Effects of stage of broiler embryo development on coccidiosis vaccine injection accuracy, and subsequent oocyst localization and hatchling quality^{1,2,3}

A. O. Sokale,* C. J. Williams,[‡] M. D. Triplett,* F. J. Hoerr,[†] and E. D. Peebles^{*,4}

*Department of Poultry Science, Mississippi State University, Mississippi State, MS 39762, USA; [†]Veterinary Diagnostic Pathology, LLC, Fort Valley, VA 22652, USA; and [‡]Zoetis Animal Health, Research Triangle Park, NC 27703, USA

ABSTRACT Control of coccidiosis in broiler chickens continues to pose challenges to commercial poultry producers, especially in an era of increased consumer demand for antibiotic-free broiler production. As a result, coccidiosis vaccines are now commonly used in rotation programs to achieve effective coccidiosis control. Inovocox EM1 vaccine (EM1) is a coccidiosis vaccine that allows for earlier immune acauisition through oocvst cycling, which reduces the effects of wild-type coccidia. The EM1 vaccine is administered to embryonated broiler hatching eggs between 18 and 19 D of incubation (doi). In the U.S., commercial broiler hatcheries vaccinate embryonated eggs at either 18.5 or 19 doi. However, it is unclear whether a difference in embryo age at the time of in ovo injection can impact the actual site of vaccine delivery. In addition, it is unclear where oocysts eventually become localized within the embryo following the in ovo injection of EM1. Therefore, the objective of this study was to determine the effects of stage of embryonic development

on the actual deposition site of the EM1 vaccine oocvsts when they are in ovo injected and to subsequently investigate the movement and eventual location of EM1 oocysts after in ovo injection. Because all eggs were injected at the same time, a 12-h difference in set time was a means to derive 18.5 and 19.0 incubation age of injection (IAN) treatments. The experimental design was a 3 injection treatment (noninjected, diluent-injected, and vaccine-injected) \times 2 IAN factorial. There was a significant main effect of IAN on site of vaccine oocvsts delivery, and subsequent hatching chick quality. Qualitative histological evaluation revealed the oral uptake of vaccine oocysts through the amnion, with their subsequent presence in the gizzard and intestinal lumen by 24 to 36 h postinjection. In conclusion, physiological development influenced the site of injection, and oocysts imbibed along with the amniotic fluid in late stage broiler embryos are subsequently transported to the gastrointestinal tract.

Key words: broiler, diseases, embryo, health, management, pathology

2020 Poultry Science 99:189–195 http://dx.doi.org/10.3382/ps/pez592

INTRODUCTION

Coccidia of the genus *Eimeria* are single-celled obligate intracellular parasites that develop and multiply in the host's intestinal epithelium. Sporulated coccidia oocysts, when ingested by the host, initiate an infection cycle that causes damage to the intestinal architecture, resulting in reduced feed utilization, impaired

⁴Corresponding author: d.peebles@msstate.edu

nutrient absorption, poor growth, high morbidity and mortality, and an increased susceptibility to other diseases such as necrotic enteritis (Williams, 2005; Mc-Dougald and Fitz-Coy, 2008; Opengart, 2008; Li et al., 2010). Embryonated broiler hatching eggs are vaccinated between 18 and 19 D of incubation (doi) with a live Inovocox EM1 coccidiosis (EM1) vaccine, which is a nonattenuated coccidiosis vaccine that contains a controlled dose of Eimeria acervulina, Eimeria maxima, and Eimeria tenella. Administration of the EM1 vaccine initiates an immune response through the replication of coccidia, which allows the bird to develop a natural immunity for the control of a coccidiosis infection (McDougald and Fitz-Coy, 2008; Tewari and Maharana, 2011). Commercial in ovo vaccination of late-stage chicken embryos with vaccines such as Marek's disease (MD) virus, infectious bursal disease virus, and coccidiosis has become widely accepted in the U.S. poultry industry. While efficacy of the in ovo administration of

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Received April 24, 2019.

Accepted October 2, 2019.

¹This publication is a contribution of the Mississippi Agricultural and Forestry Experiment Station.

²This material is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch project under accession number 329260.

³Use of trade names in this publication does not imply endorsement by Mississippi Agricultural and Forestry Experiment Station of these products, nor similar ones not mentioned.

