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STUDIES UPON THE POSSIBILITIES OF AVIAN INFLUENZA VIRUSES' CULTIVATION IN CHICK EMBRYOS AT DIFFERENT AGE

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Summary

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Chick embryos (CE) at different age (9- and 14-day-old) were experimentally inoculated with avian influenza viruses with different concentration. The first concentration of the strain A/duck/England/56 H11N6 was $10^{7.75}$ ELD_{50}/0.1 mL and $10^{7.75}$ EID_{50}/0.1 mL, whereas for the strain A/duck/Ukraine /1/63 H3N8 the concentration was $10^{4.87}$ ELD_{50}/0.1 mL and $10^{5.16}$ EID_{50}/0.1 mL. The second concentrations of respective strains were $10^{4.75}$ ELD_{50}/0.1 mL and $10^{4.75}$ EID_{50}/0.1 mL (A/duck/England/56 H11N6) and $10^{1.87}$ ELD_{50}/0.1 mL and $10^{2.16}$ EID_{50}/0.1 mL (A/duck/Ukraine/1/63 H3N8) (1000-fold dilution). The results showed that a more rapid and at higher percentage death was observed in 9-day-old CE than in 14-day-old CE. Inocula with lower viral concentration exhibited higher death times by 24 h in all infected chick embryos. A higher amount of haemagglutinins were detected in 14-day-old CE. Probably, this was related to delayed death and the potential for accumulation of haemagglutinins at higher quantities.

Key words: avian influenza virus, chick embryos, cultivation

INTRODUCTION

The methods using 9-day-old chick embryos (CE) are widely used for isolation and cultivation of both avian and non-avian influenza A viruses. All avian influenza A viruses (AIV) are well cultivated following allantoic infection, although in some instances of failure, infection of the yolk sac or chorio-allantoic membrane are successfully used (Woolcock *et al.*, 2001).

The studies upon AIV cultivation are in two directions: study of the possibilities of cultivation in other avian embryos apart CE (Capua *et al.*, 2002; Mutinelli *et al.*, 2003) and studies upon various factors, influencing their cultivation in CE (Rott *et al.*, 1980; Senne *et al.*, 1981; Perdue *et al.*,1989; Perdue *et al.*, 1990; Wood *et al.*,1995; Park *et al.*, 2001), such as the distribution of the virus into the embryo, determination of death time of 9-day-old CE, influence of age etc.

Investigations in the former direction such as the experiments of Capua *et al.* (2002) and Mutinelli *et al.* (2003) showed that embryo species and the pathogenicity of the AIV strain were important with regard to their cultivation. Thus, death times within 48 h (from 24 to 72 h) were evidenced in chick, turkey and Muscovy duck embryos. They were significantly delayed in Mallard duck embryos, the majority of which remained live when observed up to the 7th day despite the presence of a virus in them.

The data from the second direction allowed to make clear that low pathogenic

avian influenza viruses (LPAIV) affected the cells of the allantoic layer and did not diffuse into the embryo (Rott *et al.*, 1980; Wood *et al.*, 1995; Park *et al.*, 2001). Unlike them, the highly pathogenic AIV (HPAIV) are able to reproduce in both chorio-allantoic membrane cells and in the embryo. This difference is related to the possibility of cleavage of haemagglutinin molecule (H) to H1 and H2 by host proteases (Rott, 1992), ensuring the adsorption and penetration of the virus into the cells. The levels of haemagglutinins (assayed by haemagglutination reaction) showed no significant differences between LPAIV and HPAIV.

Senne *et al.* (1981) differentiated strains into 3 groups according to time to death in 9-day-old CE. Non-pathogenic strains provoked death from the 60th to 126th h (mean time to death 90 h), pathogenic – between hours 45 and 90 (mean 70 h) and highly pathogenic – from the 51st to the 74th h (mean 64 h). The results show that there was a significant difference between non-pathogenic and other viruses whereas the difference between LPAIV and HPAIV was not considerable.

The different sensitivity of CE is also related to their age at the time of infection (Perdue *et al.*, 1989; Perdue *et al.*, 1990). The experiments with infection of 9- and 12-day-old CE showed that 9-day-old CE died faster whereas the death in the latter occurred more slowly or did not occur at all. For HPAIV strain, such a difference was not present.

On the basis of available literature, we aimed to investigate the possibility of AIV cultivation in 14-day-old CE (non-studied up to now), to determine whether there is a difference between 9- and 14-day-old CE, the importance of viral inoculum concentration and the amount of haemagglutinins.

