Clinical and morphological studies of ducklings experimentally infected with a H6N2 strain isolate of low pathogen avian influenza A virus

I. S. ZARKOV1*, I. DINEV2

1Department of Microbiology, Infection and Parasite diseases, Faculty of Veterinary Medicine, Thracian University, Students Campus, 6000 Stara Zagora, BULGARIA.
2Department of Pathology, Faculty of Veterinary Medicine, Thracian University, Students Campus, 6000 Stara Zagora, BULGARIA.
*Corresponding author: ivanzarkov@yahoo.com

INTRODUCTION

Since 1961, when the first avian influenza virus (AIV) isolates were obtained from ostriches, it was found out that other species are not infected [1]. This fact triggered studies of HPAIV (highly pathogenic avian influenza virus) and LPAIV (low pathogenic avian influenza virus) in different avian species, including ducks, mostly 2-4 weeks old, and sometimes one or more days old ducklings infected intravenously [10, 11], cerebrally [7, 16], orally or tracheally [4, 13] and nasally [12, 20]. Most of the spontaneous and experimental cases are in a sub-clinical form with no clinical signs of disease [4]. In experiments conducted with the strain H5N7 isolated in Pennsylvania [2, 15, 19], the strains H5 and H7 [4, 14] or the LPAIV strains H11N1, H6N2 and H3N1 [13], the same results have been obtained. In other experiments loss of weight has been detected in ducklings infected intravenously [11] or cerebrally [10, 11], cerebrally [7, 16], orally or tracheally [4, 13] and nasally [12, 20].

SUMMARY

The aim of this study was to analyse the clinical and pathological signs in ducklings associated to an experimental infection with a low pathogen avian influenza virus, the H6N2 strain. For that, a total of 24 ducklings, 28 days old, were intravenously, tracheally or orally inoculated (8 birds for each inoculation route) with the H6N2 virus cultures in chicken embryos (10^3-5 ELD50 / 0.1 mL) isolated from wild ducks (Anas platyrhynchos) whereas 6 other ducklings served as negative controls. Clinical signs and mortality were daily monitored, body weights were recorded before and 3, 5, 7, 10, 14, 21 and 28 days after the virus challenge and 2 inoculated birds and 1 control were slaughtered on days 7, 14, 21 and 28 for necropsy and conventional histopathological analyses were performed on visceral organs. Clinically, significant growth retardation and feathering defects were precociously evidenced (since the 3rd-5th days post inoculation) in intravenously and tracheally inoculated ducklings than in those orally infected, were observed in heart, thymus, liver, pancreas, kidneys and lungs mainly between the 7th and the 14th days. The atrophy of lymphoid organs (thymus, bursa of Fabricius and spleen) was also noted but these lesions were never associated with a fatal issue. Compared to the literature data, these results suggest that the experimental infection of ducklings with a low pathogen avian influenza virus strain has induced a sub-clinical form of the disease coupled to vascular lesions, particularly extended and severe following intravenous or tracheal inoculation, which the major localisations would be dependent for the tissue tropism of a specific virus strain.

Keywords: Ducklings, low pathogen avian influenza A virus, H6N2 strain, experimental infection, route of inoculation, growth retardation, vascular lesions.

RÉSUMÉ

Etude clinique et anatomo-pathologique de l’infection expérimentale des canetons par un isolat d’un virus de la grippe aviaire A faiblement pathogène, de souche H6N2