Eimeria oocysts has been shown (Weber and Evans, 2003; Weber et al., 2004), there still exists unknown immunological relevance as to specific site of injection, primarily with respect to coccidia oocysts. Site of vaccine delivery in ovo has been shown to affect MD vaccination efficacy (Wakenell et al., 2002). The ideal sites of in ovo injection are the amnion, and intramuscular or subcutaneous regions of the embryo body. However, this can be influenced by embryo physiological development during embryogenesis and the accuracy of the vaccine deliverv system (Williams and Zedek, 2010; Williams and Hopkins, 2011). The position and stage of development of the embryo during embryogenesis can be determined most accurately by embryo stage scoring (Sokale et al., 2017a). The rapid development of chicken embryos during this late phase only provides a short "window of opportunity" to target the right site of injection for a specific vaccine. Therefore, to achieve optimal performance, in ovo injection must be properly coordinated with the stage of embryonic development. Previous studies have demonstrated that the stage of physiological development for optimal vaccine delivery is when the embryo's head is at the right wing with the tip of the beak tooth at the internal membrane, when internal pipping may be present or absent, when external pipping is absent, and when the yolk has begun to ascend. This is typically between 18.0 and 19.0 doi (Sokale et al., 2017a). Currently, commercial hatcheries in the U.S. vaccinate broiler embryos by in ovo injection when transferred at either 18.5 or 19.0 doi (Williams, 2007). At these times, the amnion or embryo proper are targeted for the injection of vaccines. However, vaccines may be deposited in other regions other than the intended targets (Williams, 2007).

Along with other methods used to administer a live coccidia vaccine in the early life of the bird, such as through drinking water (Williams, 1994), eye spray (Chapman, 2000), or gel (Danforth, 1998), in ovo injection has also been proven useful (Weber and Evans, 2003; Weber et al., 2004; Williams, 2007). It has been speculated in studies by Weber and Evans (2003), Weber et al. (2004) and Sokale et al. (2017a) that were based on the knowledge of pre-patent period of *Eime*ria following the oral uptake of injected oocysts, that oocysts may remain dormant in the embryo's intestine until hatch with no life cycle changes. However, it remains unknown as to the location and development of oocysts in the embryo between the time of in ovo injection and hatch. Further, although it is recommended that the EM1 vaccine be administered between 18 and 19 doi, it is unclear whether possible differences in the stage of development that the embryo is at when an in ovo injection is given will impact hatching chick quality, hatchability, site of vaccine deposition, and the efficiency of the uptake of the vaccine by the embryo. Information concerning the effects of the in ovo injection of the commercial EM1 vaccine at different periods of incubation on the actual site of injection and the subsequent movement of oocysts within the embryo have not been documented in the scientific literature. In addition, limited information is available regarding the effects of possible differences in the physiological development of embryos incubated under similar conditions, on the efficiency of in ovo vaccine delivery. Therefore, the objective of this study was to determine effects of the timing of in ovo injection of the EM1 vaccine on the embryogenesis, hatching chick quality, actual site of injection, and the location and movement of EM1 oocysts in Ross 708 broiler embryos. To the authors' knowledge, this is the first report that provides information concerning these effects and relationships.

MATERIALS AND METHODS

General

All experimental procedures were conducted under a protocol that was approved by the Institutional Animal Care and Use Committee of Mississippi State University. Ross 708 broiler hatching eggs were obtained from a single 45-wk-old commercial broiler breeder flock and were held for 2 D under standard storage conditions prior to being set. At set (0 doi), 40 eggs, which were not misshapen or cracked and within $\pm 10\%$ of the mean weight of all set eggs, were randomly assigned to each of 6 treatment groups, with each treatment occupying a single egg tray, on each of 10 incubator tray levels (2,400)total eggs), with each tray level representing a replicate unit (block). A Jamesway model PS 500 single stage incubator (Jamesway Incubator Co. Inc., Cambridge, Ontario, Canada) was used as both a setter and hatcher unit. All eggs were incubated under standard conditions (Peebles and Brake, 1987). To allow for the establishment of 18.5 and 19.0 incubation age of injection (IAN) treatments, eggs were simultaneously injected at 18.5 doi, but were set 12 h apart. A 3×2 factorial design was utilized in this study, which was comprised of 3 injection treatments (IT) and 2 IAN, for a total of 6 treatment groups. The IT were designated as: noninjected control (NIC), diluent-injected control (DIC), and EM1 vaccine-injected (VI). The eggs were candled on 18.0 doi, and any infertile eggs or those containing a dead embryo were removed and discarded (Ernst et al., 2004). Incubator dry and wet bulb temperatures were set at 37.5 ± 0.1 and 28.9 ± 0.1 °C, respectively, and monitored twice daily for the entire incubation period.

