DIFFERENT PATTERNS OF MULTIPLICATION
OF EQUINE INFLUENZA VIRUS SEROTYPES H7N7
AND H3N8 IN CHICKEN EMBRYO

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Abstract

The purpose of the study was to compare two models of multiplication patterns of equine influenza virus H7N7 – subtype A1 and H3N8 – subtype A2, after inoculation into embryonated chicken eggs through the allantoic cavity and the yolk sack. The relationship between the presence of haemagglutinin activity in allanto-amniotic fluid and the presence of M1 protein in chorioallantoic membranes, vitelline membranes, and embryos body was determined. The distribution of equine influenza virus multiplication sites in embryonated chicken eggs has revealed different patterns for A1 and A2 viruses. Virus subtype A1 multiplied better after inoculation into the yolk sack than into the allantoic cavity. Virus subtype A2 multiplied equally well after inoculation into the allantoic cavity as well as after inoculation into the yolk sack. There were cases with negative haemagglutination titers of allanto-amniotic fluid of eggs inoculated into the yolk sack with both influenza virus subtypes, though the M1 protein was detected in the vitelline membranes. Thus, the negative haemagglutination titres of allanto-amniotic fluid cannot be treated as the only indicator of virus multiplication after inoculation into the yolk sack.

Key words: equine influenza virus, isolation, embryonated chicken eggs.

Equine influenza virus belongs to the Orthomyxoviridae family type A and is represented by two serotypes: H7N7 (subtype A1) and H3N8 (subtype A2). The H7N7 strain has not been isolated from horses since 1980. H3N8 strain circulates in equine population throughout most of the world and it is regularly isolated from horses in Europe (2, 3, 15, 21, 22). Similarly to human influenza, equine influenza is a highly contagious disease. Virus is spread via aerosolised respiratory secretions. The risk of infection transmission is increased by exchanging of horses between studs, race-tracks, countries. The vaccines against equine influenza infection do not provide the protection comparable to protection stimulated after natural infection (4, 21).

In order to isolate and multiply influenza virus under laboratory conditions, embryonated chicken eggs and cell cultures are used. The reverse transcription polymerase chain reaction assay (RT-PCR) is the most sensitive to detect the virus. Virus isolation in embryonated chicken eggs is carried out routinely for surveillance and vaccine strain selection and production (5, 13, 21). The eggs are very efficient for multiplication of the virus because of their sensitivity to infection and metabolism intensity. Their advantages are: simple method of inoculation, low costs of incubation, and presence of allanto-amniotic fluid – easy and convenient source of viral particles. However, there are difficulties during isolation of wild-type strains of the virus in embryonated chicken eggs. The cause of these problems lay in too small number of infective particles of the virus in the material from infected animal, or late sample collection. The purpose of the study was to compare two models of virus inoculations: into the allantoic cavity and into the yolk sack. The relationship between the presence of haemagglutinin activity in the allanto-amniotic fluid and the presence of M1 protein in chorioallantoic membranes, vitelline membranes, and embryos was also studied. Two subtypes of equine influenza viruses (A1 and A2) were taken into consideration in the analysis.

Material and Methods

Inoculation of embryonated chicken eggs.

Eighty 10-day-old SPF embryonated chicken eggs were used for the experiment. Forty were infected with equine influenza A1 virus and the others with A2 virus. Two modes of inoculation were applied: into the allantoic cavity and into the yolk sack. The infection dose 100 EID_{50} was used independently of the way of inoculation and the subtype of influenza virus. The eggs were incubated at 37°C up to 72 h after inoculation. Embryos,
which were dead after 48 h or 72 h of incubation, and those that remained alive were cooled down to 4°C for about 2 h. The allanto-amniotic fluids, the chorioallantoic membranes, the vitelline membranes, and embryo’s bodies were collected and stored at -20°C.

**Haemagglutination assay (HA).** HA was done according to the standard of the World Organisation for Animal Health (OIE) procedures described in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2009, vol. 2, section 2.5., chapter 2.5.7.)

**Sample preparation for electrophoretic analysis.** Three volumes of sample buffer (60 mM Tris-HCl, pH 6.8; 2% sodium dodecyl sulfate (SDS), 10% glycerol, 0.001% bromphenol blue) was added to every chorioallantoic membrane and vitelline membrane (3 ml of buffer per 1g of membranes). The membranes were disintegrated (Dounce homogeniser) at 4°C and heated for 10 min at 95°C. Then the samples were sonicated (8 microns amplitude, 1 min) and centrifuged for 15 min (4,500 rpm) at 4°C. The supernatants were saved for electrophoretic analysis.

The embryo bodies were homogenised with one volume of sample buffer (1 ml of buffer per 1g of embryos). Then other 3 ml of sample buffer were added to 1 ml of the sample. The next steps: heating, sonication, and centrifugation were done as described for membrane preparation.

