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EFFECT OF *IN OVO* VACCINATION PROCEDURES ON JAPANESE QUAIL EMBRYOS (*Coturnix japonica*) AND INCUBATION PERFORMANCE

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ABSTRACT

This work aimed to evaluate the effects of *in ovo* vaccination procedures on incubation of Japanese quail eggs. The experiment was carried out in a (4 x 3) factorial design with 12 experimental treatments (4 injection days x 3 injection protocols). The injections were tested on four incubation days: at 0, 5, 10 or 15. On each injection day, the eggs were submitted to one out of three distinct injection procedures: saline injection and Newcastle disease (ND) vaccine plus saline or industrial diluent. The eggs were incubated at 37.5º C and 60% RH. All eggs and hatched quails were weighed. Unhatched eggs were opened to classify embryo mortality. Hatched quails were raised to obtain blood to evaluate antibody response against Newcastle disease virus (NDV). The injection process itself (saline) was not harmful at 10 and 15 days of incubation for Japanese quail eggs; however, in ovo vaccination with live ND vaccine (HB1 strain) is not recommended to fertile quail eggs at any incubation periods due to high levels of embryo mortality and poor post-hatch antibody titers.

KEYWORDS: eggs; *in ovo* injection; Japanese quail; Newcastle disease; vaccination.

EFEITO DOS PROCEDIMENTOS DE VACINAÇÃO *IN OVO* SOBRE EMBRIÕES DE CODORNA JAPONESA (*Coturnix japonica*) E DESEMPENHO DA INCUBAÇÃO

RESUMO

Esta pesquisa avaliou os efeitos dos procedimentos de vacinação *in ovo* na incubação artificial de codornas japonesas. Foi realizado um delineamento fatorial 4 x 3 com 12 tratamentos (4 dias de injeção x 3 protocolos de injeção). As injeções foram realizadas nos dias 0, 5, 10 ou 15 de incubação. Em cada um desses dias, os ovos foram injetados com 3 protocolos diferentes: injeção de soro fisiológico e injeção da vacina vírus vivo da doença de Newcastle (DN) com soro fisiológico ou diluente industrial. Os ovos foram incubados a 37,5°C e 60% UR. Todos os ovos e codornas nascidas foram pesados. Os ovos não eclodidos foram submetidos ao embriodiagnóstico. As codornas nascidas foram criadas para coleta de sangue e avaliação de títulos de anticorpos contra o vírus da DN. Os resultados demonstraram que a inoculação em si (soro) não foi prejudicial para os ovos com 10 e 15 dias de incubação. Verificou-se que a vacinação in ovo com o vírus vivo da DN (cepa HB1) não é recomendada para ovos férteis de codorna em nenhuma idade do embrião, devido aos elevados índices de mortalidade embrionária e pouca resposta de anticorpos após o nascimento.

PALAVRAS-CHAVE: codorna japonesa; doença de Newcastle; injeção *in ovo*; ovos; vacinação.

INTRODUCTION

In ovo technology has been studied in the last few years for administration of hormones, nutrients and vaccines. It has been widely applied for vaccination purposes. This technology is already present in 30 countries and accounts for more than 85% of broilers and 60% breeders vaccinated in United States and Canada (BERCHIERI & BOLIS, 2003).

In chicken, *in ovo* vaccines are administered to embryos on day 18 of incubation, which is normally when incubating eggs are transferred to the hatcher (LI et al., 2005). This method offers the advantages of reducing chick handling, improving hatchery manageability through automation, reducing the costs of live production and stimulating an early immune response (JOHNSTON et al., 1997). Many studies have proceeded to investigate the efficacy and safety of *in ovo* vaccination against Marek's disease (SHARMA & BURMESTER, 1982), infectious bronchitis (WAKENELL & SHARMA, 1986), infectious bursal disease (IBD) (GIAMBRONE et al., 2001), and Newcastle disease (AHMAD & SHARMA, 1992).