Injection and Experimental Layout

All egg injections were performed at 18.5 doi (corresponded to 18.5 or 19.0 IAN because of 12 h difference in time of set) using a commercial Embrex Inovoject multi-egg injection system (Zoetis Animal Health, Durham, NC). Eggs were injected through the air cell with a blunt tipped injector needle (18.4 cm length and 1.27 mm bore width) to target the amnion. The needle provided an approximate 2.49 cm injection depth from the top of the large end of the egg. As previously described by Sokale et al. (2018), a $1 \times$ dose of the EM1 vaccine (Zoetis Animal Health, Research Triangle Park, NC), containing oocysts of *E. acervulina*, *E. maxima*, and *E. tenella*, was reconstituted with sterile commercial MD vaccine diluent (Merial Co., Duluth, GA) and administered at a volume of 50 μ L per egg. In addition, during the injection process, 2 embryonated eggs from each of 6 egg trays per tray level were concurrently injected with coomassie brilliant blue G-250 (colloidal) dye for the subsequent determination of site of injection and embryo stage score (**ES**).

During the injection process, eggs belonging to the DIC group were injected first, followed by the VI group. Normal commercial needle cleansing was performed between individual injections. Intervening machine cleaning processes between IT groups were included to avoid possible cross contamination. The eggs in the NIC group were subjected to the same procedures but were not injected. Following the completion of the entire injection process, eggs were placed in hatching baskets and returned to the incubator. All eggs remained outside the incubator at room temperature during the injection and transfer processes for a maximum of 5 min. Hatching baskets (containing the injected embryonated eggs) were arranged in a treatment-replicate pattern that corresponded with the arrangement of the respective setter trays, and in a manner that prevented crosscontamination between chicks that belonged to the injected and noninjected treatment groups.

Evaluation of Site of Injection and Embryo Staging

At 18.5 doi, site of injection and ES evaluations were performed using 2 embryonated eggs from each of the 60 treatment-replicate groups (120 eggs total), as previously described by Sokale et al. (2017a). Briefly, eggs that were injected with dye were placed in air-tight bags according to their treatment-replicate designation. The embryos were then euthanized using CO_2 gas, and were then stored at 4°C for 4 h. After euthanization, each egg was carefully dissected to determine the site of dye deposition at injection and the ES. The site of injection of each egg was scored as one of the following: air cell, amnion, allantois, or body of embryo, with body of embryo injections subdivided into i.m. or s.c. injection.

The embryo stage scoring was based on a system which assigned specific scores based on identifiable developmental features. The embryos were assigned either 0 (absent) or 1 (present) for the following developmental features: internal pip, external pip, and embryo head located on its right side. Furthermore, yolk absorption was scored from 1 to 4:1 = intestine is joined to a full yolk sac via the yolk stalk; 2 = yolk stalk is absent, and the yolk sac is bi-lobed; 3 = yolk sac loses its bilobed shape; and 4 = yolk is completely absorbed. The ES scores were additive, with a maximum score of 7. For example, if an embryo had a score of 1 for internal pip, 1 for external pip, 1 for head to the right, and a score of 2 for yolk absorption, this amounted to an ES of 5 out of 7. The higher the ES, the more advanced was embryonic development, while a lower ES signifies embryos that were in an early stage of development.

Chicken Embryo Histopathology

Following the in ovo administration of the EM1 vaccine, a qualitative histological method was used to investigate the location and movement of the vaccine oocysts, using 1 embryo from 1 replicate group of the DIC and VI treatments at both the 18.5 and 19.0 IAN. All 4 embryos were sampled at 20.0 doi, which corresponded with 36 h (18.5 IAN) and 24 h (19.0 IAN) postinjection times. All 4 full-term chicken embryos (with an internalized yolk sac) were fixed in neutral buffered formalin. For histopathology analysis embryo heads were longitudinally sectioned along the central axis. Eyes were sectioned sagittally to include eyelid conjunctiva, and the embryo body underwent serial sagittal sectioning to allow for the examination of all tissues. All tissues were stained with hematoxylin and eosin and examined for evidence of coccidia replication or coccidia life cycle stages, as well as their movement throughout the embryo. The tissues were examined histologically, and all structures anatomically identified. Luminal contents were examined and identified by microscopic characteristics and classified as amniotic fluid, amniotic squames, bacteria, and structures morphologically consistent with coccidia trophozoites.