L’objectif de cette étude a été d’analyser les signes cliniques et pathologiques lors d’une infection expérimentale par une souche faiblement pathogène du virus de la grippe aviaire A (souche H6N2) chez le caneton. Au total, 24 canetons de 28 jours ont été inoculés par voie intraveineuse, trachéale ou orale (8 oiseaux pour chaque voie d’inoculation) par la souche virale H6N2 cultivée sur embryons de poulets (10^3,00 ELD50) isolée à partir de canards sauvages (Anas platyrhynchos) alors que 6 autres oisillons ont servi de contrôles négatifs. Les signes cliniques et la mortalité ont été suivis quotidiennement, les animaux ont été pesés avant et 3, 5, 7, 10, 14, 21 et 28 jours après l’inoculation virale et 2 canetons infectés et 1 contrôle ont été sacrifiés les 7, 14, 21 et 28ème jours afin de réaliser une autopsie et une analyse histologique conventionnelle sur les différentes viscères. Cliniquement, un retard de croissance significatif et un plumage défectueux ont été précocément mis en évidence (dès les 3ème et 5ème jours) chez les oiseaux inoculés par voie intraveineuse ou trachéale. Des lésions vasculaires (congestion et hémorragies, parfois associées à des zones nécrotiques), plus intenses lors d’inoculations par voie intraveineuse ou trachéale que lors d’inoculation par voie orale, ont été observées dans le cœur, le thymus, le foie, le pancréas, les reins, et les poumons, principalement entre les 7ème et 14ème jours. Une atrophie des organes lymphoïdes (thymus, bourse de Fabricius et rate) a également été constatée mais aucun cas de mortalité n’a été enregistré. Ces résultats ainsi que ceux de la littérature suggèrent qu’une infection expérimentale par une souche de virus de la grippe aviaire faiblement pathogène entraîne chez le caneton une forme subclinique de la maladie associée à des lésions vasculaires, particulièrement étendues dans le cas d’inoculations intraveineuse ou trachéale, dont les principales localisations dépendraient du tropisme tissulaire spécifique de la souche inoculée.

Mots clés : Canetons, virus de la grippe aviaire A faiblement pathogène, souche H6N2, infection expérimentale, voie d’inoculation, retard de croissance, lésions vasculaires.
[7, 16]. By contrast, clinical signs have been observed with the highly pathogenic H5N1 isolate from Hong Kong (strain A/chicken/Hong Kong/220/97 causing disease in people) in chicken, geese and emus [6] but they have not been detected in ducklings [12]. Clinical signs and/or death in spontaneous cases of infection and in studies with different isolates of H5N1 were observed in the end of 2002 in Hong Kong and recently in Vietnam, Thailand, Indonesia [18], and South Korea [21]. In spontaneously infected ducks, mild clinical signs consisted in respiratory infection, depression, diarrhoea, loss of appetite and increase in death rate up to 12% have been found. In the end of 2003 in Hong Kong and in 2004 in other Asian countries certain strains again caused death in poultry, but not in ducks, probably because of genetic changes in viruses and appearance of varieties with different biological properties [5, 8]. In an experiment using four different H5N1 strains, clinical signs were only observed with the strain Yokohama/aq-10/03 [9], when replication of the virus in the brain is critical for ducks to show neurological signs.

Despite the absence of clinical signs, pathoanatomical and pathohistological changes were usually found in ducks contrary to other avian species: PERKINS and SWAYNE [12] describe splenomegalia at the 4th-10th days post infection in 56% of cases and hyperplasia of the air sacks at the 4th-7th days post infection in 11% of cases. In spontaneous clinical cases, YONG-KUK KWON et al. [21] have detected pancreatic necrosis, splenomegalia, hepatomegalia and enlarged kidneys. Pathohistological changes have been detected in organs without pathoanatomical changes. WEBSTER et al. [17] and COOLEY et al. [4] have described pathohistological changes (focal inflammatory response with lymphocyte and macrophage infiltration) in intestines and lungs. Inflammation is considered as an efficient response for controlling the viral infection [2]. LAUDERT et al. [10] have also observed small pathohistological changes in kidneys and liver. With the H5N1 strain isolated from Hong Kong, lesions in the respiratory tract (rhinitis, lymphoplasmatic laryngitis and interstitial pneumonia in 50%, 14% and 50% of cases, respectively) as well as the atrophy of the bursa of Fabricius were evidenced. In spontaneous clinical cases in the South Korea, YONG-KUK KWON et al. [21] have recorded pathohistological changes in the brain (meningoencephalitis) and in the heart (myocarditis).

The aim of the present study was to investigate the pathohistological changes eventually associated to an experimental infection of ducks with a low pathogen A influenza virus, the H6N2 strain.

