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Effects of SEU Condition In Incubation of Embryonated Chicken Eggs Inoculated With Avian Influenza (H_9N_2) Virus by the Intra-Allantoic Route.

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ABSTRACT

Older laying breeder flocks causes various abnormalities in appearance of eggs. Big and spherical eggs are cause of small end up (SEU) position in the field and virus replication process. Thus in this study we evaluate the effect of SEU position in embryo deaths and avian influenza virus (AIV) replication cycle. 1200 embryonated chicken eggs (ECEs) from laying breeder flock at 60-65 weeks of age were divided in 3 groups. First group was in large end up (LEU) position pre and post inoculation of virus. Second group was in SEU position in pre inoculation stage and in LEU position in post inoculation stage. Third group was in SEU position pre and post inoculation. All groups were incubated for 11 day at 37.6°C and 60% humidity and turned 32 times daily. After inoculation the incubation condition was like before but it was for 3 days and without turning. In harvesting stage, the amnio-allantoic fluid (AAF) is withdrawn into the measuring cylinder. AAF from surviving embryos was tested for hemagglutination (HA) activity and egg infective dose 50 (EID_{50}). Assays were performed in three replicates. Amount of utilizable eggs in second group vs first group was 1 to 3 and in third group was 0 %. Also AAF titer in second group was significantly (p<0.05) lower than first one. Thus SEU position has important negative effect on virus replication and embryo deaths in virus growth cycle.

Keywords: SEU position, Incubation, Embryonated chicken eggs, Avian H_9N_2 Influenza virus

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INTRODUCTION

In the process of replication of avian influenza virus (AIV) vaccinal seed (H9N2), fertile eggs, as primary raw material, have the main role for culture. Fertile eggs from young flocks (35-50 weeks), both in terms of appearance including the oval shape of egg, tightening and thickening of the shell, no presence of veins and cracks and in terms of internal consistency including small volume of air chamber, albumen and yolk quality and potential quality of embryo are proper for the storage and incubation periods and have low mortality.

Sometimes consideration of high level of biosecurity conditions in egg producing farms, leads to the use of older laying breeder flocks because fertile eggs using in process of the replication of influenza virus should be negative especially for Mg (*Mycoplasma gallisepticum*), Ms (*Mycoplasma synoviae*) and influenza A genus. By the aging of laying breeder flock (60-70 weeks), variety of external and internal weaknesses and malformations appears in the egg such as thinning and weakening of the shell and increase of veins and cracks, increasing in egg weight and be spherical of its, increasing of air chamber volume and potentially vulnerable and weakness of embryo. Such eggs are sensitive to the storage and incubation conditions and because of high mortality, their final yield were reduced considerably. As well, detection of large and small ends of these eggs is difficult and may be cause setting the eggs in situation of small end up (SEU) in farm and entrance of those in the same position to process of influenza virus replication. Early data from Byerly and Olsen demonstrated that 2 to 3.5 % of fertile eggs possessed embryos with their heads in the small end of the egg [1]. Also North and Bell found that as many as 1 to 4 % of 18-day-old embryos were malpositioned in some manner [2]. The common malposition of embryos, with the head in small end of the egg, has been suggested to be due to egg orientation (e.g., set upside down), air hunger, insufficient turning frequency, improper turning angle [1,3] lack of turning during the first, but not second, week of incubation, and increased breeder flock age [4]. Also Elibol and Brake [5, 6] reported that the loss of fertile eggs from older flocks (57-61 weeks), were further classified as early dead (0 to 7 days), middle dead (8 to 17 days) and late dead (18 to 21 days) that possessed embryos with their heads away from the large end of the egg, generally in the small end of the egg, and mortality rate in this group (late-dead embryos) is higher than other groups. Such eggs has been shown to be sensitive to turning deficiencies, as a means to determine the feasibility of compensating for a reduced turning angle with increased turning frequency that improved their hatchability [5,6]. Wilson and colleagues, found a major cause of detrimental malpositions, especially in broiler eggs, is setting eggs with the SEU; it can also explain the poor hatchability (8%) found in Japanese quail eggs incubated in this position [3].