Evaluation of Somatic Characteristics

At 21.0 doi (d of hatch), all hatchlings in both the 18.5 and 19.0 IAN were pulled from the hatcher. The hatchability of injected embryonated eggs (HI) and hatching chick BW (**HBW**) were determined using 20 chicks from each of the 60 treatment-replicate groups. In addition, 2 chicks from each of the 60 treatmentreplicate groups (120 total) were wing-banded, euthanized, weighed, and necropsied, for determination of the absolute values of following 21.0 doi hatching chick quality variables: intestine (IW) and volk sac (YSW) weights, and total and yolk-free BW (YFBW). Subsequently, yolk sac (**RYBW**) and intestine (**RIBW**) weights relative to BW, and intestine weights relative to YFBW (**RIYFW**) and chick yolk free body mass (**YFBM**) were calculated. All relative weights and YFBM were expressed as percentages. The YFBM variable [(YFBW/BW) * 100] was used as an expression of the proportion of chick BW that included all tissues exclusive of the yolk sac.

Statistical Description

A randomized complete block design was used in both the setter and hatcher, with each of the 10 tray (setter) and hatching basket (hatcher) levels representing a block. All 6 treatment-replicate groups (3 IT \times 2 IAN) were equally and randomly represented in each block. The chick quality data were arranged in a 3 IT \times 2



Figure 1. Percentage amnion, i.m., and s.c. sites of injection in selected dye-injected embryos belonging to the 18.5 and 19.0 incubation age of injection (IAN) treatment groups. Across injection treatment, 2 embryos from each of 30 replicate units per IAN were used to calculate means. ^{a,b}Means within site of injection with no common superscript differ significantly ($P \leq 0.05$).

IAN factorial design to evaluate the main and interactive effects of IT and IAN on all hatching chick quality variables, using a 2-way ANOVA. Main and interactive effects of IT and IAN were viewed as fixed effects and block as a random effect. A 1-way ANOVA was used to test for IAN related differences for the site of injection and ES. Least-square means were compared in the event of significant global effects (Steel and Torrie, 1980). All variables were analyzed using the MIXED procedure of SAS software 9.3 (SAS Institute, 2012). Global and least-squares means differences were considered significant at $P \leq 0.05$. The qualitative histological findings are presented in the Results section.

RESULTS

As previously stated, a proportion of embryonated eggs were concurrently injected with dve along with all the eggs in the various IT groups, for site of injection validation. The mean site of injection for the selected embryos in the 18.5 and 19.0 IAN are shown in Figure 1. There was a significant effect of IAN (P =0.01) on mean ES. The 19.0 IAN group had a higher mean ES (3.24) compared to the 18.5 IAN group (2.44). Furthermore, there was significant effect of IAN on the amniotic (P = 0.03), as well as the s.c. (P = 0.01) and i.m. (P = 0.02) sites of injection. Out of 60 embryos injected in the 18.5 IAN group, 88.3% (53/60) were injected in the amnion, 8.3% (5/60) were injected in the i.m. region, and 3.3% (2/60) were injected in the s.c. region. In the 19.0 IAN group, 73.3% (44/60) were injected in the amnion, 21.7% (13/60) were injected in the i.m. region, and 5% (3/60) were injected in the s.c. region.

A qualitative histological method was systematically utilized to determine the location and movement of coccidia stages in the tissues (digestive tract and somatic cells) of in ovo injected embryos. Although the methodology was unable to differentiate between *Eimeria* spp. or quantify their abundance in the tissues examined, it was able to detect the presence of coccidia stages at the single time point examined (20.0 doi). Histological pictures are provided in Figure 2. In the proventriculus and gizzard, there were abundant eosinophilic homogenous ingesta (material) with suspended flattened squames. The lumen of the gizzard and intestine contained small round bodies consistent in size with coccidia sporozoites. Clusters of bacteria were also identified in the intestinal lumen of all embryos examined. There were no coccidia life cycle stages identified in any of the other tissues examined.

No difference in SEW was observed between treatment groups. There was no IT (P = 0.20), IAN (P =0.203), or IT \times IAN interaction (P = 0.482) for HI at day of hatch. There was a significant effect of IAN on HBW (P = 0.002), YSW (P = 0.001), RYBW (P =(0.001), and IW (P < 0.001) at day of hatch. The HBW, YSW, and RYBW of birds in the 18.5 IAN group were higher compared to birds in the 19.0 IAN group. However, the IW of birds in the 19.0 IAN group was higher compared to that of birds in the 18.5 IAN group. There were significant effects of IT (P = 0.045) and IAN (P =0.001) on RIBW. The RIBW of birds in the 19.0 IAN group was higher compared to birds in the 18.5 IAN group. Further, the RIBW of birds in the DIC group was higher compared with the NIC group, with the VI group being intermediate. There was a significant IT \times IAN interaction for RIYFW (P = 0.05). The RIYFW of the DIC-19.0 IAN group was highest, and the RIYFW of the NIC-18.5 IAN group was lowest, in comparison to all other treatment groups. In addition, the RIYFW values for the NIC-19.0 IAN, VI-18.5 IAN, and VI-19.0 IAN groups were not significantly different, but all 3 were significantly higher than the NIC-18.5 IAN group