Material and Methods

BIRDS AND EXPERIMENTAL DESIGN

A total of 30 crossbred Peking x Turkish Mallard (Mullary), 28 day old, were used. Birds were kept in two separate isolated 2 x 2 m rooms (13-hour regimen of day-light, constant temperature: 20°C, humidity: 70% and with a feeding and water 0.9 m long front (regulation No 13/25.04.2002)) and were not vaccinated.

Twenty-four birds were intravenously, tracheally or orally infected with 0.1 mL allantoic fluid from virus cultures in chicken embryos (8 birds for each route) whereas 6 birds were inoculated with allantoic fluid from intact chicken embryos and served as controls. The virus isolate used for the bird infection (105.00 ELD50 (embryo lethal dose 50 %) / 0.1 mL) was the 4th passage on chicken embryos of the H6N2 strain, a low pathogen A influenza virus previously isolated from wild ducks (Anas platyrhynchos) [22]. Thereafter, all ducks were fed with the same quantity and kind of food (combined corn feed for ducks) and were daily monitored for 28 days.

The experimental protocol was approved by the Bulgarian Ministry of Agriculture and Forests (No 3/27.06.2010) and was conducted following the humanity recommendations from the regulation No 25/10.06.2003 (article 20. 1, appendix 4, point B).

CLINICAL AND PATHOLOGICAL EXAMINATIONS

Ducks were daily clinically examined throughout the whole experimental period. Live body weights were measured using an electronic balance (ATEW-15, S/N: 1412274001) the day before the infection and at the 3rd, 5th, 7th, 10th, 14th, 16th, 21st and 28th days after the viral inoculation.

On days 7, 14, 21 and 28, 2 infected and 1 control birds were slaughtered (Regulation No 25/10.06.2003, article 20, appendix 4, paragraph 1, point C, permitted methods of killing birds experienced to 3 kg – breaking the spine in the neck) for pathological investigations. All visceral organs and brains were macroscopically analysed and samples from lungs, heart, spleen, liver, kidneys, pancreas, thymus, bursa, brain and duodenum were immediately removed and fixed in 10% buffered formalin for at least 2 days. Slices of 5 µm of thickness were prepared, embedded in paraffin and stained with haematoxylin and eosin by standard procedures. The preparations were examined on Leitz light microscope.

STATISTICAL ANALYSIS

The body weights were processed with STATISTICA version 6.0 (StatSoft, Tulsa, OK, USA) and were compared with controls and accordingly the route of inoculation using the One-way Analysis of Variance (ANOVA). Differences were considered as significant when P values were less than 0.05. Results are expressed as means ± standard errors.

Results

CLINICAL FINDINGS

No respiratory, digestive, secretory and nervous clinical signs were recorded in the H6N2 inoculated ducklings. However, compared to the not inoculated controls, some disturbances were observed in the body development and feathering rate since the 21st day in the intravenously infected birds (figure 1) as well as in those tracheally inoculated at a lesser extent.

In addition, as shown in Table I, the final mean body weights and the overall body weight gains in the intravenously
and tracheally inoculated ducks were significantly depressed compared to the controls (P < 0.001 for body weights and P < 0.05 for body weight gains only in the intravenously inoculated ducks) and to birds inoculated by the oral route (P < 0.05 only for body weights). The growth retardation evidenced in tracheally and intravenously inoculated ducklings by a lower body weight compared to the controls (P < 0.001) was detected since the 3rd day post inoculation and persisted over the whole experimental period although the transient body weight gains, dramatically depressed during the first 3 days period in both 2 groups (P < 0.001) and until the 10th day in intravenously inoculated birds (P < 0.001), significantly increased in tracheally inoculated birds between the 3rd and the 7th days (P < 0.05 and P < 0.001) and between the 10th and the 21st days (P < 0.05 and P < 0.001) and in intravenously inoculated ones, during the second half of the experimental period (for day 10-day 14 and for day 21-day 28: P < 0.001). In orally inoculated ducklings, significantly depressed body weights compared to controls were observed only for the second experimental period, from the 14th day (P < 0.001 for days 14 and 21, P < 0.05 for day 28).

