Influence of inoculation dose of avian H6N2 influenza A virus on virus shedding and humoral immune response of chickens after artificial experimental intravenous infection

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SUMMARY

Eighteen 30-days old chickens, divided into 3 groups (No 1, 2 and 3) of 6 birds were infected intravenously with LPAIV H6N2, isolated from wild ducks (Anas platyrhynchos). A control group (No 4) of 6 chickens was not infected. The infection was accomplished with different virus doses – group No 1 with $10^{5.25} \text{ELD}_{50} / 100 \mu L$ per bird, No 2 – with $10^{4.25} \text{ELD}_{50} / 100 \mu L$ per bird, No 3 – with $10^{3.00} \text{ELD}_{50} / 100 \mu L$ per bird. In all groups the percent of infected birds, period of virus shedding and presence of humoral immune response by HI test were monitored.

The virus reisolation showed that infection was established in chickens, infected with dose of $10^{5.25} \text{ELD}_{50} / 100 \mu L$ per bird and $10^{4.25} \text{ELD}_{50} / 100 \mu L$ per bird. More birds with virus reisolation were detected when inoculation was made with the higher virus dose (33.33% versus 16.67%), with higher number of isolates (6% versus 1.2%). Antibodies were detected in 100% of the birds, infected with a dose of $10^{5.25} \text{ELD}_{50} / 100 \mu L$ per bird and in 16.67% of the birds, infected with a dose of $10^{4.25} \text{ELD}_{50} / 100 \mu L$ per bird. Antibody titers of birds with virus reisolation varied within 8 log$_2$ - 11 log$_2$, and those of the birds without virus shedding (only the ones, infected with a dose of $10^{5.25} \text{ELD}_{50} / 100 \mu L$ per bird) – within 4 log$_2$ - 5 log$_2$, with later appearance of antibodies (since day 14 PI).

Keywords: Chickens, experiment, low pathogenic avian influenza A virus, dose, virus reisolation, antibodies.

Influence of the dose of inoculation of the virus H6N2 A de la grippe aviaire sur le dépouillement du virus et réponse immune humorale des poulets après une infection généralisée artificielle

Dix-huit poulets de 30 jours d’âge divisés en 3 groupes (No 1, 2 et 3) de 6 oiseaux ont été infectés par voie intraveineuse avec LPAIV H6N2 isolé de canards sauvages (Anas platyrhynchos). Un groupe contrôle (No 4) de 6 poulets n’a pas été infecté. L’infection a été effectuée par des doses de virus différentes – groupe No 1 par $10^{5.25} \text{ELD}_{50} / 100 \mu L$ par oiseau, No 2 – par $10^{4.25} \text{ELD}_{50} / 100 \mu L$ par oiseau, No 3 – par $10^{3.00} \text{ELD}_{50} / 100 \mu L$ par oiseau. Dans chaque groupe le pourcentage d’oiseaux infectés, la période du dépouillement du virus et la présence d’une réponse immune humorale étaient surveillés par un test HI.

Le réisolement viral a montré que l’infection avait été établie dans les poulets infectés avec les doses de $10^{5.25} \text{ELD}_{50} / 100 \mu L$ par oiseau et $10^{4.25} \text{ELD}_{50} / 100 \mu L$ par oiseau. Un plus grand nombre de réisolement viral a été possible quand l’inoculation a été effectuée par la dose virale plus haute (33.33 % versus 16.67 %), et un plus grand nombre d’isolats ont été obtenus (6 % versus 1.2 %). Des anticorps ont été détectés dans 100 % des oiseaux infectés par la dose virale de $10^{5.25} \text{ELD}_{50} / 100 \mu L$ par oiseau et dans 16.67 % des oiseaux infectés par la dose de $10^{4.25} \text{ELD}_{50} / 100 \mu L$ par oiseau. Les titres d’anticorps des oiseaux avec réisolement viral variaient entre 8 log$_2$ - 11 log$_2$, et ceux des oiseaux sans dépouillement viral (seulement les oiseaux infectés par la dose de $10^{5.25} \text{ELD}_{50} / 100 \mu L$ par oiseau) – entre 4 log$_2$ - 5 log$_2$, avec une apparition d’anticorps plus tardive (après le jour 14 PI).

Mots-clés : Poulets, expérimentation, virus A de la grippe aviaire faiblement pathogène, dose, réisolement viral, anticorps.