The concentration of proteins in homogenates of membranes and embryos was measured by colorimetric assay (Dc Protein Assay, Bio-Rad). Thirty micrograms of protein samples were used for electrophoresis.

**Electrophoresis and immunoblotting.**

Proteins were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane. The membrane was blocked at room temperature in buffer – 5% bovine serum albumin in TBST (20 mM Tris-HCl, pH 7.4; 150 mM NaCl, 0.05% Tween 20) for 12 h and next incubated with mouse monoclonal antibody for M1 protein (Sero tec) (1:10,000) for 1 h at room temperature with constant shaking. After incubation the membranes were washed three times with TBST. Fragments (Fab'); of rabbit IgG, specific for Fc fragments of mouse IgG labelled with horseradish peroxidase (Jackson Immunoresearch) were used. The conjugate was diluted 1:20,000 (in TBST) and incubation of membrane was carried out for 30 min at room temperature. After incubation, the membranes were washed three times with TBST for 5 min at room temperature. After reaction with a substrate (Amerham ECL Plus Western Blotting Detection System) results of the detection were visualised on X-ray film.

**Results**

After inoculation of influenza virus subtype A1 into allantoic cavity, M1 protein was detected in eight out of 20 samples of chorioallantoic membranes (Fig. 1A). In the sample from vitelline membranes and the embryo body, M1 protein was not detected (Figs 1B, 1C). After inoculation of influenza virus subtype A2 into allantoic cavity, M1 protein was detected in chorioallantoic membranes in all samples of inoculated chicken embryos (Fig. 1D), in three out of 20 samples of vitelline membranes (Fig. 1E), and in five out of 20 samples of embryo bodies (Fig. 1F).

After inoculation of influenza virus subtype A1 into the yolk sack, M1 protein was detected in 10 out of 20 samples of chorioallantoic membranes and in 18 out of 20 samples of vitelline membranes (Figs 2A, 2C). The M1 protein was not detected in samples from embryo bodies (Fig. 2B). After inoculation of influenza virus subtype A2, M1 protein was detected in 18 out of 20 samples of chorioallantoic membranes, in 20 out of 20 samples of vitelline membranes, and not detected in homogenates of embryo bodies (Figs 2D, 2E, 2F).

**Fig. 1.** Detection of M1 protein in particular parts of chicken embryo after inoculation into the allantoic cavity; by immunoblotting technique. A, B, C - inoculation with equine influenza virus A1, and D, E, F - inoculation of equine influenza virus A2; A, D - chorioallantoic membranes, B, E - embryos, C, F - vitelline membranes 1 - positive control (M1 protein), 2 - negative control (non infected chicken embryo), 3-22 test samples.
Table 1

<table>
<thead>
<tr>
<th></th>
<th>A1</th>
<th>A2</th>
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<tbody>
<tr>
<td>Inoculation into allantoic cavity</td>
<td>2.4240</td>
<td>2.6925</td>
</tr>
<tr>
<td>Inoculation into yolk sack</td>
<td>2.0772</td>
<td>1.9734</td>
</tr>
<tr>
<td>Geometric average of HA titer of allanto-amniotic fluid</td>
<td>2.2921</td>
<td>0.4316</td>
</tr>
<tr>
<td>SD</td>
<td>0.2921</td>
<td>0.4316</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.05</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>M1 chorioallantoic membranes</td>
<td>40%</td>
<td>50%</td>
</tr>
<tr>
<td>M1 body of embryos</td>
<td></td>
<td>25%</td>
</tr>
<tr>
<td>M1 vitteline membranes</td>
<td>-</td>
<td>90%</td>
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<td></td>
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<td>15%</td>
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<td>100%</td>
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<td>SD - standard deviation.</td>
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Results of HA of allanto-amniotic fluids of all embryonated chicken eggs in reference to results of detection of M1 proteins are presented in Table 1.

The highest HA titer demonstrated allanto-amniotic fluids from chicken embryos infected with A1 subtype into the yolk sack. M1 protein was detected also in 90% of vitteline membranes. In case of A2, the highest HA titers were obtained after inoculation into allantoic cavity and M1 protein was detected in 100% of chorioallantoic membranes.

There were two cases of chicken embryos inoculated into the yolk sack with A2 subtype when HA titer of allanto-amniotic fluids were negative and the chorioallantoic membranes did not reveal any viral proteins, whereas M1 protein was detected in vitteline membranes. The same result was obtained after inoculation of A1 virus into the yolk sack.

