virus distribution in the whole body including the kidneys and as the intestines and the kidneys are connected to the cloaca via the urethra in birds, virus particles from intestinal and renal origins would be concentrated in the cloaca leading to strong virus persistence in this anatomical site. Such a hypothesis is indirectly supported by the works of Slemons and others (1990) who isolated virus both from the cloaca (95.2%) and the kidneys (61.9%).

The higher percentage of re-isolates obtained from cloacal samples for a longer period in the four studied bird species , indicates that it is more appropriate to investigate cloacal samples for virus detection through isolation in spontaneous cases of avian influenza.

REFERENCES

1. Alexander, D.J., A review of avian influenza in different bird species. *Veterinary Microbiology* 74: 3-13, 2000.
2. Alexander D.J., Report on avian influenza in the Eastern Hemisphere during 1997-2002. *Avian Disorders* 47: 792-797, 2003.
3. Senne, D.A., Avian Influenza in the Western Hemisphere including the pacific

Island and Australia. *Avian Disorders* 47: 798-805, 2003.
4. Swayne, D.E., and Halvorson, D.A. Influenza. In Disease of poultry, 11th edn. Eds Y.M. Saif, H.J. Barnes, A.M. Fadly, J.R. Glisson. pp 135-160, 2003.
5. Fouchier R.A.M. and Munster V.J., Epidemiology of low pathogenic avian influenza viruses in wild birds, *Scientific and Technical Review/* OIE 28 (1): 49-58, 2009.
6. Otsuki, K., Kawaoka, Y., Nakamura, T., and Tsubokura, M., Pathogenicity for chickens of avian influenza viruses inoculated from whistling swans and a black-tailed gull in Japan. *Avian Disorders* 26,1: 314-320, 1982.
7. Laudert, E., Halvorson, D., Sivanandan, V., and SHAW, D., Comparative evaluation of tissue tropism characteristics in turkeys and mallard ducks after intravenous inoculation of type A influenza viruses. *Avian Disorders* 37:773-780, 1993.
8. Tumpey, T.M., Kapczynski, D.R., and Swayne D.E., Comparative susceptibility of chickens and turkeys to avian influenza A H7N2 virus infection and protective efficacy of a commercial avian influenza H7N2 virus vaccine. *Avian Disorders* 48:167-176, 2004.
9. Brown J.D., Stallknecht D.E. and Swayne D.E., Experimental infection of Swans and Geese with Highly pathogenic avian influenza virus (H5N1) of Asian lineage. *Emerging Infections Diseases*. www.cdc.gov/eid, vol. 14, 1:136-142., 2008.
10. Wood, J.M., Webster, R.C., and Netles, V.F., Host range of A/chicken/Pennsylvania/83/H5N2/ influenza virus. *Avian Disorders* 29:198-207, 1985.
11. Slemons, R.D., and Swayne, D.E., Replication of a Water fowl-origin influenza virus in the kidney and intestine of chickens. *Avian Disorders* 34, 2:277-284, 1990.
12. Shalaby, A.A., Slemons, R.D., and Swayne, D.E., Pathological studies of A/chicken/Alabama/7395/75/H4N8/ influenza virus in specific-pathogen-free laying hens. *Avian Disorders* 38, 1: 22-32, 1994.
13. Mo, L.P., Brugh, M., Fletcher, O., Rowland G.N., and Swayne D.E., Comparative pathology of chickens experimentally inoculated with avian influenza viruses of low and high pathogenicity. *Avian Disorders* 41:125-136, 1997.
14. Swayne, D.E., and Beck, J.R., Experimental study to determine of low-pathogenicity and high-pathogenicity avian influenza viruses can be present in chicken breast and thigh meat following intranasal virus inoculation. *Avian Disorders* 49: 81-85, 2005.
15. Sandhy, T., and Hinshaw, V.S., Influenza A virus infection in domestic duck. *Proceeding of the First International Symposium on Avian Influenza.* Beltsville, Maryland, United States, April 22-24, 1981. R. A. Bankowski (ed.), Carter Printing Co. Lib. Cong. Cat. Card N° 86-051243, p 93-99, 1981.
16. Lu, H., and Castro A.E., Evaluation of the infectivity, length of infection, and immune response of a low-pathogenicity H7N2 avian influenza virus in specific-pathogen-free chickens. *Avian Disorders* 48:263-270, 2004.
17. Webster, K.G., Yakhno, M., Hinshaw, V.S., Bean, W.J., and Murti, J.K.G., Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology* 84: 268-278. 1978
18. Jones Y.L. and Swayne D.E., Comparative pathobiology of low and high pathogenicity H7N3 Chilean avian influenza viruses in chickens. *Avian Disorders* 48: 119-128, 2004.
19. Zarkov, I., Manvell, R., Shell, W., and Bochev I.L., Isolation of avian influenza virus in Bulgaria. *Veterinary Record* 158, 3:106-107, 2006.
20. Anonymous, Avian Influenza, Manual of diagnostic procedures. Version adaptated. Chapter 2.7.12, 1-28. *Office International des Epizooties / OIE,* 2005.