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Original Contribution

THE IMPORTANCE OF INOCULATION DOSE OF AVIAN H6N2 INFLUENZA A VIRUS ON VIRUS SHEDDING OF *ANAS PLATHYRYNCHOS* DUCKS AFTER INDUCED GENERALISED INFECTION

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ABSTRACT
A total of 32 30-day old *Anas plathyrynchos* ducks (4 groups - No 1, No 2, No 3, No 4, each from 8 ducks - 6 infected and 2 control uninfected) were used in this experiment. Some of the birds (24 nambers) were infected intravenously with LPAIV H6N2, isolated from wild ducks (*Anas platyrhynchos*). A control group was not infected. The infection was accomplished with different virus dose – group No 1 with $10^{5.00}$ ELD_{50} /100 µL per bird, No 2 – with $10^{4.00}$ ELD_{50} /100 µL per bird, No 3 - $10^{3.00}$ ELD_{50} /100 µL per bird and No 4 - $10^{2.00}$ ELD_{50} /100 µL per bird. In all groups the portion of infected birds, period of virus shedding were monitored.
The virus reisolation showed that infection was established in ducks, infected with dose from $10^{5.00}$ ELD_{50} /100 µL per bird to $10^{3.00}$ ELD_{50} /100 µL per bird. More birds with virus reisolation (100%) were detected when inoculation was made with the higher virus dose of $10^{5.00}$ ELD_{50} /100 µL per bird and $10^{4.00}$ ELD_{50} /100 µL per bird. In dose $10^{3.00}$ ELD_{50} /100 µL per bird virus reisolation were (30 %) while in dose $10^{2.00}$ ELD_{50} /100 µL per bird virus reisolation is unsuccessful.
The highest numbers of reisolates (50% of tested samples) and for the longest time (21 days) were at the highest dose of virus.

Key words: ducks, experiment, avian influenza A virus, dose, virus re-isolation

INTRODUCTION

Wild waterfowl, in particular *Anas plathyrynchos*, are natural hosts and constitute a reservoir of the influenza A virus which can be transmitted to farm fowl, and mainly to poultry *Gallus domesticus* and turkeys *Meleagridis galopava* [1, 2]. Infection with the low-pathogenic avian influenza A virus (LPAIV) depends on the avian species, the duration of shedding and virus re-isolation from the oropharynx and the cloaca widely vary [3-5]. The most often measured infection features are the percent of virus-shedding birds and the period of virus shedding [6, 7]. The Influenza A virus in experiments has been isolated from 8.3% [8] to 45.7% [3] of the ducks. It was generally stated that the virus shedding lasted 4-7 days [3] but few studies have reported longer shedding periods: until 30 days [9]. Furthermore, shedding in oropharynx is considered to last longer than in cloacae [5].

Only LU & CASTRO (2004), CAPUA *et al.* (2004) and PILLAI et al. (2010) have studied the influence of virus dose on the presence of infection but in domestic birds. LU & CASTRO (2004) detect no shedding when chickens are inoculated with $10^{0.7\text{-}2.0}$ mean embryo lethal doses (ELD_{50}) of virus per bird. Chickens were successfully infected with $10^{4.7\text{-}5.7}$ ELD_{50}/500 µL per bird and were shedding virus up to 2 wk post inoculation (PI). In the turkey experiment immune response has not been observed at a dose of $10^{2.0}$ ELD_{50}/100 µL per bird. It has been detected at a dose of $10^{4.0}$ ELD_{50}/100 µL per bird with a little number of reisolations (3.3 %). More reisolates (26.6 %)

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were obtained at virus dose of $10^{6.0}$ ELD_{50}/100 μL per bird - CAPUA *et al.*, 2004.

In another experiment PILLAI et al. (2010) found that different isolates of wild waterbirds best replicated in domestic ducks and less in chicken. In isolates from ducks minimum infectious dose (MID_{50}) to infect ducks is lower.