PATHOLOGICAL FINDINGS

No macroscopic and microscopic lesions were detected in the control group (not inoculated ducklings).

The intensity of gross lesions, prevailing in the period between the 7th and the 14th days after viral exposure, was low to moderate in orally and tracheally inoculated ducks and was high in ducks intravenously infected by the virus. Ecchymoses and petechial to marked and not clearly demarcated haemorrhages were seen in the epicardium, myocardium and endocardium in 50% of the infected ducklings (figure 2), independently on the length of exposure. The haemorrhages were localized in the left and right coronary walls, both in the atrium and the ventricle areas. Persistent hyperaemia of the parenchymal organs (liver, spleen, kidneys and lungs) was also observed.

<table>
<thead>
<tr>
<th>Body weight (kg)</th>
<th>Controls</th>
<th>Orally inoculated</th>
<th>Tracheally inoculated</th>
<th>Intravenously inoculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>0.830 ± 0.004</td>
<td>0.838 ± 0.002</td>
<td>0.835 ± 0.003</td>
<td>0.837 ± 0.003</td>
</tr>
<tr>
<td>3 day</td>
<td>0.948 ± 0.003a</td>
<td>0.955 ± 0.003a</td>
<td>0.872 ± 0.004b</td>
<td>0.871 ± 0.003b</td>
</tr>
<tr>
<td>5 day</td>
<td>1.054 ± 0.004a</td>
<td>1.052 ± 0.004a</td>
<td>0.978 ± 0.004b</td>
<td>0.930 ± 0.003b</td>
</tr>
<tr>
<td>7 day</td>
<td>1.155 ± 0.002a</td>
<td>1.156 ± 0.003a</td>
<td>1.116 ± 0.003b</td>
<td>1.023 ± 0.002b</td>
</tr>
<tr>
<td>10 day</td>
<td>1.453 ± 0.004a</td>
<td>1.433 ± 0.004a</td>
<td>1.361 ± 0.003b</td>
<td>1.182 ± 0.004c</td>
</tr>
<tr>
<td>14 day</td>
<td>1.696 ± 0.004a</td>
<td>1.680 ± 0.004b</td>
<td>1.603 ± 0.005b</td>
<td>1.421 ± 0.004c</td>
</tr>
<tr>
<td>21 day</td>
<td>2.292 ± 0.003a</td>
<td>2.263 ± 0.004b</td>
<td>2.198 ± 0.007bc</td>
<td>1.859 ± 0.005c</td>
</tr>
<tr>
<td>28 day</td>
<td>2.558 ± 0.003a</td>
<td>2.543 ± 0.004b</td>
<td>2.407 ± 0.005c</td>
<td>2.296 ± 0.004d</td>
</tr>
</tbody>
</table>

Weight gain (%) | Controls | Orally inoculated | Tracheally inoculated | Intravenously inoculated |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0-Day 3</td>
<td>14.22 ± 0.56a</td>
<td>13.96 ± 0.20a</td>
<td>4.43 ± 0.38b</td>
<td>4.06 ± 0.26b</td>
</tr>
<tr>
<td>Day 3-Day 5</td>
<td>11.18 ± 0.39a</td>
<td>10.10 ± 0.20a</td>
<td>12.20 ± 0.21a</td>
<td>6.77 ± 1.43b</td>
</tr>
<tr>
<td>Day 5-Day 7</td>
<td>9.58 ± 0.34a</td>
<td>10.16 ± 0.25b</td>
<td>14.11 ± 0.21c</td>
<td>10.00 ± 0.38ab</td>
</tr>
<tr>
<td>Day 7-Day 10</td>
<td>25.80 ± 0.16a</td>
<td>23.96 ± 0.15b</td>
<td>21.95 ± 0.05b</td>
<td>15.54 ± 0.55c</td>
</tr>
<tr>
<td>Day 10-Day 14</td>
<td>16.72 ± 0.80a</td>
<td>17.24 ± 0.22ab</td>
<td>17.78 ± 0.45b</td>
<td>20.22 ± 0.46c</td>
</tr>
<tr>
<td>Day 14-Day 21</td>
<td>35.14 ± 0.22b</td>
<td>34.70 ± 0.13b</td>
<td>37.12 ± 0.43a</td>
<td>30.82 ± 0.92c</td>
</tr>
<tr>
<td>Day 21-Day 28</td>
<td>11.61 ± 0.20b</td>
<td>12.37 ± 0.00c</td>
<td>9.51 ± 0.57a</td>
<td>23.51 ± 0.35d</td>
</tr>
<tr>
<td>Day 0-Day 28</td>
<td>208.2 ± 0.66b</td>
<td>203.4 ± 0.28a</td>
<td>188.3 ± 21.4ab</td>
<td>174.3 ± 0.4b</td>
</tr>
</tbody>
</table>