Newcastle disease (ND) is a highly contagious viral disease of poultry and other bird species caused by specified viruses of the avian paramyxovirus type I (APMV-I) belonging to the family Paramyxoviridae (MAYO, 2002).

Studies within the last few years have shown, however, that only few live vaccines that are routinely administered to hatched chicks may also be injected into embryonated eggs during the late stages of embryonic development without a lethal effect (MAST & MEULEMANS, 2003). NDV strains of low virulence such as the Bl strain (AHMAD & SHARMA, 1992) and NDV clone-30 (MEBATSION et al., 2001), which are routinely administered to hatched chicks, cannot be employed for *in ovo* vaccination in their current form due to their embryonic lethality. In order to attenuate NDV strains, different approaches have been applied. Hitchner Bl derived NDV strain is recommended for *in ovo* vaccination, mutated by the chemical agent ethyl methanesulfonate (AHMAD & SHARMA, 1992). According to LIMA et al. (2004), vaccination programs can efficiently eradicate this NDV in quails.

However, the *in ovo* vaccination procedures against ND for Japanese quail eggs have not been studied. Thus, the objective of this research was to evaluate the effect of *in ovo* vaccination procedures on incubation performance in Japanese quail embryos (*Coturnix japonica*), analyzing the effect of the inoculation itself as well as the vaccinal virus on the embryos.

MATERIAL AND METHODS

Eighty Japanese quails (*Coturnix japonica*) were used for egg collections. They were housed in experimental battery cages in the Laboratório de Estudos Ornitológicos da Universidade Estadual do Ceará. Three females and one male were lodged in each cage (25 x 25 x 20cm). The birds were 12 weeks old and averaged 90% egg production. Water and balanced feed were supplied *ad libitum* according to NATIONAL RESEARCH COUNCIL (1994). They were also submitted to 17 hours/day of light. All the collected eggs were selected verifying egg shape, extreme sizes and eggshell integrity by candling.

A total of 600 quail eggs was divided into a (4 x 3) factorial design with 12 experimental treatments (4 injection days x 3 injection types), each group had 50 eggs. Other 150 eggs were also incubated without any injection (Control group).

The *in ovo* injections were tested at four incubation days: at 0 day (prior to incubation), 5 days, 10 days or 15 days of incubation (transference day).

On each injection day, the experimental eggs were submitted to three distinct injection procedures: saline injection, Newcastle vaccine plus saline injection or Newcastle vaccine plus industrial diluent injection.

The eggs were removed from incubators and the *in ovo* injection was performed at room temperature in a clean lab. First the eggs were disinfected with ethyl alcohol (70%) and then they were placed with their large end up. This part of eggshell was holed with a sterile hollow neddle (0.8mm diameter) to allow the injection of 0.025mL of inoculum. The eggs were injected by an adjustable micropipette with plastic tips adapted with hollow neddles (0.7mm diameter). The inoculum was delivered at 4-5mm deepth from eggshell surface. The injection procedure made a hole in the eggshell with an area of approximately 0.5mm² and then the eggs were placed again in the incubator machines.

All treatments consisted of injection of 0.025mL of a solution as follows: saline, vaccine plus saline or vaccine plus industrial diluent.

Saline: Physiological saline solution 0.9% Sodium Chloride. Na+ 154 mEq/L, Cl- 154 mEq/L. Total osmolarity of 308 milliosmoles per liter and 6.0 pH.

Vaccine: live freeze-dried vaccine of Newcastle disease, Hitcher B1 strain (HB1).

Industrial diluent: Sterile diluent for vaccination by eye drop route consisting of water treated by reverse osmosis and inert dye. Both vaccine and diluent were made by the same manufacturer.

Live freeze-dried vaccines were rehydrated just before the use for injection into quail eggs with industrial diluent or saline according to each experimental treatment. The vaccine dilution was prepared by mixing 1000 doses of vaccine with 30 mL of diluent. All procedures from storage to manipulation of injected substances were carried out according to manufacturers' recommendations.

All eggs were identified individually and weighed on their collection day and on the 15th day of incubation to verify egg weight loss. All hatched quails were weighed individually after hatching. Weight measurements were obtained with a precision balance (0.001g).