The aim of this study was through experimental infection of *Anas plathyrynchos* duck to determine the percent of virus-shedding birds, the period of re-isolation and the connection of these parameters with the inoculation dose of the H6N2 virus, isolated from the same species.

MATERIAL AND METHODS

1. Virus and inoculum preparation

The low-pathogenic avian influenza A virus (LPAIV) of the H6N2 subtype obtained from a wild duck *Anas plathyrynchos* was used [11]. Allantoic fluid was collected after inoculation of LPAIV (H6N2 subtype) into the allantoic sac (100 μL) of 5 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 test (HA) [12]. Samples with haemagglutinin titres of 7 log_2 were stored at – 85 ^{0}C until their dilution, titration and use in the experiment.

For titration, we prepared 3 diluted (1:10, 1:100 и 1:1000) in Minimal Essential Medium (MEM) viral suspensions. Every suspension was diluted from 10^{-1} to 10^{-10} in MEM and 100 μL of each dilution was inoculated into the allantoic cavity of eight 9-days old chicken embryos (CE). The calculation of ELD_{50}/100 μL was accomplished according to the method of Reed & Muench, 1938.

The three viral inoculation suspensions had titers of $10^{5.00}$ ELD_{50} /100 μL, $10^{4.00}$ ELD_{50} /100 μL, $10^{3.00}$ ELD_{50} /100 μL and $10^{2.00}$ ELD_{50} /100 μL.

2. Birds and protocol design.

A total of 32 30-day old *Anas plathyrynchos* duck (4 groups each from 8 duck - 6 infected and 2 control uninfected) were used in this experiment. First infected group was intravenously inoculated with 100 μL allantoic fluid with a virus titer of $10^{5.00}$ ELD_{50} /100 μL per bird, second with 100 μL allantoic fluid with a virus titer of $10^{4.00}$ ELD_{50} /100 μL per bird, third group with 100 μL allantoic fluid with a virus titer of $10^{3.00}$ ELD_{50} /100 μL per bird and fourth group with 100 μL allantoic fluid with a virus titer of $10^{2.00}$ ELD_{50} /100 μL per bird. Uninfected control groups was inoculated intravenously with 100 μL per bird of allantoic fluid from non-infected CE.

The 5 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 was administered to the birds.

Cloacal and oropharyngeal swabs from all the infected and uninfected birds were collected on day 0 (before infection) and on days 3, 5, 7, 10, 14, 21 and 28 post infection (PI). Consequently, 336 samples were stemming from infected birds (168 from cloaca and 168 from oropharynx) and 256 samples from uninfected birds (from the controls, n = 208) and prior to infection – day 0 (n = 48).

3. Virus re-isolation

A 10 % (w/v) suspension of the samples was prepared in MEM (pH 7.2-7.4), supplemented with Penicillin G (2.10^6 U/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 2 hours and the allantoic fluid was collected. The presence of the haemagglutinating virus and the titre of viral haemagglutinins were determined by the haemagglutination (HA) test. Serial dilutions (1:2 – 1:4096, 50 μL aliquots) of the allantoic fluids were prepared in a micro plaque with phosphate - buffered saline and 50 μL of a 1% hen erythrocyte suspension were added to each well. The highest dilution of the allantoic fluid hindering the spot-like agglutination of erythrocytes corresponded to the haemagglutinating viral titre. The haemagglutinins from the H6 isolates were identified by the haemagglutination inhibition (HI) test using a chicken monospecific hyper immune serum diluted to 1:256 [1]. 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 haemagglutinin.

RESULTS

We did not detect any ducks symptoms of disease both in the infected and control groups. The total number of re-isolations from ducks, infected with virus of titer $10^{5,00}$ELD$_{50}$ /100 µL per bird was 21 from cloaca (up to 21 day PI) and 8 from oropharynx (up to 10 day PI), infected with virus of titer $10^{4,00}$ELD$_{50}$ /100 µL per bird was 14 only from cloaca (up to 10 day PI), infected with virus of titer $10^{3,00}$ELD$_{50}$ /100 µL per bird was 2 only from cloaca (up to 3 day PI) **(Table 1)** and infected with virus of titer $10^{2,00}$ELD$_{50}$ /100 µL per bird was unsuccessful.