A little information is available about the effect of position of eggs during the incubation (before and after virus inoculation) impact on replication of avian influenza virus (AIV) (H9N2) in allantoic cavity, mortality rate of embryos and viral titer of amnio-allantoic fluid (AAF) yielded from the eggs. In this study we evaluated the influence of SEU position (before and after virus inoculation) on mortality rate of embryos and growth cycle of virus.

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MATERIAL AND METHODS

Viral stock preparations

Standard vaccine strain AIV (A/Chicken/Iran/99/H9N2) was inoculated into 11-day-old specific pathogen free (SPF) embryonated chicken eggs (ECEs). The eggs were observed for 24-72 hrs post inoculation according to the Manual of Standards for Diagnostic Tests and Vaccination [7]. The AAFs of the inoculated eggs were collected and centrifuged at 1200 rpm for 30 minute.

The egg infective dose 50 (EID_{50}) was calculated by Reed and Muench method in 11-day-old embryonic SPF eggs [8].

Hemagglutination assay (HA) was performed in V-bottom 96-well plates with 1% chicken red blood cell as described by Burleson et al [9].

Study design

1200 one-day-old ECEs from Hy-line laying breeder flock at 60-65 weeks of age, that were negative for Mg (*Mycoplasma gallisepticum*), Ms (*Mycoplasma synoviae*) and influenza A genus, were selected.

All eggs were disinfected according to the Animal Production and Health Paper [10]; then they were divided into 3 groups: First group was in large end up (LEU) position pre and post inoculation of virus. Second group was in SEU position in pre inoculation stage and in LEU position in post inoculation stage. Third group was in SEU position pre and post inoculation.

All groups were incubated (Petersime model 576) for 11 day at 37.6°C and 60% humidity and turned 32 times daily. Incubator was cotrolled 3 times daily and registerred all items. At the 11^{th} day, all groups were candled and early death embryos (infertile eggs and dead embryos) were removed [10], then the 10^{-5} dilution of the H9N2 virus work seed (EID_{50} = 9.8 log10 and HA titer = 10 log2) was inoculated into ECEs at the rate of 0.1 ml/ECE, via intra-allantoic way [7]. All eggs were sealed with wax. After inoculation, the incubation condition was like before but it was for 72 hrs and without turning. Eggs were candled daily and early death embryos in first 24 hrs were removed.

In the next step, the eggs were chilled at 4 ˚C for 24 hrs to kill the embryo and to reduce the contamination of the AAF with blood during harvesting [10].

In harvesting stage, after removing the shell and shell membranes at the blunt end of the eggs, the AAF samples were withdrawn into the small measuring cylinders by pressing the suction bulb until the total volume of each egg AAF was obtained [11]. Each group AAF volume was measured accurately. The mortality rates of eggs were identified exactly in each group, during the process. Mean of extracted fluid volume from extractable eggs in each group and

mean of extracted fluid volume from total eggs in each group were calculated. AAF samples of each group were tested for HA activity as described by Burleson et al and for EID_{50} by Reed and Muench method [8,9]. Assays were performed in three replicates.

Statistical analysis

Data were analyzed using SPSS (version 17 for Windows, SPSS Inc., Chicago, IL, USA), and comparisons were made using the descriptive statistics and one way ANOVA tests.

RESULTS

Table 1 shows the results of three replicates performed on all groups. In 11^{th} day candling (before inoculation), percentage of infertile eggs and dead embryos eggs was similar in second and third groups, but higher than the first group. Total rate of mortality in the first, second and third groups was 24.75%, 76% and 100% respectively. In the second and third groups, 44% and 50.25% of mortalities were detected during the AAF harvesting stage respectively. Thus amount of utilizable eggs (capable for virus replication) in second group vs first group was 1 to 3 and in third group was 0 % ($p<0.05$).

Also this paper results showed that mean of extracted AAF volume from extractable eggs and mean of extracted AAF volume from total eggs in first group was 2.5 and 8 times in compare with the second group respectively. Did not extracted any AAF from third group. AAF titer in second group (EID_{50}=8.9 log10 and HA=8 log2) was significantly lower than first one ($p<0.05$).