Eggs were incubated in horizontal position by automatic hatcheries with temperature of 37.5°C, relative humidity of 60% and egg turning every 2 hours. On the 15th day of incubation (360h) the eggs were transferred to the hatcher, which maintained the same conditions of humidity and temperature, but without turning. After hatch the quails were housed in an experimental poultry facility. They were reared up to 15 days, in experimental cages according to each treatment. Each cage was supplied with water and balanced feed *ad libitum* according to NATIONAL RESEARCH COUNCIL (1994), 24 hours of light and heat. After 15 days, all quail chicks were euthanized to perform the blood collections.

Eggs that failed to hatch were opened for macroscopic observation, thus they were classified according to time of embryonic mortality. They were staged as infertile-early death embryo, which were the eggs with true infertility, pre-incubation mortality or initial stage mortality. The embryos that presented mortality in the intermediate development stage were classified as intermediate embryo death. Unhatched eggs classified as final embryo death were the ones with late stage mortality or pipped eggs with dead embryos. This classification was according to PEDROSO et al. (2006), who classified the embryo mortality in quail chicks as early death embryos (1 up to 4 days), intermediate (5 up to 15) and late death embryo (16 up to 18 days).

Hatched quails were reared separately according to each experimental group up to 15 days, when blood collections were made in order to assess the antibody response against Newcastle disease vaccination.

The birds were euthanized by decapitation and blood samples were collected from jugular vein. Sera were separated, identified and frozen at -20ºC until the serological tests were performed.

Serological analysis was performed by haemagglutination inhibition test (HI) according to ALLAN & GOUGH´s (1974) methodology. The test was done in V-bottomed microtitre plates with 96 wells. It started with the addition of 25 µL of phosphate buffered saline-PBS (pH 7.2) in each well of the microtitre plates. A multichannel pipette was used to perform serial twofold dilutions of each serum sample along the row by transferring 25 µL of fluid from one well to the next. 25 µL of ND virus antigen at a concentration of 4 haemagglutination units was added to each well. The side of plates was tapped gently to mix, and then they were covered and allowed to stand at room temperature (26ºC) for 30 minutes. Afterwards, 25 µL of a 1% suspension of red blood cells was added to each well. The side of plates was tapped gently to mix again, and then they were covered and allowed to stand at room temperature for 45 minutes for the appearance of a pattern of haemagglutination. The agglutination pattern was read and the titers were recorded as the highest dilution of serum that caused complete inhibition of haemagglutination.

HI results from individual birds were expressed as the reciprocal of the end point serum dilution. Three rows of wells were left as controls: the first row contained a known NDV antiserum (positive control), the second row contained NDV antigen alone (negative control) and the third row contained normal saline with chicken red blood cells (reagent control).

A total of 600 quail eggs was divided into a (4 x 3) factorial design with 12 experimental treatments (4 injection days x 3 injection types). Each experimental group (n=50) consisted of five replicates of 10 eggs. Additional 150 eggs were not part of the factorial design but were used as control. Data from factorial design for the different analyzed variables (hatchability, egg weight loss, hatch weight and embryo mortality) was analyzed by the PROC MIXED from SAS v.9.0 (2002) and the means were compared by PDIFF statment adjusted by Tukey allowing all pairwise comparisons. Letters for statistical differences were obtained by the Macro PDMIX.800. A second statistical approach was applied to compare the control group results with each of the other 12 experimental treatments. This analysis was carried out using PROC GLM and the means were compared with Dunnett's test.

Statements of significance were based on $P<0.05$.

RESULTS AND DISCUSSION

Figure 1 shows the hatch rates of Japanese quail eggs that were in ovo injected at four different periods of incubation with three different solutions.