Table 1. *Results of virus reisolation and viral shedding period from pooled cloacal and oropharyngeal swab samples from ducks, challenged with different doses of isolate of influenza A virus H6N2*

Inoculation dose[a]	Samples	3d.p.c.[b]	5 d.p.c.	7d.p.c	10d.p.c.	14d.p.c	21d.p.c	28d.p.c.	vsp[e]
$10^{5,00}$	Cloacal	5[c]	4	4	3	3	2	0	8.43
	Oropharyngeal	2	0[d]	1	0	0	0	0	4.38
$10^{4,00}$	Cloacal	2	4	4	4	0	0	0	3.76
	Oropharyngeal	0	0	0	0	0	0	0	0
$10^{3,00}$	Cloacal	2	0	0	0	0	0	0	2.00
	Oropharyngeal	0	0	0	0	0	0	0	0
$10^{2,00}$	Cloacal	0	0	0	0	0	0	0	0
	Oropharyngeal	0	0	0	0	0	0	0	0

Legend : a – ELD$_{50}$ /100 µL per bird
b - d.p.c., days post challenge
c – number of isolates
d – No virus isolated
e - viral shedding period

Reisolation was successful from 100 % of ducks infected with virus of titer $10^{5,00}$ELD$_{50}$ /100 µL per bird on day 5 and infected with virus of titer $10^{4,00}$ELD$_{50}$ /100 µL per bird on day 7. Only 30% of ducks infected with virus of titer $10^{3,00}$ELD$_{50}$ /100 µL per bird was positive on day 3 PI.

The reisolation ratio in cloacal samples (n = 42) taken from the bird infected with virus of a titer $10^{5,00}$ELD$_{50}$ /100 µL per bird reached 50 %, and the re-isolation ratio in all tested samples (n = 84) reached 34.5 %. The reisolation ratio among birds, infected with virus of a titer $10^{4,00}$ELD$_{50}$ /100 µL per bird (n = 84) was 16.7 % for the whole experiment, and the ratio in the cloacal samples only (n = 42) was 33.3 %. The reisolation ratio among birds, infected with virus of a titer $10^{3,00}$ELD$_{50}$ /100 µL per bird (n = 84) was 2.4 % for the whole experiment (4.8% in the cloacal samples only - n = 42).

No viruses were re-isolated from the healthy ducks population.

The mean period of virus shedding was 8.43 days for cloacal samples, 4.38 days for oropharyngeal samples from birds, infected with virus of a titer $10^{5,00}$ELD$_{50}$ /100 µL per bird, 3.76 days for cloacal samples from birds, infected with virus of a titer$10^{4,0}$ ELD$_{50}$ /100 µL per bird and 2.00 days for cloacal samples from birds, infected with virus of a titer$10^{3,00}$ ELD$_{50}$ /100 µL per bird.

DISCUSSION

After an experimental low dose ($10^{2.00}$ ELD$_{50}$ /100 µL per birds) inoculation of ducks with H6N2 LPAIV, isolated from *Anas platyrhinchos*, the parameters measured indicated that no infection was detectable. Thus, induction of infection in ducks appears to require a level of virus administration greater than $10^{3.00}$ ELD$_{50}$ /100 µL per bird. When challenging with virus doses of $10^{4.00}$ ELD$_{50}$ /100 µL and $10^{5.00}$ ELD$_{50}$ /100 µL infection was demonstrated by virus re-isolation in all ducks. Dose importance for the infection was demonstrated also by the differences in re-isolate numbers and periods of re-isolation, with percentage odds in favour of the higher dose. Infectious doses between $10^{2.00}$ ELD$_{50}$ /100 µL per bird and $10^{3.00}$ ELD$_{50}$ /100 µL per bird were not used in this study; thus, the infectivity of this dose range remains unknown, but these findings suggest that LPAIV

concentration is critical for the establishment of a productive infection in a ducks.

