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Original Contribution

SHEDDING OF THE AVIAN INFLUENZA A H6N2 SUBTYPE VIRUS ISOLATE IN *NUMIDA MELEAGRIS*

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ABSTRACT
An experimental intravenous infection of 7 guinea fowl (Numididae meleagris) with Influenza A virus (AIV) isolate has been performed. The results indicated that over the entire period of observation (21 days) the virus was reisolated in all infected birds from the cloaca and the oropharynx. The maximum percentage of positive birds was observed by the 7th post infection day. Reisolation was established in 51.2 % of studied cloacal and 41.9 % oropharyngeal samples, with their number varying with time. The major part of samples was found on the 7th post infection day – 92.9 % of all tested samples. The average period of virus shedding from the cloaca was 5.4 days, and from the oropharynx - 4.6 days.

Key words: Avian influenza virus, H6N2 subtype, shedding, Numididae meleagris.

INTRODUCTION

From the 8600 known avian species, Influenza A virus (AIV) infection has been established in 106 species (1), with irregular distribution. Wild waterfowl are the principal vector of the disease. They spread the virus among domestic fowl (2). From domestic fowl species, chickens (*Gallus domesticus*), turkeys (*Meleagridis galopava*), ducks (*Anas spp*.), goose (*Anser spp*.) are most frequently affected – (3). AIV infecting birds are divided into 2 groups according to their pathogenicity – high-pathogenicity (HPAIV) and low-pathogenicity (LPAIV). LPAIV strains with H5, H7, H9 could mutate into highly pathogenic termed highly pathogenic notifiable avian influenza (HPNAI). Due to the highest number of strains, the infection in birds is more commonly with low pathogenic strains.

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It is established that LPAIV infection depends on the avian host species, as shown from experimental infection studies (4-10). Thus, in an experiment with an H13N2 isolated from gulls, (6) reisolated the virus from 51.4% of infected turkeys, 45.7 % of infected ducks, but did not isolate it from chickens. After experimental infection of turkeys and chickens with LPAIV H7N2, (7) were found more isolates among turkeys.

In birds experimentally infected with different LPAIV strains, the period of carriership and shedding varies within a broad range (4-7). In ducks, the carriership of the virus was in 8.3 % to 45.7% of birds, and the shedding continued for 4–7 days (6). The respective percentage in chickens was from 0% to 95.2%, and viral shedding lasts 5 to 14 days (11, 12), wild gooses carrying the virus are between 1% and 30% (13) until the 8th day (14).

In Bulgaria, LPAIV have been isolated in wild and domestic birds. Most commonly, isolates were of the H6N2 type (15-17).

In experimental studies with H6N2, the virus was reisolated in 100% of infected ducks until the 5th day after infection, and the shedding of the virus lasted until the 21st post infection day, in goose the reisolation was positive in 67% of infected birds until the 10th post infection day for a 10-day period, in turkeys – 56% until the 7th day for a 10-day period, and in chickens – in 33% until the 7th day for a 5-day period. The reisolation in all birds was performed from the cloaca and the oropharynx. The reisolation from the cloaca was more significant and more prolonged. In ducks, the reisolation was until the 21st day from the cloaca and until the 10th day from the oropharynx, in goose – respectively until post infection days 10 and 5; in turkeys – until days 10 and 7 and in chickens – until days 5 and 3 (8, 9).

Guinea fowls (*Numididae*) are a bird family from the order *Galliformes*, which also includes chickens, turkeys, pheasants, partridges and other AIV-sensitive representatives. Guinea fowl are encountered in a wild state in Africa and domesticated – on a global scale (18). From the known 6 guinea fowl species, the common guinea fowl (*Numida meleagris*) is most widely distributed.

Guinea fowl reared under domestic conditions are most commonly in places with close contact with other bird species. The first literature data for spontaneous infections of guinea fowl with AIV date back to 2000 (3), showing that they could be infected from other birds and from their part, is a source of infection for other birds. All data for infection of guinea fowl available so far are related to HPAIV and HPNAI.