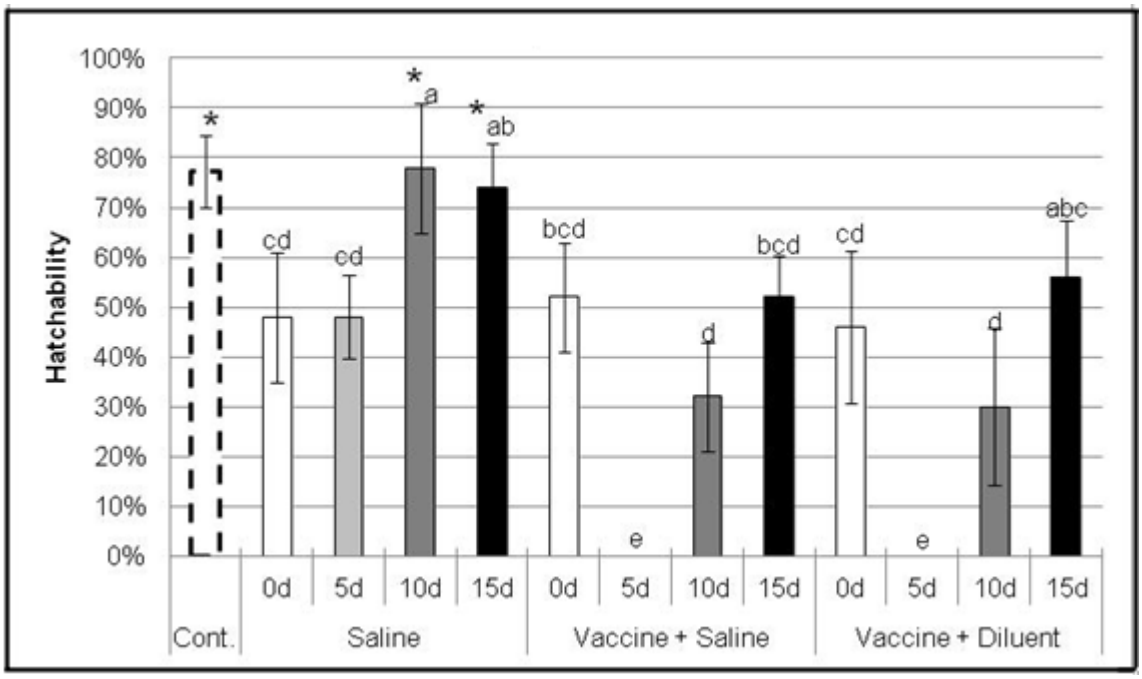

Figure 1 - Hatchability of a Japanese quail eggs submitted to different protocols of *in ovo* injection at various days of incubation.

[a,b,c,d,e] Means with different superscripts differ significantly ($p < 0.05$).

* Means with asterisk do not differ from control group ($p<0.05$).

The results showed that eggs that received *in ovo* injection before the incubation (0 day) presented similar hatch rates. This shows that at this period the injection of live vaccine of ND is not detrimental to hatchability when compared to the group that received only saline. At this period the embryo is still in its early stages of development and almost all egg's volume is filled by albumen and yolk. According to BURLEY & VADEHRA (1989) the albumen proteins present non-specific defenses against microorganisms and probably against virus. This could be the reason why the live virus was not able to affect the egg hatchability compared to the eggs that received saline. The process of *in ovo* injection before incubation even with saline solution was harmful to embryo survival, since the hatch rates of injected eggs were lower than the control group ($p<0.05$).

The hatchability of eggs injected with saline at 5 days was similar to the ones injected before the incubation. However, this hatch rate was completely different to the eggs injected at 5 days with vaccine which had 0% hatchability. This result shows that the *in ovo* injection with live vaccine of ND (B1) was lethal to quail embryos at 5 days of development. This finding is in accordance with some studies that have shown that only few live vaccines that are routinely administered to hatched chicks may also be injected into embryonated eggs during the late stages of embryonation without a lethal effect (MAST & MEULEMANS, 2003). NDV strains of low virulence, such as the Bl strain (AHMAD & SHARMA, 1992) and NDV clone-30 (MEBATSION et al., 2001), which are routinely administered to hatched chicks, cannot be employed for *in ovo* vaccination in their current form due to their embryonic lethality.