The comparative data of other authors, who have isolated field strains and conducted similar experiments with LPAIV reveal differences in bird species sensitivities to infection. So LAUDERT *et al.* (1993) have not succeeded to infect chickens with H13 isolate from wild waterfowl. In our experiments [14] in chickens with the same isolate infections was established with a dose of $10^{3.16}$ ELD_{50} /100 µL per bird.

Infections of chickens with chicken isolate LPAIV H7N2 by LU & CASTRO (2004), turkeys with LPAIV H7N3 by CAPUA *et al.* (2004) and PILLAI et al. (2010) in experiment of ducks with dusks isolates have yielded minimal infection dose as our experiment with ducks. In all three experiments with virus doses of $10^{2.00}$ ELD_{50}/100 µL or $10^{2.30}$ ELD_{50}/100 µL per bird do not find any infection through re-isolation attempts.

For re-isolation sources cloaca and oropharynx are used, because LPAIV replicates in the respiratory and digestive systems. The specific localization of LPAIV strains is due to the presence of trypsin-like enzymes, necessary for the absorption and penetration of virus into target cells. Use of higher virus doses ($10^{5.00}$ ELD_{50} /100 µL per bird in our study, $10^{5.7}$ ELD_{50} /100 µL per bird in the study of LU & CASTRO (2004) and $10^{6.0}$ ELD_{50} /100 µL in the study of CAPUA *et_al.* (2004) and PILLAI et al. (2010) upon the number of birds, yielding re-isolates and upon re-isolation period. In all experiments with higher doses the number of reisolates is higher and for a longer period.

REFERENCES

1. Alexander, D.J., A review of avian influenza in different bird species, *Veterinary Microbiology* 74: 3-13, 2000.
2. Björn, O., Munster, V., A. Wallensten, A., J. Waldenström, J., Osterhaus, A., and Fouchier, R., Global patterns of influenza Avirus in wild birds. *Science*, 312: 384 – 388, 2006.
3. 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.
4. Manvell, R.., Jorgensen, P., O. Nielsen, O., and Alexander., D., Experimental assessment of the pathogenicity of two avian influenza A H5 viruses in ostrich chicken (Struthio camelus) and chickens. *Avian Pathology*, 27: 400 – 404, 1998.
5. 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.
6. Capua, I., Terregino, C., Cattoli, G., and Toffan, A., Increased of vaccinated turkeys to experimental infection with an H7N3 low-pathogenicity avian influenza virus. *Avian Pathology*, 33: 2, 158 – 163, 2004.
7. 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..
8. 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.
9. 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
10. Pillai, S.P.S., Pantin-Jackwood, M.., Suarez, D. L., Saif, Y.M.., and Lee, C.-W. , Pathobiological characterization of low-pathogenicity h5 avian influenza viruses of diverse origins in chickens, ducks and turkeys. *Archiv Virology*, 1- 13, 2010.
11. Zarkov, I., Manvell, R., Shell, W., and Bochev I.L., Isolation of avian influenza virus in Bulgaria. *Veterinary Record* 158, 3:106-107, 2006.
12. Anonymous, Avian Influenza, Manual of diagnostic procedures. Version adaptated. Chapter 2.7.12, 1-28. *Office International des Epizooties / OIE*, 2005.
13. Reed, L. J. and Muench, H.., A simple methods of estimating fifty percent endpoints. *Journal of Hygiene*, 27: 493-497, 1938.
14. Zarkov, I.., and Bochev, I., Influence of inoculation dose of avian H6N2 influenza A virus on virus shedding and humoral immune response of chickens after artificial experimental intravenous infection. *Revue Medicine Veterinare*, 159: 10, 489 - 493, 2008.