The high extent of spread of HPAIV H5N1 among birds has led to establishing infection in guinea fowl as well (19). In Kenya, guinea fowl isolates are available in H5N1 outbreaks (20). In Italy, an emerging infection with HPAIV H7N7 has affected 7 bird species – chickens, turkeys, emus, goose, ducks, pelicans and guinea fowl (21). The most recent data justify the performance of a monitoring survey for the presence of AIV in birds reared in domestic conditions, including guinea fowl (22), and from some of them, a H9N2 strain was isolated.

In an experiment with HPAIV H5N1 in guinea fowl, all birds died within 2 to 5 days (23).

From literature data, no information about guinea fowl infection with LPAIV is available. After the acknowledged considerable occurrence of the H6N2 strain in Bulgaria, infection of guinea fowl is quite possible. Therefore, our aim was to investigate the possibility for infection of guinea fowl with a H6N2 AIV isolate and to follow out the periods of virus carriership and shedding.

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) . 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). Samples with haemagglutin titres of 1:128 were stored at -84°C until used in the experiment (24).

BIRDS AND PROTOCOL DESIGN

Seven one yare birds 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 = 2). 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 antibiotics 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 and 21 post infection (P.I.). Consequently 68 samples were obtained from infected birds and 38 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.

Reisolation was successful from both cloacal and oropharyngeal samples of infected guinea fowl **(Figure 1).**

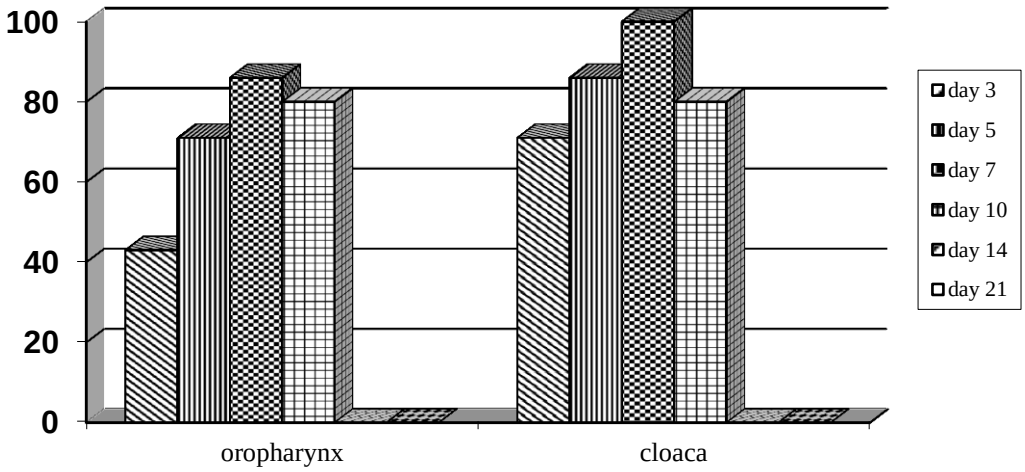

Figure 1. Cumulative percentages of positive cloacal and oropharyngeal samples from guinea fowl infected with Influenza A viral strain H6N2 by days

Infected guinea fowl were virus-positive until the 10th post infection dat. Reisolation occurred in 51.2% (22 birds) of tested cloacal and 41.9% (18 birds) from oropharyngeal samples. The total number of reisolates varied with time. It increased until the 7th day up to 92.9 % of tested samples and by the 10th showed a slight reduction up to 80%. Equal number of cloacal and oropharyngeal reisolates were found on the 5th and 10th day after the challenge. On the other sampling days, more reisolates were observed in cloacal samples. Maximum positive result was obtained only from cloacal samples by the 7th post infection day. From both sites, reisolation was established on all studied days with positive result. The number increased until the 7th day with maximum result of 6 positive out of 7 tested birds. For the cloaca only, positive results were detected on all intervals of the study. They were most numerous on the 3rd post infection day – 3 birds. Afterwards, they persisted on the same level (1 infected bird) until the end of the experiment. Reisolates from the oropharynx only were established on day 10 – 2 birds, and on days 3 and 5 – 1 bird.