MATERIALS AND METHODS

Viruses

Two reference viral strains were used: A/duck/Ukraine/1/63 H3N8 and A/duck/England/56 H11N6. The viruses were selected on the basis of titrations for determination of fifty percent egg lethal dose (ELD_{50}) and fifty percent egg infectious dose (EID_{50}). Strains with the highest and lowest values were chosen. The titres for A/duck/England/56 H11N6 were $10^{7.75}$ ELD_{50}/0.1 mL and $10^{7.75}$ EID_{50}/0.1 mL, whereas those for A/duck/Ukraine/1/63 H3N8 were $10^{4.87}$ ELD_{50}/0.1 mL and $10^{5.16}$ EID_{50}/0.1 mL.

One part of the experiment was performed with inocula with high concentration of viruses. The infection was done with undiluted viruses at 0.1 mL with titres $10^{7.75}$ ELD_{50}/0.1 mL and $10^{7.75}$ EID_{50}/0.1 mL for A/duck/England/56 H11N6 and $10^{4.87}$ ELD_{50}/0.1 mL and $10^{5.16}$ EID_{50}/0.1 mL for A/duck/Ukraine/1/63 H3N8. In the second part of the trial 1000-fold diluted inocula in 0.1 mL amounts were used. The diluted viruses had titres as followed: $10^{4.75}$ ELD_{50}/0.1 mL and $10^{4.75}$ EID_{50}/0.1 mL for A/duck/England/ 56 H11N6 and $10^{1.87}$ ELD_{50}/0.1 mL and $10^{2.16}$ EID_{50}/0.1 mL for A/duck/Ukraine/1/63 H3N8.

Chick embryos

In the experiments, 9- and 14-day-old chick embryos were used. The infection was done in 10 CE. In order to obtain more confident results, the inoculations were repeated three times.

Infection and incubation of CE

The infection was done in the allantoic cavity. Infected CE were submitted to stationary incubation at 36 ^{o}C. They were observed at 24-h intervals up to the 144th

hour. All dead CE and at the end, all live ones, were stored for 2 h at 4 °C and afterwards the embryos were opened and allantoic fluids (AF) were individually collected.

Haemagglutination (HA) reaction

The reaction was performed with AF of infected CE with 1% chick erythrocytes by the method of Salk (1944), improved as an microvariant by Alexander (1982) with minimum positive results at 1:4 titres.

Detection of results

Death of CE: The minimum death time of CE (MinDT) was determined following ovoscopy and registration of death time. The obtained data (as percentage) were used for graphic presentation of MinDTs (profile of death) in used strains.

The mean death time (MDT): It was determined using the results of the 10 inoculated CE from the triple performance by summarizing the data for MinDT for all CE and then, dividing it to the total number of dead CE.

For both tests (MinDT and MDT) the results were accepted only after positive haemagglutination as a confirmation of specific deaths.

HA reaction: The mean arithmetic titres of haemagglutinin were determined (MAT). These were the sum of HA reaction values of AFs of each CE divided to the number of studied AFs.

RESULTS AND DISCUSSION

Cultivation of viruses in CE after inoculation with viral inocula at a high concentration (indiluted inoculum)

In 9-day-old CE, the inoculation of both strains resulted in 100% death that occurred at various time intervals (Fig. 1).

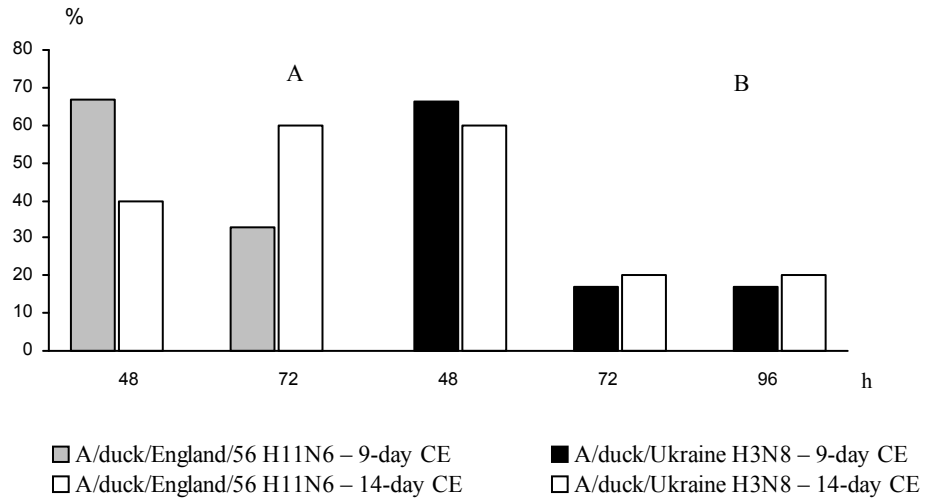

Fig. 1. Distribution of dead chick embryos (CE) after infection at the age of 9 days and 14 days with 0.1 mL inocula containing high concentrations of viruses A/duck/England/56 H11N6 (A) and A/duck/Ukraine/1/63 H3N8 (B).

