



Original Contribution

SURVIVAL OF AVIAN INFLUENZA VIRUS H6N2 IN FAECAL SAMPLES OF CHICKENS EXPERIMENTALLY INFECTED

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ABSTRACT

The persistence of infectivity of avian influenza virus (AIV) subtype H6N2 with initial titre $10^{4.00}$ EID₅₀/100 µl was investigated in faeces of experimentally infected separately reared chickens under conditions of preserved humidity, ambient temperatures of 4 °C, 15 °C, 22 °C and pH 7.6.

At the temperature of 15 °C and constant humidity, infectious virus was detected until the 6 day but not after a repeated inoculation on day 9. Virus titre reduction rate was slower until day 2 – by $10^{0.5}$ EID₅₀/100 µl and faster between days 2 and 4 (by $10^{1.17}$ EID₅₀/100 µl); then slowed down between days 4 and 6 – by $10^{0.83}$ EID₅₀/100 µl and after the 6th day – the reduction was by $10^{1.55}$ EID₅₀/100 µl until zero titre by the 9 day.

At 4 °C and preserved humidity, the virus persisted until the 25 day with a titre of $10^{0.5}$ EID₅₀/100 µl. Reduction of titres occurred with less than $10^{0.1-0.26}$ EID₅₀/100 µl by day 2 and 12, with more than $10^{0.36-1.47}$ EID₅₀/100 µl between days 12 and 25 until zero titres by day 30.

At 22 °C and preserved humidity the virus was detected up to the 2nd day with a titre of $10^{1.0}$ EID₅₀/100 µl. Between days 1 and 2 titres declined by $10^{1.5}$ EID₅₀/100 µl until zero titres by day 4.

Key words: chickens, faeces, experiment, avian influenza A virus, virus re-isolation

INTRODUCTION

An important part of infection of new susceptible host is the time that infective avian influenza viruses could survive outside the host in faeces and nasal secretion. The time of survival of viruses shed with excreta depends on the environmental conditions, and particularly on ambient temperature and humidity.

Some assessments carried out to determine the period of time when viruses preserve their infective properties lack enough information about the environmental conditions. For

instance, Fitchner (1986) (1) affirms that during the Pennsylvania outbreak of a highly pathogenic avian influenza in 1983-85, caused by a H5N2 virus in chickens, the agent was detected in wet manure under field conditions after 105 days. Utterback (1984) (2) concluded that the virus could be detected in concentrations up to $10^{7.0}$ EID₅₀/g and could survive for more than 44 days, but in both studies, the changes in ambient humidity and temperature were not reported although it could be assumed that variations were present.

The effect of ambient temperature upon the persistence of an infectious virus was documented at a better extent.

In faeces of ducks, experimentally inoculated with H3N6, the virus maintained infectious titres for 14 days at 0 °C, but a significant

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decline of titres was observed over the next 14 days. The viruses retain infectivity in faecal material for at least 30 days at 4 °C and for 7 days at 20 °C (3). In wet manure, the virus (H5N1) persists 4-6 days at 37 °C, 7 days at 25 °C, and more than 20 days at 4 °C (4).

Experiments mixing faeces with chick embryo-cultivated virus were conducted by Songersam et al., 2005 (5); Chumpolbanchorn et al., 2006 (6); Jawad, 2011 (7). Added to sterile chicken faeces, the H5N1 virus ($2.38 \times 10^{5.25}$ EID₅₀/g) is inactivated for 15 min at 40 °C and does not persist after 1 day at 25 °C (6). The same virus persists for up to 10 days at room temperature in fresh chicken faeces (5). Jawad, 2011 provided evidence for a different term of persistence in different avian influenza viral strains (H4N6, H5N1, H6N8) with various initial titres ($10^{4.14}$ TCID₅₀/100 µl; $10^{5.00}$ TCID₅₀/100µl; $10^{5.32}$ TCID₅₀/ 100µl) at different temperatures (30 °C, 20 °C, 10 °C, 0 °C and -10 °C) in closed flasks (preserved humidity). It was concluded that at high temperatures, viruses persisted for 2 days, at 20 °C for 4–7 days, at 10 °C for weeks (2-3), and at 0 °C and -10 °C – for months.