The eggs injected at 10 and 15 days with saline presented hatch rates similar to control, showing that the injection procedure was not detrimental to quail embryos at 10 and 15 days of development. However, eggs injected with live vaccine at 10 and 15 days had lower hatchability than the control group. Interestingly, the embryos at 15 days of development seemed to be more resistant to live vaccination compared to embryonated eggs vaccinated at 10 days, since those showed lower hatch rates compared to the older ones. The results indicate that the HB1 strain cannot be applied to *in ovo* vaccination to quail eggs due to its high impact on hatchability. It is relevant to mention that on the transference day (15 days for quails and 18 days for

chickens), Japanese quail embryos seemed to be more resistant than chicken embryos regarding the *in ovo* vaccination with a lentogenic NDV strain since chicks were shown to present up to 100% mortality (SARAVANABAVA et al., 2005). Maybe a process of attenuation of this strain could produce a better hatchability for quail eggs. Different approaches have been applied in order to attenuate NDV strains. AHMAD & SHARMA (1992) described a Hitchner Bl derived NDV strain for *in ovo* vaccination, mutated by the chemical agent ethyl methanesulfonate. La sota strain has also been attenuated to *in ovo* vaccination by selection of scape mutants with monoclonal antibodies (MAST et al., 2006).

The use of saline solution or industrial diluent to rehydrate the freeze-dried vaccine did not promote differences among the groups of eggs vaccinated at the same period. Thus, it was verified that both saline solution and industrial diluent have similar effects on hatch rate concerning their use to *in ovo* vaccination against ND.

Figure 2 shows the percentile of weight loss of Japanese quail eggs that were in ovo injected at four different periods of incubation with three different solutions.