For A/duck/England/56 H11N6, death occurred at MinDT from 48 to 72 h and for A/duck/Ukraine/1/63 H3N8 – at MinDT from the 48th to the 96th h. The percentages of dead CE for both viruses were the highest by hour 48 (66.67%) and differed by hour 72 (33.33 % for A/duck/England/56 H11N6 and 16.67 % for A/duck/Ukraine/1/63 H3N8). By hour 96, the percentages were 0 % and 16.67 %, respectively.

In 14-day-old CE, the overall death rate percentage of both strains was preserved (100 %). The time of its occurrence for A/duck/England/56 H11N6 (with MinDT between hours 48 and 72) was also nearly the same as A/duck/Ukraine/1/63 H3N8 (with MinDT between hours 48 and 96). A difference was observed only in the percentage of killed CE. They were lower by hour 48 (40 % for A/duck/England/56 H11N6 and 60 % for A/duck/Ukraine/1/63 H3N8) and higher at later time intervals.

The MDT for 9-day-old CE infected with A/duck/England/56 H11N6 was 56 h, whereas for CE infected with A/duck/Ukraine/1/63 H3N8: 60 h. The respective values for 14-day-old CE were 62.4 h for both strains.

HA were observed in all CE at both ages and for both strains. The individual titres for embryos infected with A/duck/England/56 H11N6 ranged between 1:16 to 1:64, and those for CE inoculated with A/duck/Ukraine/1/63 H3N8 were between 1:8 and 1:64. MAT in 9-day-old CE were slightly lower than that in 14-day-old CE for both strains. The values were 1:24 (9-day-old) and 1:48 (14-day-old) for A/duck/England/56 H11N6 and 1:22 (9-day-old) and 1:30 (14-day-old) for A/duck/Ukraine H3N8.

The results showed no substantial difference between the MinDT in 9- and 14-day-old CE inoculated with high concentration of viruses. The percentage of deaths was higher in the early hours (hour 48) at both ages. The MDT was shorter in 9-day-old embryos for both strains. Regardless of the close reciprocal haemagglutinin titres, MAT was slightly higher in 14-day-old CE. We assume that this was due to the higher percentage of CE that survived longer (later death).

Our results using viral inocula with high concentrations, although with some minor differences, confirmed the conclusions of Perdue *et al.* (1989; 1990) for a faster death in 9-day-old CE. This was determined by detection of MinDT and MDT. The differences came from the fact that in 9-day-old CE the death percentage was higher at early hours and therefore, the MDT was shorter, although the overall death occurred at the same time intervals.

Our results evidence that 14-day-old CE (other authors used CE up to the age of 12 days) could as well be used for infection with AIV.

Cultivation of viruses in CE after inoculation with viral inocula at a low concentration (inoculum diluted 1:1000)

The death rate in 9-day-old CE for both strains was 100%. The MinDT was between hours 72 and 96 for both viruses. The dead CE infected with A/duck/ England/56 H11N6 were 75 % by hour 72 and 25 % by hour 96 (Fig. 2). The majority (83.33 %) of CE infected with A/duck/Ukraine/1/63 H3N8 died by hour 72.

A 100% death rate in 14-day-old CE was observed only after infection with A/duck/England/56 H11N6. The other strain (A/duck/Ukraine/1/63 H3N8) resulted in 83.33% death rate. A difference was present in the MinDT of both strains. For A/duck/England/56 H11N6 it was by hour 72 (50% dead) and hour 96 (same