The weight gains were calculated for a given period following the formula: weight gain = 100 x (Wj-Wi)/Wi where Wj was the weight measured on day j and Wi the weight measured on day i.

Different superscripts a,b,c,d in the same row indicate significant differences (P < 0.05 or more) between groups.

TABLE I: Variations in body weights and in growth rate in 28 days old ducks inoculated by the H6N2 strain (10^5.00 ELD50/0.1mL) and in not inoculated ducks (controls, n = 6) according to time and to the inoculation route (n = 8 for each modality). Results are expressed as means ± standard errors.
throughout the experiment. Numerous petechial haemorrhages were found out bilaterally in the thymus gland in 50% of the ducklings between the 7th and the 14th days post inoculation (figure 3).

Histologically, mostly perivascular congestions and haemorrhages were observed between the 7th and the 14th days after virus inoculation in most visceral organs (lungs, heart, spleen, liver, kidneys, pancreas, thymus and bursa) from intravenously and tracheally infected birds (figure 4). Furthermore, haemostasis and haemorrhages were seen in the epicardium from all infected ducks whatever the route of inoculation and focal petechial inflammatory proliferations were observed in 75% of intravenously infected ducklings and in 62.5% of those tracheally infected (figure 5). Inter- and intratubular haemorrhages and dystrophic degenerative changes in the epithelium of the straight outlet tubules were observed in kidneys from tracheally and intravenously inoculated ducks. Intensive haemorrhages in the thymus parenchyma prevailed in intravenously and tracheally infected birds. Congestion and parenchymatous dystrophy in liver were observed in all inoculated birds independently on the length of exposure. Additionally, haemorrhages of varying severity were detected in intravenously and tracheally infected ducks and besides, acute haemorrhagic infarctions containing hyaline drops were evidenced in two cases (figure 6). Numerous necrosis areas with various sizes were detected in the pancreatic parenchyma in 50% of the intravenously and tracheally infected ducklings (figure 7) on day 21. Some of these zones were besides infarcted with haemorrhages. In some outlet tubules adjacent to the necrotic areas, cystic dilatation was observed in intravenously and tracheally infected ducklings. In addition, follicles in the bursa of Fabricius from all infected birds and the white pulp in spleen from intravenously or tracheally inoculated ducks were severely atrophied on day 21 and on day 7, respectively.

**Figure 2:** Ecchymoses and haemorrhages (arrow) in the myocardium and endocardium of the left ventricle from a duck 28 days after the tracheal infection with the H6N2 strain (10^5.00 ELD_{50}/0.1mL).

**Figure 3:** Numerous petechial haemorrhages (arrow) in all parts of the thymus gland from a duck 8 days after tracheal infection with the H6N2 strain (10^5.00 ELD_{50}/0.1mL).

**Figure 4:** Congestion and haemorrhages (arrows) in lung from a duck 8 days after tracheal infection with the H6N2 strain (10^5.00 ELD_{50}/0.1mL), haematoxylin and eosin, bar = 4 µm.

