Effects of humidity and temperature on avian influenza virus H6N2 persistence in faecal samples from experimentally infected ducks (Anas platyrynchos)

I. S. ZARKOV*, V. S. URUMOVA

Department of Microbiology, Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, Trakia University of Stara Zagora, Students Campus, 6000, Stara Zagora, BULGARIA

*Corresponding author: ivanzarkov@yahoo.com

SUMMARY
The persistence of infectivity of avian influenza virus (AIV) subtype H6N2 was investigated in faeces collected 5 days after experimental infection (10^1-2 EID50/100 μL) of 4 ducks (Anas platyrhynchos) according to the humidity of the samples and to the ambient temperature. For that, the weights of the ball-shaped and thin-layer faeces (0.2 cm thickness) were determined for 3 days and the bacterial microflora and the virus titres using haemagglutination test on allantoic fluids from 6-9 days old chicken embryos inoculated with faecal samples were measured in parallel. In a second step, the H6N2 survival for 18 days according to the ambient temperatures (4°C, 15°C and 22°C) was determined in faecal samples keeping at constant humidity. Whereas the microflora was preserved, the virus was no more detected in thin layer faeces after a 24 hour drying (corresponding to a reduction weight of 72%) whereas virus titres were still around 10^5 EID50/100 μL in ball shaped faeces (with a reduction weight of 42.7%). When faecal humidity remained constant, the H6N2 virus persisted until 14, 6 and 2 days at 4°C, 15°C and 22°C, respectively. These results suggest that in natural conditions, the persistence of the avian influenza A virus H6N2 in the environment may be promoted by humidity and low temperatures as in winter months.

Keywords: ducks, feces, avian influenza A virus H6N2, virus re-isolation, virus titre, humidity, ambient temperature.

RESUME
Effets de l'humidité et de la température sur la persistance du virus de la grippe aviaire H6N2 dans les féces de canards (Anas platyrhynchos) expérimentalement infectés

La persistance du caractère infectieux du virus de la grippe aviaire H6N2 a été évaluée dans les fèces récoltées 5 jours après inoculation (10^1-2 EID50/100 μL) de 4 canards (Anas platyrhynchos) en fonction de l'humidité des échantillons et de la température ambiante. Pour cela, les poids d'échantillons fécaux en boule ou étalés sur une fine épaisseur (0.2cm d'épaisseur) ont été mesurés en parallèle. Dans une seconde étape, la survie du virus H6N2 en fonction de la température ambiante (4°C, 15°C et 22°C) a été déterminée sur une durée de 18 jours dans des échantillons fécaux maintenus à une humidité constante. Le virus n'a plus été détecté dans les échantillons fécaux étalés en couche mince au bout de 24 heures (correspondent à une dessiccation de 72%) alors que les titres viraux étaient encore de l'ordre de 10^5 EID50/100 μL dans les échantillons en boule (présentant une dessiccation de 42.7%). Lorsque l'humidité des échantillons est restée constante, le virus H6N2 a persisté dans les fèces pendant 14 jours à 4°C, 6 jours à 15°C et 2 jours à 22°C. Ces résultats suggèrent que dans les conditions naturelles, l'humidité et de faibles températures comparables à celles observées en hiver favoriseraient la persistance du virus de la grippe aviaire H6N2 dans le milieu extérieur.

Mots-clés : canards, féces, virus A de la grippe aviaire H6N2, ré-isolation virale, titre viral, humidité, température ambiante.

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 [4]. The time of survival of viruses shed with excreta depends on the environmental conditions, and particularly on ambient temperature and humidity [4].

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 [5] affirms that during the Pennsylvania outbreak of a highly pathogenic avian influenza in 1983-1985, caused by a H5N2 virus, the agent was detected in wet manure under field conditions after 105 days. UTTERBACK [11] concluded that the virus could be detected in concentrations up to 10^7 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 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 [12]. 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 [13].

Experiments mixing faeces with chick embryo-cultivated virus were conducted by SONGERSAM et al. [10] and CHUMPOLBANCHORN et al. [4]. Added to sterile chicken faeces, the H5N1 virus (2.38x10⁵⁵ EID₅₀/g) is inactivated for 15 minutes at 40°C and does not persist after 1 day at 25°C [4]. The same virus persists for up to 10 days at room temperature in fresh chicken faeces [10].

BEARD et al. [3] 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 (Specific Pathogen Free) H7N2-infected chickens (10⁻⁰⁷ EID₅₀/g), LU et al. [6] 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 degrees of persistence in faeces of infected SPF birds, in birds from poultry farms, birds reared in BSL-2 (Biosafety Level 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 birds, 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.

Virus-mixed fresh faeces placed on eggshells and egg trays and dried, did not exhibit an infectious virus after 24 hours [4]. The present study aims to estimate the effects of temperature and humidity on persistence of avian influenza virus isolate H6N2 in faecal samples obtained from experimentally infected ducks (Anas platyrhynchos).