Beard et al., 1984 (8) performed experiments with faeces from H5N2 AIV-infected chickens placed at 4 °C and established preserved infectiveness for 30 - 35 days, while at 20 °C the infective properties were preserved for only 7 days. In SPF H7N2-infected chickens ($10^{7.00}$ EID₅₀/g), Lu et al., 2003 (9) demonstrated a difference in the survival of infectious virus in faeces at various temperatures (56 °C, 37 °C, 28-30 °C, 15-20 °C and 4 °C). The authors reported various terms of persistence in faeces of infected SPF birds, in birds from poultry farms, birds reared in BSL-2 biocontainment facility, from commercial birds and in non-inactivated and inactivated faecal samples. In non-inactivated faeces at ambient temperature of 15–20 °C the virus is preserved for up to 19 days in SPF птици, 4 days in birds from BSL-2 biocontainment facility and for less than 2 days in commercial farm samples. At 4 °C, the survival duration was over 23 days and over 20 days, respectively.

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 platyrhynchos* was used (10). Allantoic fluid was collected after inoculation of LPAIVA (H6N2 subtype) into the allantoic sac (100 µl) of 3-9-day old chickens embryos (CE). Embryos were observed daily for 96 hours (when all were dead). Allantoic fluid (ALF) derived from them was explored by the haemagglutination test (HA) (11), and stored at –85 °C until titration of virus in ALF and use in the experiment.

Titration of virus in ALF, was prepared after dilution of ALF from $10^{-0.5}$ to $10^{-6.0}$ in Minimal Essential Medium (MEM) and 100 µl of each dilution was inoculated into the allantoic cavity of 6 -9-days old CE. The calculation of EID₅₀/100 µl was accomplished according to the method of Reed & Muench, 1938 (12).

2. Birds, faecal material, virus re-isolation and titration.

Four one -year -old chickens separately kept were used in experiment. Birds were intravenously infected with 100 µl allantoic fluid with a virus titre of $10^{5.25}$ EID₅₀/ 100 µl per bird (13). Faecal material received by the 3 day after inoculation of birds was employed in the experiment after preliminary testing with a commercial immunoassay for rapid detection of influenza A antigen (Directigen Flu A antigen, BD).

In the experiment faecal material (10 g), placed in three closed containers for keeping initial humidity, and at temperature 4 °C, 15 °C, 22 °C. Out of each faecal sample, 0.5 g were used for titration of virus at days 0, 1, 2, 4 in temperature 22 °C; at days 0, 1, 2, 4, 6, 9 in temperature 15 °C; at days 0, 1, 2, 4, 6, 9, 12, 16, 19, 22, 25, 30 in temperature 4 °C. A suspension of samples 1:5 ($10^{0.5}$ dilution) was prepared in MEM (pH 7.2-7.4), supplemented with Penicillin G (2.10^6 U/L), Streptomycin (200mg/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 (1000g, 4 °C for 10 minutes) the supernatant was diluted to $10^{6.0}$. From each

dilution 100 µl was inoculated into the allantoic sac of 6-9 day-old chicken embryos (CE). The inoculated 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. A haemagglutinating virus was

determined by the haemagglutination (HA) test (11).

RESULTS

In faeces spread onto a thin layer, infectious virus was not detected as early as the day that followed (Figure 1).