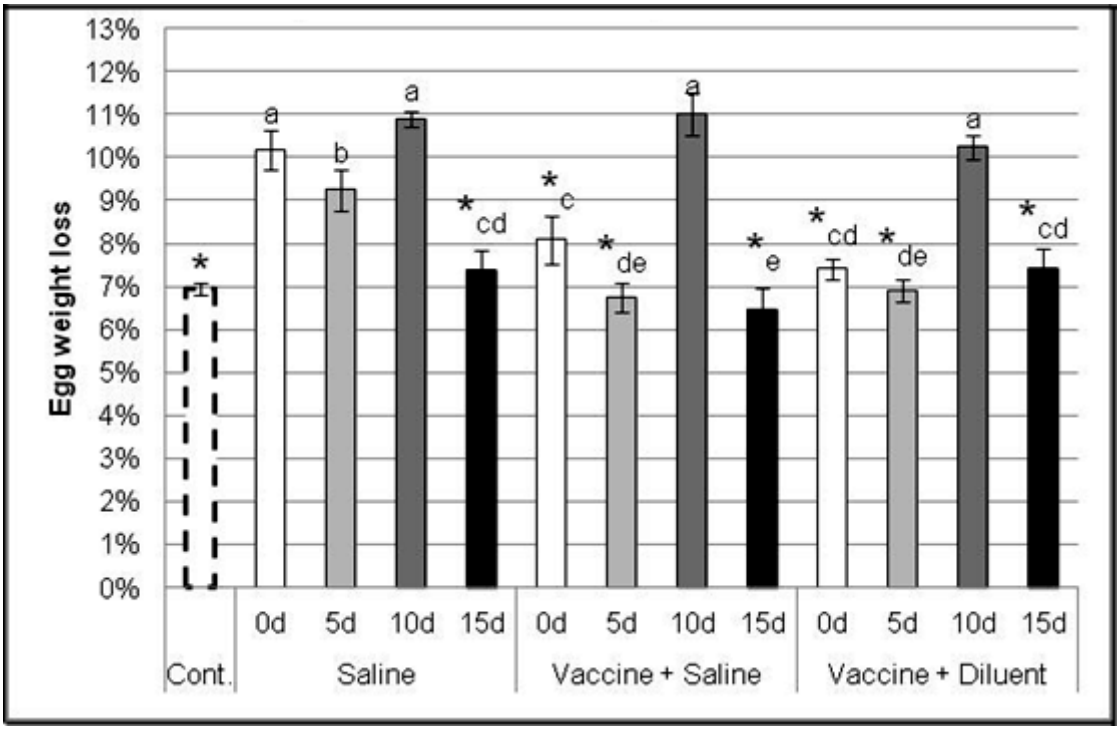

Figure 2 - Egg weight loss of Japanese quail eggs submitted to different protocols of *in ovo* injection at various days of incubation.
[a,b,c,d,e,] Means with different superscripts differ significantly ($p < 0.05$).
* Means with asterisk do not differ from control group ($p<0.05$).

The egg weight loss is an important parameter for incubation. It has been used to estimate vital gas exchange (PAGANELLI et al., 1978; RAHN et al., 1979) and has been correlated with the rate of embryonic metabolism and development (RAHN & AR, 1980; BURTON & TULLET, 1983). AR & RAHN (1980) examined the loss of mass in eggs during incubation and evidences showed that this was essentially due to loss of water. The water vapor conductance is related to the pore area and thickness of eggshell (RAHN & AR, 1974).

Some of the treatments had an egg weight loss similar to eggs in control group, while others had a higher egg weight loss. That can be partially explained by the hole that was made to perform the injection of solutions. The process of *in ovo* injection into the egg creates a hole with approximately $0.5mm^2$ that increases the conductance area of eggshell. Without artificial holes, the gas exchange is made only by the pores that are naturally distributed on eggshell.

It was not found any statistical difference for eggs injected at 15 days, thus, this process does not interfere when *in ovo* injection is performed at the later stages of embryo development.

In general, the eggs injected at 10 days of

incubation presented higher levels of weight loss compared to eggs that were injected earlier (0 and 5 days) and later (15 days). The increase of weight loss and therefore the conductance between the egg and the environment can be particularly interesting for poultry embryos at the later stages of incubation for a better performance of gas exchange and respiration. In chicken eggs, the *in ovo* injection performed by 16 gauge (1.6mm diameter) needles provide an addition of 25 to 30% of relative pore volume, which can be considered as a physiological advantage (BERCHIERI & BOLIS, 2003).

Figure 3 shows the chick/egg weight ratio (%) of Japanese quail eggs that were *in ovo* injected at four different periods of incubation with three different solutions.