DISCUSSION

The results of present study showed that detection of large and small ends of the eggs from older laying breeder flock is difficult and may be cause setting the eggs in situation of SEU in farm and entrance of those in the same position to process of influenza virus replication. This situation increased the mortality rate of embryos in various stages of process.

Also these results indicated that more than 75% of eggs that candled in incubation stage and corrected to LEU situation, are considerably effective in process of virus replication. According to Cain and Abbote (1971) study, approximatey all species of birds lay eggs with a certain angle in their nest while the small end is down[12]. Also during artificial incubation, LEU position improved the growth of embryo and increased fertile hatchability of eggs as compared with SEU position [5,13]. Results of present study as well indicated that setting the eggs in situation of LEU during incubaction (pre and post virus inoculation), decreased the embryonic mortality and improved the mean of extractable AAF volume.

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Table 1: Interaction of small end up (SEU) and large end up (LEU) positions of eggs with mortality rate of eggs and extractable AAF volume and titers (Three replicates mean values ± SD).

Group Number	Total eggs (No.)	Mortality (%. and No.)						Total extractable eggs (%. and No.)	Mean of extracted AAF volume (ml)		HA (Log2)	EID_{50} (Log10)
		Pre inoculation candling		24 hr post inoculation candling	48 hr post inoculation candling	During AAF harvesting	Total mortality		From extractable eggs	From total eggs		
		Dead embryos	Infertile eggs									
First Group	400	9^{a}±52 2.2±13	3^{a} ±18 0.7±4.5	2.6^{a}±2 0.6±0.5	2.6^{a}± 5 0.6±1.25	22±6.2^{a} 1.5±5.5	12^{a}±99 3±24.75	12^{a}±301 3± 75.25	0.25^{a}±10 .1	0.1^{a}±7.6	10^{a}	9.67^{a}
Second Group	400	14^{ab}±59 3.5±14.75	4^{ab}±22 1±5.5	4.5^{b}±19 1.1±4.75	9.1^{b}±28 2.2±7	176±14.5^{b} 3.6± 44	18.3^{b}±30 4 4.5 ±76	^{b}3.±1896 4.5 ±24	0.89^{b}±4	0.1^{b}±0.9	8^{b}	8.9^{b}
Third Group	400	8.8^{b}±64 2.2±16	5.1^{b}±20 1.2±5	7^{c}±44 1.7±11	12.1^{c}±71 3±17.75	27.7^{c}±201 6.9±50.25	0^{c}±400 0±100	0^{c}±0 0±0	–	–	–	–

First group: eggs were in LEU condition pre and post inoculation of virus.

Second group: eggs were in SEU condition in pre inoculation stage and in LEU condition in post inoculation stage.

Third group: eggs were in SEU condition pre and post inoculation.

^{a,b,c} in each column: difference between letters indicates significant differences ($p < 0.05$).

Ralph and Moraes et al., reported that the Japanese quail eggs (*Coturnix japonica*) incubated at vertical position with SEU presented the highest level of egg weight loss during incubation, had a smaller number of hatched eggs and had a higher rate of

embryo deaths compared to the other eggs incubated at vertical position with LEU [14-16]. Wilson and colleagues as well found the most important cause of harmful malpositions, is setting eggs with the SEU; it can also explain the poor hatchability (8%) found in Japanese quail eggs incubated in this position [3]. In this study we have shown that the incubation of eggs with SEU position before virus inoculation increased the embryonic mortality to 51.25% during the AAF harvesting stage. According to Elibol and Brake the loss of fertile eggs from older flocks, were further classified as early, middle and late deads that possessed embryos with their heads in the small end of the egg, and mortality rate in the last group is higher than other groups [6]. In this paper we did not survey of embryos malpositions, but our results were similar to other researches about low level of quality and high rate of mortality during storage and incubation the eggs from older flocks (60-65 weeks).

This study found that the candling of eggs at start of incubation stage and corrected to LEU situation, by reducing the embryonic mortality and improving the extractable AAF volume, can highly effect in process of virus replication. Because of low quality of eggs from older flocks, pre incubation storage conditions [6], long-term storage [16] and position of eggs during storage [3, 17] are effective on reach to upward results. It seems that in next studies, by research on eggs from younger flocks (35-50 weeks) can raise the accuracy of tests.

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