Introduction

Avian influenza A viruses have been isolated from 105 bird species, belonging to 12 orders [3]. Birds have different susceptibility to these viruses. It is most often detected after experimental infection of domestic birds with low pathogenic strains of avian influenza A viruses (LPAIV) [2, 10, 17]. The most often measured infection features are the percent of infected birds, period of virus shedding and the presence of subtype specific antibodies against haemagglutinin [4, 6]. The percent of infected chickens varies from 0% [5], 4% - 43% [16] up to 80% - 100% [4, 6, 7, 13]. The period of virus shedding lasts 5-7 days [9, 11, 14, 15, 16], 12 - 14 days [7, 8] or 28 - 31 days [13], with most intensive shedding in the first week. In some cases the shedding from the oropharynx
lasts longer than the cloacal [13, 16], in other it’s equal to the cloacal [15], in still other cases it is shorter than it [7, 8, 11].

LU & CASTRO (2004) have studied the influence of virus dose on the infection in chickens and CAPUA et al. (2004) in turkeys. The authors have studied the presence of virus in infected birds, its shedding and the immune response after experimental infections. LU & CASTRO (2004) detect no shedding when chickens are inoculated with 10^{0.7-2.0} ELD_{50} (mean embryo lethal doses causing a 50% death rate in inoculated chicken embryos) of virus per bird. At this dose level, the immune response was not detected by the hemagglutination-inhibition (HI) test. Chickens were successfully infected with 10^{4.7-5.7} ELD_{50}/500 µL per bird and were shedding virus up to 2 wk post inoculation (PI). In these challenged birds the antibody levels as measured by the HI test spiked within 1 - 2 wk. In the turkey experiment an 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 ratio of reisolations (3.3%) and low titers of antibodies (up to 6 log2). More isolates (26.6%) were obtained at virus dose of 10^{6.0} ELD_{50}/100 µL per bird and the antibody titers reached 8 log2.

The aim of this study was through experimental infection of chickens to determine the percent of infected birds, the period of reisolation, the immune response by HI test and the connection of these parameters with the inoculation dose of the H6N2 virus, isolated from a wild duck of the Anas platyrhynchos species.

Materials 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 [18]. Allantoic fluid was collected after inoculation of LPAIV (H6N2 subtype) into the allantoic sac of five 9-day old chicken embryos (CE). Embryos were observed daily for 120 hours PI (when all were dead). Allantoic fluid derived from them was explored by haemagglutination test (HA) [1]. Samples with haemagglutinin titres of 7 log_{2} were stored at – 80 °C until their dilution, titration and use in the experiment.

For titration, we prepared 3 initial dilutions of viral suspensions (1:10, 1:100 and 1:1000) in Minimal Essential Medium (MEM). Every suspension was diluted from 10^{-1} to 10^{-10} in MEM. A 100 µL aliquot 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^{5.25} ELD_{50}/100 µL, 10^{4.25} ELD_{50}/100 µL and 10^{3.00} ELD_{50}/100 µL.

BIRDS AND PROTOCOL DESIGN

A total of 24 30-day old chickens (4 groups of 6 chickens) of the Decalp breed were used in this experiment. The first group was intravenously infected with 100 µL allantoic fluid with a virus titer of 10^{5.25} ELD_{50}/100 µL per bird, second with 100 µL allantoic fluid with a virus titer of 10^{4.25} ELD_{50}/100 µL per bird and third group with 100 µL allantoic fluid with a virus titer of 10^{3.00} ELD_{50}/100 µL per bird. A fourth group (uninfected control group) was inoculated intravenously with 100 µL per bird of allantoic fluid from non-infected CE. The intravenous route of infection was chosen for the quick and broad spreading of the virus into the entire organism of the host.

The 4 groups of infected and uninfected birds were kept separately in 16 m² 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 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, 252 samples originated from infected birds (126 from cloaca and 126 from oropharynx, 84 of each group – 42 equally from cloaca and oropharynx) and 132 samples from uninfected birds (- from the controls (n = 96) and from the birds determined for infection prior to it (on day 0 - n = 36).

Blood samples (n = 120) were obtained on days 0, 7, 14, 21, 28. Their total number from the healthy population was 48 (30 from the non-infected controls and 18 from the infected groups on day 0). A total of 72 blood samples were obtained from the infected population (24 from each group).