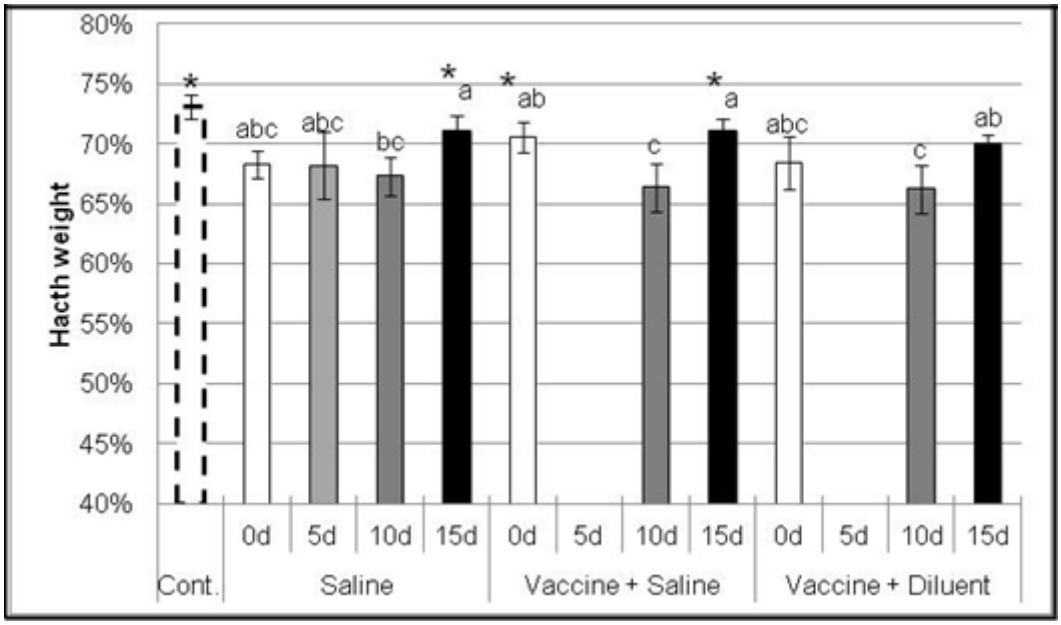

Figure 3 - Hacth weight of Japanese quail submitted to different protocols of *in ovo* injection at various days of incubation.
[a,b,c,d] Means with different superscripts differ significantly ($p < 0.05$).
* Means with asterisk to not differ from control group ($p<0.05$).

The weight of chicks at hatch can be affected by several factors, including species, breed, egg nutrient levels, egg environment, egg size (WILSON, 1991), weight loss during incubation period, weight of shell and other residues at hatch (TULLET & BURTON, 1982), shell quality and incubator conditions (PEEBLES et al., 1987). In this experiment, the hatch weight was affected by different factors, such as the procedures of *in ovo* injection. Most *in ovo* injection treatments promoted a reduction in hatch weight compared to the eggs that were not injected (control group). The eggs injected at 10 days of incubation were the ones that presented the lower chick/egg weight ratio. We believe that this lower hatch weight for eggs injected at 10 days can be related to their weight loss during incubation. Increased weight loss and reduced hatch weight may promote detrimental effects on survival of newly hatched quails, since these parameters may lead to dehydration. Small chicks have higher surface area to weight ratios and are therefore more easily dehydrated than larger chicks. Dehydration has been reported to be associated with higher mortality of chicks from young breeders (WYATT et al., 1985).

The eggs inject at 15 days of incubation were among the ones that seemed to be the least affected by *in ovo* injection, concerning hatch weight, which might indicate that the older the embryo (past 10 days of development) at the time of *in ovo* injection the higher will be the hatch weight, probably due to a lower egg weight loss.

Eggs injected with ND live vaccine or saline solution were not statistically different when compared in the same injection day, showing that the *in ovo* vaccination with Newcastle disease virus did

not alter initial weight of hatched Japanese quails. The exception were the eggs vaccinated with live ND virus at 5 days, which did not survive; therefore, they were not measured for hatch weight.

Figure 4 shows the frequency of embryo mortality of Japanese quail eggs that were *in ovo* injected at four different periods of incubation with three different solutions.