Material and methods

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 [14]. Allantoic fluid was collected after inoculation of LPAIV (H6N2 subtype) into the allantoic sac (100 µL) of 3-9-day old chicken 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) [1], 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 10⁻⁰⁶ to 10⁻⁷ 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 (50% embryo infections dose)/100 µL was accomplished according to the method of REED and MUENCH [7].

BIRDS, FAECAL MATERIAL, VIRUS RE-ISOLATION AND TITRATION

Four 1 year old ducks (Anas platyrhynchos) separately kept were used in the experiment. Birds were intravenously infected with 100 µL allantoic fluid with a virus titre of 10⁻¹⁵ EID₅₀/100 µL per bird [16]. Faecal material obtained at the 5th 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 FlA antigen, BD) [15]. Homogeneous combined faecal material from all birds (17.68 g, pH: 7.8) was investigated for presence of bacterial cells and for E. coli. Ten-fold dilutions from 10⁻¹ to 10⁻¹⁰ were prepared, and 1 mL of each dilution was inoculated on Mac Conkey agar (Difco,UK), Trypticase soy agar (Difco, UK), and on blood agar NCIPD, Sofia). After incubation at 37°C, plates inoculated with a sample dilution yielding between 30 and 300 colonies were read. Colony counts were determined as the average of the counts on two plates inoculated with the selected dilution.

The first experiment was conducted with faecal material (9.45 g), placed in open Petri dishes, at controlled of total humidity and temperature 15°C, after weighing the sample after 0, 1, 2 and 3 days. This temperature and humidity of about 60% were very close to the temperature and humidity in spring and autumn seasons, in Bulgaria.

Faecal samples were divided in two parts, one was ball-shaped and the other part was spread in a thin layer with a height of 0.2 cm. Out of each faecal sample, 0.5 g were used for titration of virus on days 0, 1 and 2. A suspension of samples 1:5 (10⁻⁵ dilution) was prepared in MEM (pH 7.2-7.4), supplemented with Penicillin G (2.10³ U/L), Streptomycin (200 mg/L), Polymyxin B (2.10³ U/L), Gentamicin sulfate (250 ml/L), Nystatin dehydrate (0.5x 10⁻³ 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. From each dilution 100 µL was inoculated into the allantoic sac of 6-9 days 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 [1].

In the second experiment the same procedure was followed, but the faecal material was placed in three closed containers for keeping initial humidity, and at 3 temperatures (4°C, 15°C and 22°C). Virus titres were determined on days 0, 1, 2, 4, 6, 8, 10, 14 and 18.

Statistical analysis

Results were submitted to ANOVA (One Way Analysis of Variance, GraphPad InStat3). Differences were considered statistically significant at p ≤ 0.05 level.
Results

In studied faecal samples, the presence of conventional bacterial microflora (total bacterial counts $0.2 \times 10^{10}$/g and enterobacteriae $0.7 \times 10^9$/g) was detected.

The initial sample weight in the first experiment with ball-shaped faeces was 4.95 g (100 %). After one-day stay, the weight decreased to 57.3% of the initial weight representing a reduction by 42.7%. After 2 days, the resultant weight was 30% corresponding to reductions by 70% and 49.5% compared to the days 0 and 1, respectively. At the 3rd day, the faecal weight continued to decline, reaching 20.7% (the corresponding reductions were 79.3%, 64% and 28.7% compared to the days 0, 1 and 2, respectively) (figure 1). The faeces spread in thin layer (0.2 cm thickness) showed a reduction of the weight up to 28% of the initial value as early as the following day and appeared as a thin crust. The reduction by the 2nd and 3rd days was up to 19% and 14% of the initial weight, respectively (figure 1).

Lack of infectious virus was established at the 2nd day in ball-shaped faeces. In this sample type, virus titres rapidly declined after one-day stay from an initial value of $10^{4.00}$ EID$_{50}$/100 μL to $10^{0.75}$ EID$_{50}$/100 μL (reduction by $10^{3.25}$ EID$_{50}$/100 μL). In faeces samples spread onto a thin layer, virus titres have declined more rapidly, no infectious particle being detected on day 1 (figure 2). Changes in faecal weights and virus titres according to the time between the samples with ball-shape and samples spread in thin layer were highly significant ($p < 0.01$).