Figure 2. Histological pictures of the location and movement of coccidia sporozoites in the gastrointestinal tissues of broiler embryos that were injected with Inovocox EM1 vaccine on d 19.0 of incubation and sampled on d 20.0 of incubation (a) gizzard squames, (b) gizzard sporozoite, (c) intestinal squames with evidence of bacterial contamination possibly due to embryo sub-optima fixation in buffered formalin (d) intestinal sporozoites.

and significantly lower than the DIC-19.0 IAN group. There was a significant main effect of IAN on YFBM (P = 0.001). The YFBM of birds in the 19.0 IAN group was higher than that of birds in the 18.5 IAN group. For reference, the means for all the hatching chick quality variables evaluated are presented in Table 1.

DISCUSSION

Use of a live oocyst vaccine in broilers has become a widely accepted means of controlling coccidiosis. The commonly used live oocyst vaccines can be administered to chicks at hatch using spray cabinets (Chapman et al., 2002; Williams, 2002), or to late stage embryos by in ovo injection (Williams, 2007). The live nonattenuated vaccine, Inovocox EM1, is recommended for the immunization of healthy broiler embryos between 18.0 and 19.0 doi for the prevention of coccidiosis. Therefore, the accuracy of injection, the movement of oocysts within the embryo, and their subsequent effects on broiler embryogenesis and hatching chick quality were investigated in eggs that were set and injected 12 h apart (18.5 or 19.0 IAN) under the same incubation conditions.

A previous report by Sokale et al. (2017a) showed the importance of ES in accurately determining embryo physiological development. In the current study, embryos in the 19.0 IAN group had a higher ES in comparison to those in the 18.5 IAN group. This indicates that at the time of in ovo injection, there were developmental differences between the 2 groups, with embryos in the 19.0 IAN group showing more advanced physiological developmental characteristics. These advancements were mostly recognized in the positioning of the head and the occurrence of pips through the eggshell membrane and the eggshell proper (Sokale et al., 2017a). Further, our findings indicate that IAN affected the proportion of amniotic injections, the ideal in ovo target site for the vaccine. At the time of in ovo injection, the accuracy of vaccine deposition into the amnion was 15% greater in embryos in the 18.5 IAN group in comparison to those in the 19.0 IAN group. Studies have shown that the success of an in ovo injection that is associated with the imbibing of vaccine by the embryo is dependent on the amount of amniotic fluid that is present during the late-stage of development (Zhai et al., 2011b; Bello et al., 2014; Sokale et al., 2017a). Williams (2007) reported that when embryos are between 17.5 doi and 19.0 doi + 4 h, they are at a physiological stage that is optimal for in ovo injection into the amnion. When the volume of amniotic fluid is reduced, there is a higher chance of vaccine deposition in sites other than the amnion, such as the i.m. and s.c. regions (Jochemsen and Jeurissen, 2002; Sokale et al., 2018). This is supported by a previous study (Sokale et al., 2018) in which a significantly higher percentage of injections occurred in the i.m. and s.c. regions (81.5 and 11.7%), in comparison to the amniotic region

Table 1. Hatching chick quality variable means in the 18.5 and 19.0 incubation age of injection (IAN), and noninjected control, diluent-injected control, and vaccine-injected injection type (IT) groups.¹