The percentage of positive birds attained maximum results (100% positive) on the 7th day after the challenge, and was preserved until the 10th day **(Figure 2)**. In the preceding periods (days 3 and 5), the percentage was 86 %. The bird with conditional number 2 was positive on the 7th day after infection.

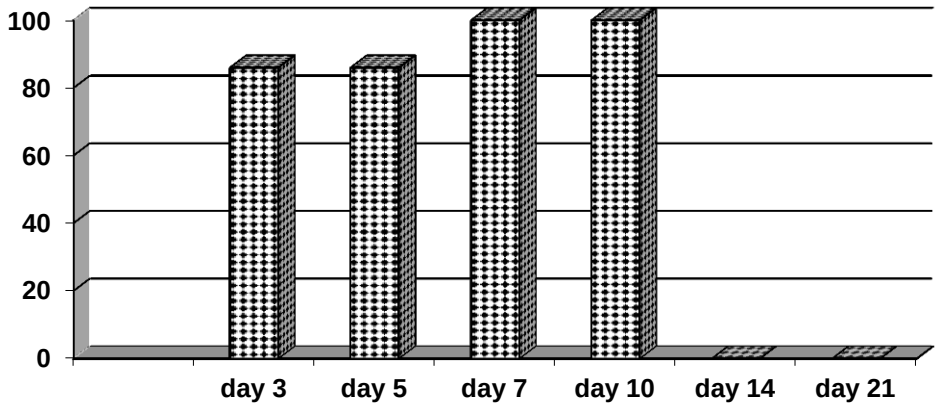

Figure 2. Percentage of positive guinea fowl infected with Influenza A virus strain H6N2

Cumulative data for virus shedding periods from cloacal and oropharyngeal samples of guinea fowl are shown in **Table 1.** The average period of virus shedding from cloacal samples was 5.4 days, and from oropharyngeal – 4.6 days

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Table 1. *Period of shedding of the virus from cloacal and oropharyngeal samples from guinea fowl experimentally infected with Influenza A virus H6N2 strain*

Shedding period (days)														
Birds	from the cloaca							From the oropharynx						
Days→	33	55	7	110	114	221	**Average period**	33	55	7	110	114	221	**Average period**
Numida meleagris	55	66	77	44	00	00	**5.4**	33	55	66	44	00	00	**4.6**

DISCUSSION

In the present study, the LPAIV H6N2 subtype virus was successfully reisolated from previously intravenously infected birds of species *Numida meleagris*. The results showed that all birds were positive, and they were comparable to data obtained in ducks from the *Anas plathyrynchos*species. The experimental data in guinea fowl differed from results in goose (67 % positive), turkeys (56% positive) and chickens (33% positive) – (8, 9). These data show that ducklings (the natural host) and *Numida meleagris* are much more sensitive to the virus than the 3 other avian species whereas chickens are much more resistant.

Regardless of the percent of positive birds after infection with LPAIV H6N2 subtype virus, the results for virus carriership periods were different. The maximal length of the virus re-isolation period was shorter in the recipient species (5 days in chickens and 10 days in goslings, turkey poults and Numida meleagris) than in the original host species (until 21 days in ducklings). It reflected upon the the mean virus shedding periods - 10.6, 7.0, 5.4 and 4.3 days in ducklings, turkey poults, goslings and Numida meleagris and chickens respectively. 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 parallel, the mean virus shedding period was greater in the cloacae, than in the oropharynx as experiments with ducklings, turkey poults, goslings and chickens.

These results were in accordance with previous studies (25, 4), although some researchers observed a longer persistence of the virus (7 days) and a higher titre in oropharyngeal samples (7). 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) (26) 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 5 studied bird species, indicates that it is more appropriate to investigate cloacal samples for virus detection through isolation in spontaneous cases of avian influenza.

CONCLUSIONS

An experimental infection with isolate of Influenza A virus (AIV) has been performed on 7 guinea fowls (Numididae meleagris). On the seventh day of contamination all birds have been with infection. Reisolation was established in 51.2 % of studied cloacal and 41.9 % oropharyngeal samples.

The average period of virus shedding from the cloaca was 5.4 days, and from the oropharynx - 4.6 days.

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