VIRUS REISOLATION AND ANTIBODY DETECTION METHOD

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 µg/mL), Nystatin dehydrate (0.5.10^{6} U/L), Sulphamethoxazole (0.2 g/L) and fetal 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 hyperimmune 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.
The presence of antihemagglutinins in the blood sera was detected by the standard procedure with a viral hemagglutinin of 8 hemagglutinating units (1 hemagglutinating unit - the highest dilution of virus yielding hemagglutination) of the H6N2 strain. The sera with antibody titers equal or higher than the 1:8 dilutions were considered positive [1]. Antibodies were expressed as log2 mean HI titers for each collection each week from each group.

**Results**

We did not detect any clinical symptoms of disease both in the infected and the control groups of chickens.

The total number of reisolations from chickens, infected with virus of a titre $10^{5.25} \text{ ELD}_{50} /100 \mu L$ per bird was 5 - 4 from cloaca and 1 from oropharynx (table 1). In most (n=4) of the 6 infected chickens from this group the reisolation was unsuccessful (66.67%). It was successful from two chickens - on day 3 PI (one cloacal and one oropharyngeal sample - chicken No10), day 5 PI (two cloacal samples - No8, No10) and on day 7 PI (one cloacal sample - No10). The reisolation ratio of cloacal samples (n = 42) taken from this group reached 9.5%. No viruses were reisolated from the healthy chicken population between days 10 and 28 PI. The mean period of virus shedding was 5 days for cloacal samples, 3 days for oropharyngeal samples and the reisolation ratio of all tested samples (n = 84) reached 6.0%.

Only one reisolation from cloaca on day 3 PI was obtained from chickens infected with virus dose $10^{4.25} \text{ ELD}_{50} /100 \mu L$ per bird (16.67%). It was from chicken No 4. The reisolation ratio of all samples from these birds (n = 84) was 1.2% and the ratio for the cloacal samples only (n = 42) was 2.4%. The mean period of virus shedding was 3 days for cloacal samples. Virus isolates yielded hemagglutinating titers from 7 log2 up to 9 log2. Lower titers were displayed by the cloacal isolates on days 3 and 5 PI and by the oropharyngeal isolates.

<table>
<thead>
<tr>
<th>Inoculation dose$^a$</th>
<th>Samples</th>
<th>3 d.p.i.$^b$</th>
<th>5 d.p.i.</th>
<th>7 d.p.i.</th>
<th>10 d.p.i.</th>
<th>14 d.p.i.</th>
<th>21 d.p.i.</th>
<th>28 d.p.i.</th>
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<tr>
<td>$10^{5.25}$ Cloacal</td>
<td>1/No10$^c$</td>
<td>2/No8,10</td>
<td>1/No10</td>
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<tr>
<td>Oropharyngeal</td>
<td>1/No10</td>
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<td>$10^{4.25}$ Cloacal</td>
<td>1/No4</td>
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<td>$10^{3.00}$ Cloacal</td>
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$^a$ – ELD$_{50}$ /100 µL per bird

$^b$ - d.p.i., days post infection

$^c$ – number of isolates / ordinal number of bird (Geometric mean titers by HA test)

$^d$ – No virus isolated

**TABLE 1** : Virus reisolation from pooled cloacal and oropharyngeal swab samples, taken from control chickens and chickens, challenged with isolate of influenza A virus H6N2.

**FIGURE 1** : Geometric haemaglutinin titres of chickens, infected with different doses of Influenza A virus H6N2.
No viruses were reisolated from chickens, infected with virus dose of $10^{3.00}$ ELD$_{50}$/100 µL per bird and from the control chickens.

On day 7 PI two chickens (No 8 and 10 with virus reisolation) out of 6 infected with virus dose $10^{5.25}$ ELD$_{50}$/100 µL per bird were positive by HI (33.33%). Their geometric antibody titres were 8 log$_2$ and 11 log$_2$. Later (day 14 PI) all the infected chickens yielded positive results (100%) (Figure 1). Their titres were 4 log$_2$ (4 chickens with No 7, 9, 11, 12 without virus reisolation), 9 or 10 log$_2$. On days 21 and 28 PI the HI titers of the 4 chickens without virus isolation remained within 4 log$_2$ – 5 log$_2$, and the titers of birds No 8 and 10 – within 9 log$_2$ - 11 log$_2$. For the entire experiment GMT varied within 3 log$_2$ – 3.5 log$_2$ for the birds without virus reisolation, and it was 9.25 log$_2$ for birds with virus reisolation.

Only one of the birds, infected with $10^{4.25}$ ELD$_{50}$/100 µL per bird (No 4), yielded antibodies and isolates (16.67%). This positivity remained till the end of the experiment (Figure 1). The hemagglutination titer of this bird was 8 log$_2$, then it rose to 10 log$_2$ (on day 14 PI) and then dropped to 9 log$_2$ (on days 21 and 28 PI). GMT of this bird for the whole experiment was 9.25 log$_2$.