	Treatments	IAN	HI ² (%)	$\begin{array}{c} \rm HBW^2 \\ \rm (g) \end{array}$	${ m BW^3}$ (g)	YSW ³ (g)	YFBW ³ (g)	RYBW ³ (%)	IW ³ (g)	RIBW ³ (%)	RIYFW ³ (%)	YFBM ³ (%)
	Noninjected control	18.5	86.99	47.51	48.54	7.44	41.10	15.26	1.80	3.71	4.37 ^c	84.74
		19.0	87.14	46.42	46.95	5.81	41.14	12.27	2.08	4.44	5.05^{b}	87.73
	Diluent-injected control	18.5	84.80	47.02	46.49	6.88	39.61	14.81	1.87	4.02	$4.72^{b,c}$	85.19
		19.0	88.93	46.38	46.13	6.10	40.03	13.13	2.20	4.77	5.49^{a}	86.87
	Vaccine-injected	18.5	83.71	47.09	46.54	6.93	39.60	14.89	1.90	4.09	4.81 ^b	85.11
		19.0	84.83	46.35	46.61	5.89	40.73	12.49	2.02	4.37	4.99 ^b	87.51
	SEM		1.809	0.310	0.65	0.40	0.56	0.78	0.05	0.12	0.13	0.78
IT	Noninjected control		87.06	46.96	47.74	6.62	41.12	13.77	1.94	4.07^{b}	4.71^{b}	86.23
	Diluent-injected control		86.86	46.70	46.31	6.49	39.82	13.97	2.04	4.39^{a}	5.10^{a}	86.03
	Vaccine-injected		84.27	46.72	46.57	6.41	40.17	13.69	1.96	$4.23^{a,b}$	$4.90^{a,b}$	86.31
	SEM		1.35	0.22	0.46	0.28	0.39	0.55	0.03	0.09	0.09	0.55
IAN	18.5		85.17	47.21 ^a	47.19	7.08^{a}	40.10	14.99 ^a	1.85 ^b	3.94^{b}	4.63 ^b	85.01 ^b
	19		86.97	46.38 ^b	46.57	5.93^{b}	40.63	12.63 ^b	2.10^{a}	4.53^{a}	5.18^{a}	87.37^{a}
	SEM		1.15	0.18	0.37	0.23	0.32	0.45	0.03	0.07	0.07	0.45
<i>P</i> -values IT		0.200	0.649	0.074	0.862	0.064	0.937	0.123	0.045	0.013	0.937	
	IAN		0.203	0.002	0.247	0.001	0.250	0.001	0.001	0.001	0.001	0.001
$\mathrm{IT} imes\mathrm{IAN}$			0.482	0.744	0.422	0.553	0.622	0.704	0.099	0.110	0.050	0.704

^{a-c}Means within a variable with no common superscript differ significantly ($P \leq 0.05$). Bold values represents statistical significance.

¹Hatchability of injected eggs (HI), Hatchling BW (HBW), Chick BW (BW), yolk sac weight (YSW), yolk-free BW (YFBW), yolk sac weight as a percentage of BW (RYBW), intestine weight (IW), intestine weight as a percentage of BW (RIBW), intestine weight as a percentage of yolk-free BW (RIYFW), yolk-free body mass (YFBM).

 $^{2}20$ birds in each of 60 treatment-replicate units were used to calculate each mean for HI and HBW.

 $^{3}2$ birds from each of 60 treatment-replicate units were used to calculate the means of each hatching chick quality variable.

(6.8%), when embryos were in ovo injected at 19.0 doi. In the current study, there is clear indication that a 12 h window of incubation could influence the proportion of in ovo injections into the amnion of late stage embryos.

There is currently no published literature concerning the movement, localization, and development of oocysts within the chicken embryo following in ovo injection of the EM1 vaccine into the amnion. Previous reports by Weber et al. (2004) and Sokale et al. (2017a) suggested that following the in ovo injection of live coccidia oocysts and their oral uptake, oocysts may remain dormant in the intestine of the embryo. Furthermore, a previous report (unpublished data) demonstrated that following in ovo injection of E. maxima oocysts at 18.0 doi, life cycle stages of coccidia were detected in the amniotic fluid, gizzard, and intestine, and the number of oocysts recovered from the amnion gradually decreased through hatch. In this study, a qualitative histological investigation of the movement of oocysts within the embryo revealed the oral uptake of EM1 vaccine oocysts through the imbibition of the amniotic fluid. The oral uptake of the vaccine oocysts via the amnion resulted in the presence of life cycle stages of coccidia in the gizzard and intestine. However, there was no coccidia stage detection in any of the nongastrointestinal tissues of the embryos examined. This was unexpected, considering that in ovo injection also occurred in the s.c. and i.m. regions of the embryo. It is possible that these sites of injection may not be suitable for the survival or subsequent viability of coccidia. Based on these evidences, it can be suggested that following the in ovo injection of the EM1 vaccine, oocysts are imbibed by the embryo, and transported via the amniotic fluid into the gastrointestinal tract.

In the current study, it was shown that the EM1 vaccine did not adversely affect hatchability, as the hatchability in the VI group was not significantly different from either the NIC or DIC groups. This is supported by previous studies in which coccidia oocysts were injected in ovo (Weber and Evans, 2003; Sokale et al., 2017a). Upon evaluating hatching chick quality in this study, apparent differences in chick quality were observed that were due to a 12 h difference in time of set (18.5 and 19.0 IAN). A higher HBW of chicks in the 18.5 IAN group may be associated with a greater amount of residual yolk due to a higher retention of moisture in the yolk sac and body tissues. The yolk sac accounts for 15 to 25% of total hatchling BW (Jamroz et al., 2004). Yolk sac moisture content can be altered by factors such as: in ovo application of exogenous substances which influence the bird's utilization of residual volk nutrients (Uni et al., 2005; Moran, 2007; Zhai et al., 2011a,b; Bello et al., 2013; Sokale et al., 2017b), incubation length (van de Ven et al., 2011), incubation temperature and relative humidity (Pulikanti et al., 2012), and total egg moisture loss (Peebles et al., 2005). Higher IW, RIBW, RIYFW, and YFBM in chicks from the 19.0 IAN may be associated with an increased rate of moisture loss, higher internal temperature, and a more rapid

rate of metabolism (Zhai et al., 2011b; Pulikanti et al., 2012) in the embryos belonging to this group.