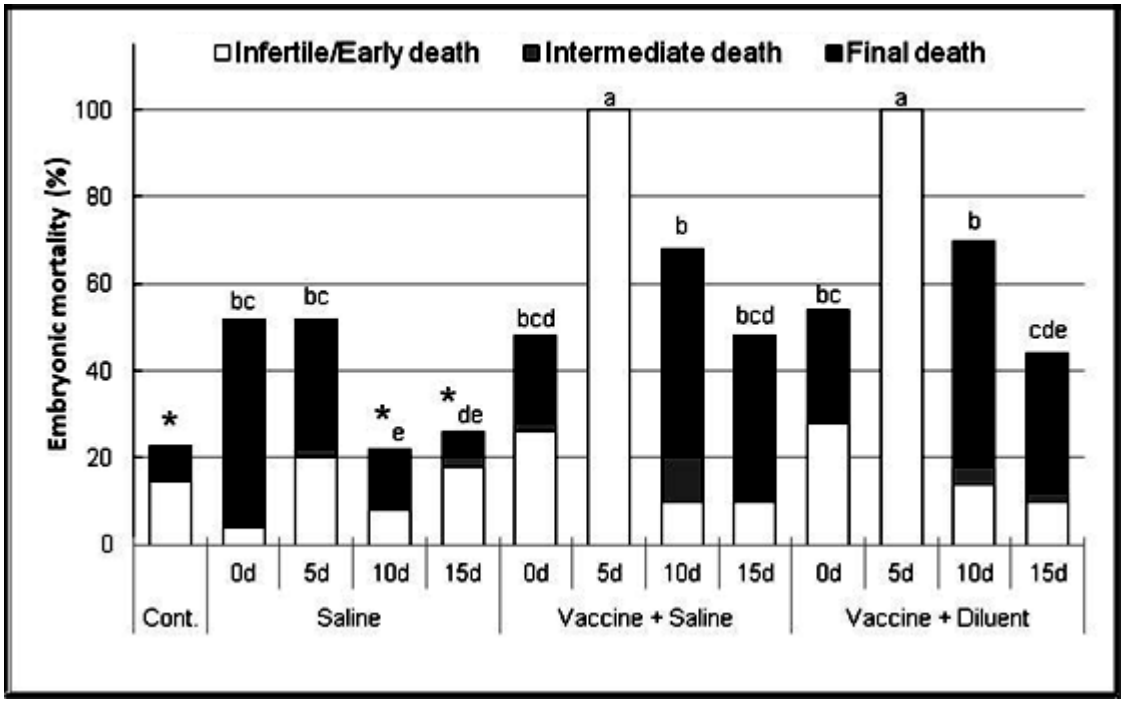

Figure 4 - Frequency of embryonic mortality of Japanese quail eggs submitted to different protocols of *in ovo* injection at various days of incubation.
[a,b,c,d,e] Means with different superscripts differ significantly ($p<0.05$).
* Means with asterisk do not differ from control group ($p<0.05$).

Differences on embryonic mortality were not found between eggs injected on the same day with vaccine plus saline or diluent. Most injection treatments results differed from the control group (not submitted to any injection procedure). The only groups that did not differ significantly were the ones injected at 10 or 15 days with saline. Most injected groups seemed to have higher levels of final death than infertile/early death. On the other hand, the eggs injected with saline at 15 days and the ones injected with vaccine at 0 and 5 days showed infertile/early death as the main reason for failure to hatch. The intermediate embryo death was the least relevant classification of unhatched eggs and did not appear in all groups. These observations are similar with those from chicken eggs that do not present relevant intermediate mortality, since there are two phases of increased embryonic mortality during incubation: the first phase occurs during the first week of incubation and the second phase during the last week (JASSIM et al., 1996). The process of *in ovo* injection can increase embryo mortality since the hole made in the eggshell provides the opportunity to microbial infection. When the hole is made at the later stages of embryo development the time of exposure to a microbial challenge is decreased and the embryo is more prepared to face a microbial challenge, but when the process is done in the earlier stages of incubation, the exposure time is longer and the embryo has not a well developed immune system (BERCHIERI & BOLIS, 2003), thus, the process can be detrimental to quail embryo survival.

The hatched quails from all treatments were bled at 15 days of age to evaluate antibody titers against Newcastle disease virus. In general, the birds did not respond well. Eggs injected at 0 day did not have quails with antibody against NDV. The eggs injected at 5 days of incubation with vaccine had 0% of hatchability. The eggs injected with ND vaccine at 10 and 15 days presented 8.7% and 6.9% of hatched quails with antibodies against Newcastle disease. These poor levels of response to *in ovo* vaccination can be explained by the use of an aggressive and inadequate NDV strain for *in ovo* vaccination

purpose. Furthermore, it is necessary to study more the injection site in quail eggs since different locations can promote varied *in ovo* vaccination responses. For example, it was verified that the injection of Marek's vaccine in air cell provided no protection for chicks, when it was applied in allantoic fluid the protection ranged from 25 to 50%, and when it was applied in amniotic fluid the protection was 90% (BERCHIERI & BOLIS, 2003).

CONCLUSION

The injection process itself (saline) is not harmful at 10 and 15 days of incubation for Japanese quail eggs; however, *in ovo* vaccination with live Newcastle vaccine (HB1 strain) is not recommended to fertile quail eggs at any incubation period due to high levels of embryo mortality and poor post-hatch antibody titers.

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