No antibodies were detected by HI in chickens, infected with virus dose of $10^{3.00}$ ELD$_{50}$/100 µL per bird and contact birds through all the experiment.

**Discussion**

After an experimental low dose ($10^{3.00}$ ELD$_{50}$/100 µL per birds) inoculation of chickens with H6N2 LPAIV, isolated from *Anas platyrhinchos*, the measured parameters indicated that no infection was detectable. Furthermore, immune response in these birds could not be detected by HI serology. Thus, the induction of infection in chickens appears to require a level of virus administration greater than $10^{3.00}$ ELD$_{50}$/100 µL per bird, for instance $10^{4.25}$ ELD$_{50}$/100 µL per bird. When challenging with virus doses of $10^{4.25}$ ELD$_{50}$/100 µL and $10^{5.25}$ ELD$_{50}$/100 µL infection was demonstrated by virus reisolation and positive HI serology. The dose importance for the infection was demonstrated also by the differences in isolate numbers, periods of reisolation and number of seropositive birds, with percentage odds in favour of the higher dose. Infectious doses between $10^{3.00}$ ELD$_{50}$/100 µL per bird and $10^{4.25}$ ELD$_{50}$/100 µL per bird were not used in this study; so the infectivity of this dose range remains unknown, but the findings suggest that LPAIV concentration is critical for the establishment of a productive infection in a chicken.

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. LAUDERT et al. (1993) have not succeeded to infect chickens with H13 isolate from wild waterfowl. The experiments of MUTINELLI et al., (2000) and WERNER et al. (2000) prove that turkeys are highly susceptible to AIV infection in comparison to other sensitive bird species. Infections of chickens with chicken isolate LPAIV H7N2 by LU & CASTRO (2004) and of turkeys with LPAIV H7N3 by CAPUA et al. (2004) have yielded minimal infection dose results that differ from ours. In both H7 experiments with virus doses of $10^{2.00}$ ELD$_{50}$/500 µL per bird for the chickens and $10^{2.00}$ ELD$_{50}$/100 µL per bird for the turkeys the authors do not find any signs of infection after attempts for reisolation and antibody detection. In our experiment we obtained similar results with higher infection dose ($10^{3.00}$ ELD$_{50}$/100 µL per bird), albeit we used more optimal route of inoculation (intravenous) than those used in the others experiments (intranasal - CAPUA et al., 2004; intranasal, conjunctival, oral, intramuscular - LU & CASTRO, 2004). Although we have not used other bird species, we suppose that bird species sensitivity to the virus used is also of importance for the minimal infecting dose.

Our experiments with virus dose of $10^{4.25}$ ELD$_{50}$/100 µL per bird, in accordance to the findings of LU & CASTRO (2004) (LPAIV H7N3) using $10^{4.7}$ ELD$_{50}$/500 µL per bird and CAPUA et al. (2004) using $10^{4.0}$ ELD$_{50}$/100 µL, were successful in causing an infection, evidenced by virus reisolation and presence of antibodies. For reisolation 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 availability of trypsin-like enzymes, necessary for the absorption and penetration of virus into the target cells. The use of higher virus doses ($10^{5.25}$ ELD$_{50}$/100 µL per bird in our study, $10^{5.7}$ ELD$_{50}$/500 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)) influences upon the number of birds, yielding isolates and upon the reisolation period. In all experiments with higher doses, the number of reisolates is higher and for a longer period (correspondingly 33.33% reisolates and shedding until day 7 PI in our study). The summarized results for active reisolation of LU & CASTRO (2004) are 80% reisolates up to day 9 PI, and results of CAPUA et al. (2004) are 60% reisolates up to day 10 PI for tracheal samples and up to day 7-20 (depending on dose) for cloacal samples.

Antibodies detected by HI studies in experimental birds and different virus doses reveal, that higher titers are reached when the virus dose is higher, confirmed by our and other experiments. In chicken inoculation with two different doses ($10^{5.7}$ ELD$_{50}$/500 µL per bird and $10^{4.7}$ ELD$_{50}$/500 µL per bird) causing infection, LU & CASTRO (2004) detect a difference in antibody titers of 1 log$_2$ in favor of the higher dose, while we detect a higher difference (2 log$_2$). We also observe a significant titer difference (from 4 log$_2$ to 6 log$_2$) both in birds with and without virus reisolation.

**References**


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