In conclusion, injection treatment (vaccine- and diluent- injection) did not affect broiler hatchability. However, chick quality characteristics were affected with a 12 h difference in embryo IAN. Further, the accuracy of injection into the amnion was lower in embryos belonging to the 19.0 IAN group. Histological analysis of embryos at 24 to 36 h post-in ovo injection of the EM1 vaccine revealed oral uptake of the vaccine through the amnion with the presence of coccidia life cycle stages in the gizzard and intestinal lumen. The effects of this outcome on broiler performance remain to be reported.

ACKNOWLEDGMENTS

We express our appreciation for the financial support of Zoetis Global Animal Health, the expert technical assistance of Sharon K. Womack, and for the assistance of the intern, graduate and undergraduate students of the Department of Poultry Science at Mississippi State University.

REFERENCES

- Bello, A., W. Zhai, P. D. Gerard, and E. D. Peebles. 2013. Effects of the commercial in ovo injection of 25-hydroxycholecalciferol on the hatchability and hatching chick quality of broilers. Poult. Sci. 92:2551–2559.
- Bello, A., R. M. Bricka, P. D. Gerard, and E. D. Peebles. 2014. Effects of commercial in ovo injection of 25-hydroxycholecalciferol on broiler bone development and mineralization on days 0 and 21 posthatch. Poult. Sci. 93:1053–1058.
- Chapman, H. D. 2000. Practical use of vaccines for the control of coccidiosis in chicken. World's Poult. Sci. J. 56:7–20.
- Chapman, H. D., T. E. Cherry, H. D. Danforth, G. Richards, M. W. Shirley, and R. B. Williams. 2002. Sustainable coccidiosis control in poultry production: the role of live vaccines. Int. J. Parasitol. 32:617–629.
- Danforth, H. D. 1998. Use of live oocyst vaccines in the control of avian coccidiosis: experimental studies and field trials. Int. J. Parasitol. 28:1099–1109.
- Ernst, R. A., F. A. Bradley, U. K. Abbott, and R. M. Craig. 2004. Egg candling and breakout analysis for hatchery quality assurance and analysis of poor hatches. University of California, Division of Agriculture and Natural Resources. pub. 8134.
- Jochemsen, P., and S. H. M. Jeurissen. 2002. The localization and uptake of in ovo injected soluble and particulate substances in the chicken. Poult. Sci. 81:1811–1817.
- Jamroz, D., T. Wertelecki, A. Wiliczkiewicz, J. Orda, and J. Skorupi´nska. 2004. Dynamics of yolk sac resorption and posthatching development of the gastrointestinal tract in chickens, ducks and geese. J. Anim. Physiol. Anim. Nutr. (Berl.) 88:239–250.
- Li, G., H. S. Lillehoj, K. W. Lee, S. I. Jang, P. Marc, G. Cyril, D. Ritter, D. A. Bautista, K. Phillips, A. P. Neumann, T. G. Rehberger, and G. R. Siragusa. 2010. An outbreak of gangrenous dermatitis in commercial broiler chickens. Avian Pathol. 39:247– 253.
- McDougald, L. R., and S. H. Fitz-coy. 2008. Coccidiosis. Pages 1067– 1080 in Diseases of Poultry, 12th ed. Y. M. Saif, A. M. Fadley, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, eds. Wiley-Blackwell Publishing, Ames, Iowa.
- Moran, E. T., Jr. 2007. Nutrition of the developing embryo and hatchling. Poult. Sci. 86:1043–1049.