**Figure 5:** Focal perivascular proliferation areas (arrows) in the myocardium from a duck 14 days after tracheal infection with the H6N2 strain (10^5.00 ELD_{50}/0.1mL), haematoxylin and eosin, bar = 4 µm.
Discussion

The experimental infection of ducklings with an isolate of avian influenza A virus strain A/duck/Bulgaria/05 H6N2 (a low pathogen virus) resulted in delayed growth development and in extensive vascular lesions in thymus, heart, lungs, liver, pancreas, bursa of Fabricius and kidneys, particularly in intravenously or tracheally inoculated birds, but did not cause death. By contrast, no clinical signs and only moderate growth retardation were evidenced in orally infected ducks and some vascular lesions were only detected in heart, liver, and bursa of Fabricius.

The growth retardation in ducks observed in the present study was more pronounced than in previous studies also using low pathogen avian influenza viruses [7, 11, 16] and was accompanied with feathering disturbances (no reference concerning this clinical sign was found), leading to important economic losses. These alterations of the general health status probably result from the virus induced atrophy of the peripheral immune organs (bursa, thymus, and spleen). Indeed, a low thymus function coupled to the low secretion of thymus hormones may induce slow growth, retarded development and cachexia as well as loss of feathers [3] and more tardily to atrophy of spleen, liver and bursa of Fabricius [12], as observed in the present study since the 7th day and until the end of the experiment.

LAUDERT et al. [10] demonstrated that pathohistological changes induced by 11 various avian influenza virus strains from different sources (water fowls, pheasants and turkeys) were mainly located in the spleen (86%), in the liver and the bursa of Fabricius (57%), kidneys (43%) and in lungs (28.5%) but they failed to detect any lesions in the thymus, the heart, the intestines and the pancreas contrary to the results obtained here. PERKINS and SWAYNE [12] and COOLEY et al. [4] in experimental infections induced with high pathogen avian influenza viruses, the H5N1 strain and other H5 strains, respectively, detected major changes mainly in the respiratory tract (rhinitis, laryngitis, interstitial pneumonia, aero-sacculitis) and in the spleen and they observed no lesions in the other organs. Histologically the induced lesions consisted in epithelium hyperplasia and tissue infiltrations with lymphocytes, macrophages and plasmatic cells. By contrast, the respiratory lesions did not prevail in the current study. However, the lesions in the heart, pancreas, liver, spleen and kidneys induced by the LPAIV, H6N2 strain were similar to those described in spontaneous infections with subtype H5N1 in ducks [9, 21]. The histopathological discrepancies noted between the different studies conducted in ducks are related to the type of avian influenza virus strain and to the possibility of virus re-isolation, leading to different localisation of the virus induced lesions according to the tissue tropism of the specific virus strain [10]. A possible explanation for the observed pathological changes and weight changes following intravenous and tracheal entry of virus compared with oral is part of eliminating the virus from a number of protective barriers of the digestive system (low pH, neutralization effect of immunity in lining of the gastrointestinal tract).

As a conclusion, even if the experimental infection with a low pathogen avian influenza virus, the H6N2 strain, only lead in ducklings to not organ specific clinical signs, such as growth retardation and feathering defect, the virus inoculation mainly by the intravenous and the tracheal route has induced a systemic disease involving the heart, the liver, the pancreas, the kidneys and the lymphoid organs (thymus, bursa of Fabricius and the spleen) and the lungs at a lesser extend, whereas damage in the respiratory tract prevailed in the case of spontaneous and experimental infections with high pathogen viruses. Discrepancies in localisation of the lesions induced by various low pathogen virus strains occurred, depending probably from the viral tissue tropism. Further investigations using immunohistochemistry would be required in order to specify the virus localisation according to its type.

FIGURE 6: Haemorrhagic infarct (arrow) in liver in a duck, 14 days after an intravenous inoculation with the H6N2 strain (10^5.00 ELD_{50}/0.1mL), haematoxylin and eosin, bar = 3 µm.

FIGURE 7: Haemorrhagic necrosis (arrow) due to infarction in pancreas in a duck, 21 days after an intravenous inoculation with the H6N2 strain (10^5.00 ELD_{50}/0.1mL), haematoxylin and eosin, bar = 4 µm.
DUCKLING INFECTED WITH H6N2 STRAIN

References