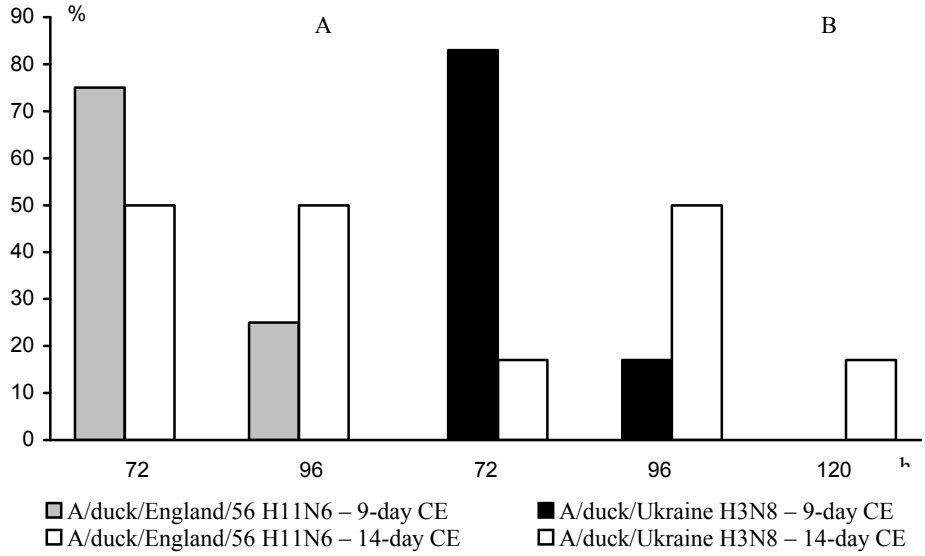

Fig. 2. Distribution of dead chick embryos (CE) after infection at the age of 9 days and 14 days with 0.1 mL inocula containing 1000-fold dilution of viruses A/duck/England/56 H11N6 (A) and A/duck/Ukraine/1/63 H3N8 (B).

percentage). For A/duck/Ukraine/1/63 H3N8 death occurred by hours 72, 96 and 120. The deaths by hour 96 prevailed (50%), whereas the percentages for the other time intervals were equal (16.67 %).

MDT in 9-day-old CE was 80 h for A/duck/England/56 H11N6 and 76 h for A/duck/Ukraine/1/63 H3N8. The MDT observed in 14-day-old CE were higher for both strains: 84 h for A/duck/ England/56 H11N6 and 96 h for A/duck/ Ukraine/1/63 H3N8.

HA reaction was observed in 100% of 9- and 14-day-old CE infected with A/duck/England/56 H11N6 and in 100% and 96.67 % of 9- and 14-day-old CE infected with A/duck/Ukraine/1/63 H3N8, respectively. The HA titres in 9-day-old CE infected with the A/duck/England/56 H11N6 strain were between 1:4 to 1:64 and MAT was 1:33.5, whereas in 14-day-old CE HA titres ranged between 1:4 and 1:512 and MAT was 1:116.75. Higher HA values were observed in 14-day-old CE infected with A/duck/Ukraine/1/63 H3N8. With equal titres for both ages between 1:8 and 1:32, MAT in 14-day-old CE was 1:20.8 vs 1:17 in 9-day-old CE.

The results for both viral strains showed clearly and definitely a higher susceptibility of 9-day-old CE infected with lower viral concentration compared to that in both ages after employing viral inocula with higher concentration. Similar conclusions are also reported by Perdue *et al.* (1989; 1990). Furthermore, in our experiment, the infection with one of strains (A/duck/Ukraine/1/63 H3N8) did not result in death in 3.33% of 14-day-old CE. A prolonged MinDT was observed in CE infected with A/duck/Ukraine/1/63 H3N8 (72^{nd} – 96^{th} h and 72^{nd}–120^{th} in 9- and 14-day-old CE, respectively. In this experiment, similarly to that using viruses at higher concentration, the percentages of CE killed by both strains were higher at early time intervals and the MDT in 9-day-old CE was shorter.

Comparing the data of undiluted and diluted viruses, considerable variations in the MinDT could be noticed. When diluted, both viruses exhibited MinDT delay by 24 h in both ages of CE. The delay was both for the early hours of death (48 h for undiluted vs 72 h for diluted) and the late hours of death occurrence (72 h vs 96 h for A/duck/England/56 H11N6 and 96 h vs 120 h for A/duck/Ukraine/1/63 H3N8). These fact reflected upon MDT. In both strains used at both concentrations, it was shorted in 9-day-old CE. The cause for that, also evidenced by other authors (Perdue *et al.*, 1990) is still not clear. It is hypothesized that complex causes as change in CE anatomy after the 10^{th} day of life, the appearance of new barriers (septa) that impede the distribution of non-pathogenic influenza viruses (used by us), reduced level of proteases necessary for haemagglutinin cleavage, appearance of harmful components as urates, inactivating the virus, could be involved (Perdue *et al.*, 1990).

The HA values showed higher MAT in 14-day-old CE for both strains. In our opinion, the cause was the later occurrence of death of CE allowing a higher accumulation of haemagglutinins.

CONCLUSIONS

The avian influenza A viruses used in our experiment could be cultivated in CE at the age of 9 to 14 days. A more rapid death occurred after infection with viruses at a higher concentration, whereas the lower concentrations prolonged the survival by 24 hours.

In 9 day-old CE, infected with both undiluted and diluted viral inocula, the death occurred faster than in 14-day-old CE. Higher MAT of haemagglutinins were observed in 14-day-old CE vs 9-day-old CE.

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