Discussion

There are many factors influencing the isolation of influenza virus in embryonated chicken eggs. The first step essential for viral infection of cell is binding of viral HA to host cell surface glycan receptors. Two main factors: cell receptors specificity and viral HA structure determine the effective viral infection (20). It has been shown that different cell types contain different amounts, types, and linkages of sugar chains by using sialyl linkage-specific lectins (1, 7, 9, 11, 17). Cells...
from particular parts of embryo show different expression of N-glycans types. Recent studies, based on the analysis with multi-dimensional high pressure liquid chromatography (HPLC) mapping and matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), showed that molar percentages of α 2-3 sialic acid (SA) linkage in chorioallantoic and amniotic membranes were 27.2 and 15.4, respectively. Molar percentages of α 2-6 SA linkage in chorioallantoic membranes were 8.3 and 14.2 in amniotic membranes (18). Thus, the affinity of HA to cell receptors is characteristic and depends on the particular species of animals. Human influenza viruses prefer glycan receptors α 2-6 SA, so they are more efficiently isolated by inoculating samples into the amniotic, rather than the allantoic cavity of embryonated chicken eggs (6, 10). Molar percentages of sulfated glycans, recognised by human influenza virus, were 3.8 in chorioallantoic membranes and 12.7 in amniotic membranes (18). Equine influenza virus similar to avian influenza prefers α 2-3 SA, although the virus should prefer chorioallantoic rather than amniotic membranes. The study comprised inoculation of two strains of equine influenza virus (A1 and A2) into the allantoic cavity and into the yolk sac. The distribution of equine influenza virus multiplication sites in embryonated chicken eggs has revealed different patterns after inoculation with A1 and A2 viruses. The data showed that equine influenza virus subtype A1 multiplies definitely better in embryonated chicken eggs after inoculation into the yolk sac than into the allantoic cavity. Equine influenza virus subtype A2 multiplies equally well in embryonated chicken eggs after inoculation into the allantoic cavity, and after inoculation into the yolk sac. Inoculation of influenza virus into the yolk sac seems to be the more effective way for virus isolation than typical inoculation into the allantoic cavity. Different ways of inoculation seems to be the most effective method during isolation of viruses. The trials of isolation of avian or swine influenza viruses using three inoculation ways – into the allantoic cavity, into the yolk sac, or onto the chorioallantoic membrane showed that the inoculation into the yolk sac or onto chorioallantoic membrane were the most effective. Thus, combined use of three methods of virus inoculation increases the number of virus isolations (8, 19, 23, 24).

The differences found during A1 and A2 subtype of equine influenza virus multiplication may come from the heterogeneity of HA structures. In case of avian influenza viruses, the differences of virus spread within chicken embryo are based on different activation of the haemagglutinin by proteolytic cleavage. The haemagglutinin of pathogenic strains is cleaved in cells of each layer whereas the haemagglutinin of non-pathogenic strains is cleaved only in the allantoic epithelium. Thus, the spread of non-pathogenic strains is restricted in the embryonated chicken eggs (12, 14, 16). The isolation of influenza virus in embryonated chicken eggs is usually confirmed by haemagglutination assay of allanto-amniotic fluid. The average haemagglutination titers of allanto-amniotic fluids were higher in case of inoculation with A1 subtype into the allantoic cavity as well as into the yolk sac, in comparison to the inoculation with A2 subtype. However, despite of the presence of haemagglutinin in allanto-amniotic fluid after infection with A1 subtype into allantoic cavity, M1 viral protein was detected only in 40% of chorioallantoic membranes, and other elements of chicken embryo did not show the presence of any viral proteins. The allanto-amniotic fluids from chicken embryos infected with influenza virus A2 subtype had lower haemagglutination titer, than in case of A1 infection but viral proteins were detected in 100% of chorioallantoic membranes and in 50% of embryo bodies.

M1 viral protein was not detected in most of the analysed parts of embryo bodies after inoculation with equine influenza virus subtype A1 into the allantoic cavity in spite of the presence of haemagglutination activity in the allanto-amniotic fluids.

In case of infection with influenza virus A2 subtype haemagglutination activity is considered as the indicator of virus multiplication, and is confirmed by M1 presence in the membranes of embryos. Two cases with negative haemagglutination titres of allanto-amniotic fluid of embryonated chicken eggs infected into the yolk sack with influenza virus subtype A2 were demonstrated. In chorioallantoic membranes there were no viral proteins but in the vittelal membranes viral proteins were detected. Detection of viral proteins in vittelal membranes with negative haemagglutination titers of allanto-amniotic fluid and lack of viral proteins in the chorioallantoic membranes was also proved in case of inoculation of A1 subtype. It follows the results showing that negative haemagglutination titres of allanto-amniotic fluid cannot be treated as the only indicator of virus multiplication after inoculation into the yolk sac. This observation is significant for the interpretation of the results of strains isolation, particularly when the results concern the specific multiplication of some strains of influenza virus after inoculation into the yolk sac.

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References