At a constant humidity (experiment 2, figure 3), the H6N2 virus titres abruptly declined at 22°C and virus was no more detected since the 4th day: the reduction rate was approximately linear during the first 2 days by $10^{0.5}$ EID$_{50}$/100 μL/day, then (between the days 2 and 4), the virus titres decreased more slowly (reduction rate: $10^{0.5}$ EID$_{50}$/100 μL/day). At 15°C, the virus titres slowly declined for the first 2 days (reduction rate: $10^{0.25}$ EID$_{50}$/100 μL/day) whereas the loss in virus particles increased during the 4 following days (the reduction rate calculated between the 2nd and the 6th days was $10^{0.5}$ EID$_{50}$/100 μL/day) and the maximal decrease in the H6N2 titres was obtained between the 6th and the 8th days (reduction rate: $10^{0.75}$ EID$_{50}$/100 μL/day). On day 8 no virus was detected in faecal samples at 15°C. At 4°C, the virus persisted until the 14th day with a titre of $10^{0.5}$ EID$_{50}$/100 μL. Reduction of virus titres occurred by levels (0-2 days; 4-6 days and 10-14 days) and the reduction rate between 2 levels has never exceeded $10^{0.5}$ EID$_{50}$/100 μL/day. The virus was not detected in faecal samples only on day 18. Extremely

![Figure 1: Changes in the weight according to time of combined faecal samples obtained on the 5th day after experimental infection of 4 ducks (Anas plathyrynchos) with avian influenza virus H6N2 (105.25 EID50/100 μL) at ambient temperature of 15°C, air humidity 60%, preserved microflora, naturally reduced humidity of ball-shaped and thin-layer faeces (0.2 cm thickness).](image1)

![Figure 2: Changes in the H6N2 virus titres according to time of combined faecal samples obtained on the 5th day after experimental infection of 4 ducks (Anas plathyrynchos) with avian influenza virus H6N2 (105.25 EID50/100 μL) at ambient temperature of 15°C, air humidity 60%, preserved microflora, naturally reduced humidity of ball-shaped and thin-layer faeces (0.2 cm thickness).](image2)

![Figure 3: H6N2 virus titres in combined faecal samples obtained on the 5th day after experimental infection of 4 ducks (Anas plathyrynchos) with avian influenza virus H6N2 (105.25 EID50/100 μL) at constant humidity and preserved microflora according to the ambient temperatures of 4°C, 15°C and 22°C.](image3)
significant differences in virus titres were noticed (p < 0.001) according to the time between the samples which were kept at different temperature -4 °C, 15° C, 22 °C in closed containers.

Discussion

It is known that the primary site of virus replication in ducks is the gastrointestinal tract [8, 9], where the shedding of the virus into the environment begins from the infected Muscovy duck excretes 6.4 g faecal mass per hour with virus concentrations attaining 10⁷.8 EID₅₀/g. Over 24 hours, one duck excretes 153.6 g faecal mass (approximately 10¹⁰ EID₅₀/g virus) [12]. These amounts together with high virus titres predetermine the virus-contaminated faeces as a main source of transmission to susceptible birds [3]. The period of virus shedding should be also considered.

Other studies with the same virus have shown that ducks experimentally infected with the same isolate shed the virus up to 21 days (in average 10.6 days for the group of infected ducks) [16]. It is now clear that the titre of the virus shed by one bird was 10⁴.00 EID₅₀/100 µL 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.

In this study, these investigations were extended to the circumstances that native faeces excreted by ducks could fall onto a solid ground and for elucidating the effect of some principal factors as drying and seasonal average ambient temperature within temperate climate conditions upon the survival of the infectious virus. In agreement with previous reports [3, 5, 6, 10], the present experiments confirmed that low ambient temperatures as in winter months resulted in more prolonged persistence of the virus in the faeces. Regardless of this general finding, there were significant differences in individual survival times between the present trials and other reported studies [2, 3, 5, 6, 12, 13], which could be attributed to different experimental designs, the different used viral strains and the initial titres. For instance, a longer survival of infectious virus particles (around 20-35 days) at 4°C of different viral strains than 14 days in the present study was established by others [3, 6, 12, 13]. At higher temperatures (10°C and 15-20°C), the virus persistence varied from less than 2 days in faeces from commercial chickens and non-inactivated samples [6], whereas in the present study, the H6N2 virus was detected until the 6th day in faecal samples at 15°C. When the ambient temperature was comprised between 20-25°C, BEARD [2] also observed a viral persistence in faecal samples which did not exceed 2 days as in the present study but the WHO [13] recorded longer viral survival (4-7 days).

SONGSERM et al. [10] failed to detect H5N1 strain virus after drying for 24 hours at 20°C in faecal samples with initial titre of 10².26-3.52 EID₅₀/g. However, for the first time, the persistence of virus infectivity have been investigated after drying (reduction of humidity) depending on the thickness of faecal masses and reduction of their weight: the present study clearly demonstrate that at a constant higher humidity in the core of ball-shaped faecal samples than in thin-layer faeces (0.2 cm thickness), leading to a reduction in weight of 42.7% (ball-shaped faeces) instead of 72% (thin-layer faeces), the virus could be still detected after a one-day stay albeit a substantially reduction in titre. When faeces were spread on thin layer (allowing a rapid drying), the virus infectivity was rapidly lost.

Having in mind that ducks dwell water basins and areas adjacent to water basins (i.e. with higher humidity), it could be suggested that faeces excreted on the shore preserved the infectious virus for a longer period than the rapid drying reproduced in our study. Viruses could be additionally protected by the crust formed on the surface of ball-shaped faeces, allowing a longer maintenance of moisture in their core.

References