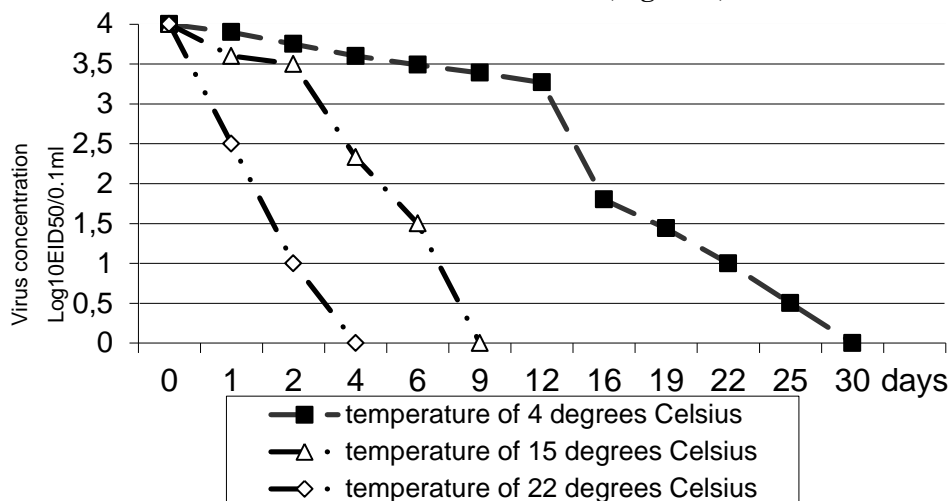


Figure 1. Virus titres of H6N2 avian influenza virus in faecal sample obtained on the 3 day after experimental infection of chickens at constant humidity at ambient temperatures of 4 °C, 15 °C and 22 °C.

Reduction of titres at constant humidity and 15 °C occurred at various rates, with least reduction between days 0 and 2 – from $10^{4.00}$ EID₅₀/100 µl to $10^{3.5}$ EID₅₀/100 µl (reduction by $10^{0.5}$ EID₅₀/100 µl). Between days 2 and 4, the reduction of titres was by $10^{1.17}$ EID₅₀/100 µl (up to $10^{2.74}$ EID₅₀/100 µl by the 4th day); between days 4 and 6 – by $10^{0.83}$ EID₅₀/100 µl (up to $10^{2.5}$ EID₅₀/100 µl by the 6th day) and after the 6th day – by $10^{1.5}$ EID₅₀/100 µl (zero values by the 9 day).

At 4 °C, the virus persisted until the 25th day with a titre of $10^{0.5}$ EID₅₀/100 µl. Reduction of titres with less than $10^{0.1 - 0.26}$ EID₅₀/100 µl occurred between days 2 to 12, and with more than $10^{0.36 - 1.47}$ EID₅₀/100 µl: between days 12 to 25 until zero titres by day 30.

At 22 °C the virus was detected up to the 2nd day ($10^{1.0}$ EID₅₀/100 µl) and it was not present by the 4th day. Between days 0 and 1, and days 1 and 2 titres declined by $10^{1.5}$ EID₅₀/100 µl.

DISCUSSION

It is acknowledged that the primary site of virus replication in poultry is the gastrointestinal tract

[10, 11], where the shedding of the virus into the environment begins from. In the infected chickens re-isolations of virus were achieved in 33% of the infected chickens in the first 3 days after experimental inoculation. Re-isolates constituted 4% of all tested samples (13). An infected bird releases a large amount of faecal mass per day and large amounts of virus in them. These amounts together with the high virus titre predetermine the virus-contaminated faeces as a main source of transmission to susceptible birds (8).

The period of virus shedding should be also considered. Other studies of ours with the same virus have shown that chickens experimentally infected with the isolate shed the virus up to 5 days (13). It now became clear that the titre of the virus shed by one birds was $10^{4.00}$ EID₅₀/100 µ evidencing the large amount of virus excreted by a single infected bird.

The next important issue is where are the faeces excreted (in the water or on the ground) and which factors would have an impact for preserving virus infectivity. It was shown that the same strain, with lower initial titre ($10^{1.63}$

EID₅₀/100 µl) shed in surface waters, was mainly influenced by pH and salt concentrations. Under optimal pH, different concentration of salts and presence of microflora, the infectivity of the virus is preserved for 3 - 12 days (14).

In support of data reported by others, our experiments confirmed that low ambient temperatures (winter months) resulted in more prolonged persistence of the virus. Regardless of this general finding, there were significant differences in individual survival times in our trials and reported studies, which could be attributed to different experimental design, the different used viral strains and the initial titres. For instance, at 4 °C, other researchers (3 - 4, 8 - 9) using different viral strains, established close to our results an infectious virus (20 - 35 days). At higher temperatures (15-20 °C and 10 °C), similar to our value of 15 °C, the studies of Lu et al., 2003 and Jawad, 2011 the persistence varied from less than 2 days (faeces from commercial chickens and non-inactivated samples) to 2-3 weeks (Jawad, 2011), which were very different in both directions from our data (6 days). At 20 °C - 25 °C (in our trial, 22 °C) the persistence of 2 days was equal to that reported by us and Beard, 1984, but 2-3 times lower than the data of Jawad, 2011 (4-7 days) and Webster, 1978 (7 days).

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