- Opengart, K. 2008. Necrotic Enteritis. Pages 872–879 in Diseases of Poultry, 12th ed. Y. M. Saif, A. M. Fadley, J. R. Glisson, L. R. McDougald, L. K. Nolan, and D. E. Swayne, eds. Wiley-Blackwell Publishing, Ames, Iowa.
- Peebles, E. D., and J. Brakes. 1987. Eggshell quality and hatchability in broiler breeder eggs. Poult. Sci. 66:596–604.
- Peebles, E. D., R. W. Keirs, L. W. Bennett, T. S. Cummings, S. K. Whitmarsh, and P. D. Gerard. 2005. Relationships among prehatch and posthatch physiological parameters in early nutrient restricted broilers hatched from eggs laid by young breeder hens. Poult. Sci. 84:454–461.
- Pulikanti, R., E. D. Peebles, W. Zhai, L. W. Bennett, and P. D. Gerard. 2012. Physiological relationships of the early post-hatch performance of broilers to their embryo and eggshell characteristics. Poult. Sci. 91:1552–1557.
- SAS Institute. 2012. SAS Proprietary Software Release 9.3. SAS Inst. Inc., Cary, NC.
- Sokale, A. O., W. Zhai, L. M. Pote, C. J. Williams, and E. D. Peebles. 2017a. Effects of coccidiosis vaccination administered by *in ovo* injection on the hatchability and hatching chick quality of broilers. Poult. Sci. 96:541–547.
- Sokale, A. O., W. Zhai, L. M. Pote, C. J. Williams, and E. D. Peebles. 2017b. Effects of coccidiosis vaccination administered by *in ovo* injection on Ross 708 broiler performance through 14 days of post-hatch age. Poult. Sci. 96:2546–2551.
- Sokale, A. O., C. J. Williams, T. S. Cummings, P. D. Gerard, A. Bello, and E. D. Peebles. 2018. Effects of *in ovo* injection of different doses of coccidiosis vaccine and turn-out times on broiler performance. Poult. Sci. 97:1891–1898.
- Steel, R. G. D., and J. H. Torrie. 1980. Principles and procedures of statistics. A Biometrical Approach. 2nd ed. McGraw-Hill, New York, NY.
- Tewari, A. K., and B. R. Maharana. 2011. Control of poultry coccidiosis: changing trends. J Parasit. Dis. 35:10–17.
- Uni, Z., P. R. Ferket, E. Tako, and O. Kedar. 2005. In ovo feeding improves energy status of late-term chicken embryos. Poult. Sci. 84:764–770.
- van de Ven, L. J. F., A. V. van Wagenberg, M. Debonne, E. Decuypere, B. Kemp, and H. van den Brand. 2011. Hatching system and time effects on broiler physiology and posthatch growth. Poult. Sci. 90:1267–1275.
- Wakenell, P. S., T. Bryan, J. Schaeffer, A. Avakian, C. Williams, and C. Whitfill. 2002. Effect of *in ovo* vaccine delivery route on Herpesvirus of Turkeys/SB-1 efficacy and viremia. Avian Dis. 46:274–280.
- Weber, F. H., and N. A. Evans. 2003. Immunization of broiler chicks by *in ovo* injection of *Eimeria* tenella sporozoites, sporocysts, or oocysts. Poult. Sci. 82:1701–1707.
- Weber, F. H., K. C. Genteman, M. A. LeMay, D. O. Lewis, Sr., and N. A. Evans. 2004. Immunization of broiler chicks by in ovo injection of infective stages of Eimeria. Poult. Sci. 83:392–399.
- Williams, R. B. 1994. Safety of the attenuated anticoccidial vaccine 'Paracox' in broiler chickens isolated from extraneous coccidial infection. Vet. Res. Comm. 18:189–198.
- Williams, R. B. 2002. Anticoccidial vaccines for broiler chickens: Pathways to success. Avian Pathol. 31:317–353.
- Williams, R. B. 2005. Intercurrent coccidiosis and necrotic enteritis of chickens: rational, integrated disease management by maintenance of gut integrity. Avian Pathol. 34:159–180.
- Williams, C. 2007. In ovo vaccination for disease prevention. Int. Poult. Prod. 15:7–8.
- Williams, C. J., and A. S. Zedek. 2010. Comparative field evaluations of in ovo applied technology. Poult. Sci. 89:189–193.
- Williams, C. J., and B. A. Hopkins. 2011. Field evaluation of the accuracy of vaccine deposition by two different commercially available in ovo injection systems. Poult. Sci. 90:223–226.
- Zhai, W., L. W. Bennett, P. D. Gerard, R. Pulikanti, and E. D. Peebles. 2011a. Effects of in ovo injection of carbohydrates on somatic characteristics and liver nutrient profiles of broiler embryos and hatchlings. Poult. Sci. 90:2681–2688.
- Zhai, W., P. D. Gerard, R. Pulikanti, and E. D. Peebles. 2011b. Effects of in ovo injection of carbohydrates on embryonic metabolism, hatchability, and subsequent somatic characteristics of broiler hatchlings. Poult. Sci. 90:2134–2143.