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In ovo vaccination of chicken eggs

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1 Introduction

The vaccination method to safely present a live antigen in an efficacious dosage to the developing chicken embryo advanced from the research laboratories of the US Department of Agriculture in the 1980s to the commercial poultry industry during the early 1990s with the development of the Embrex®[1] Inovoject® egg injection system. Following the commercial inception in 1993, the application of vaccines in ovo has grown to encompass over 35 different vaccine types (see Table 1) and an application scope including over 95% of North American broiler chickens. Additionally, commercial in ovo vaccination is in broiler production in more than 35 countries worldwide with an excess of 24 billion eggs vaccinated annually. The success of the equipment application as a veterinary medical process is due to many factors; however, two basic principles drive the technology. One, the mass application with individual dosage control during administration delivers a uniform flock vaccination in commercial production at an ergonomic and rapid rate. Two, the process serves to safely deliver to the broiler egg biologically active vaccines at the earliest possible time of embryonic development, limiting stress from the procedure and preparing the chick 'immunologically' for several disease challenges that may occur later in life, during the grow-out period. From these two basic principles, the application scope has continued to grow, expand and adapt

[1] Embrex®, Inovoject®, Bursaplex® and Magniplex® are registered trademarks of Zoetis, Inc., Parsippany, NJ, USA.

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Table 1 USDA licensed in ovo vaccines by manufacturer as of December 2021

	Manufacturer				
Vaccine type	Boehringer Ingelheim	Ceva	Huvepharma	Merck	Zoetis
HVT	+	+			
SB1	+				
HVT SB1		+			
CVI988				+ +	+
HVT CVI988					+
HVT-IBD	+	+			+
HVT-ND	+	+		+	+
HVT-ILT		+		+	
HVT-IBD-ND	+	+		+	
HVT-IBD SB1	+	+		+	
HVT-ILT SB1				+	
HVT-ND-ILT				+	
HVT-IBD CVI988		+			
HVT-ND CVI988		+			
HVT-ND SB1		+		+	
HVT-IBD SB1 CVI988		+			
FP	+				
FP-ILT		+			
FP-ND	+				
IBD				+	
IBD plus AB	+				+
Coccidia			+		

to regional priorities of disease pressure and different hatchery environments across the global commercial poultry industry. Many factors continue to interact to both challenge and define the technology, and it is the goal of the author to describe and detail how these factors shaped and continue to evolve. Additionally, there are adjacent considerations that are present and will be discussed to fully understand the impact and scope of embryonic vaccination in the chicken egg.

2 History and evolution of in ovo vaccination

The inoculation of embryonated chicken eggs has been used for many decades in different evaluative and production capacities targeting the developing embryo as a 'bio-reactor' or growth medium for viral and bacterial replication.

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There has been a wide range of use in immunological and virology, as the embryonated egg serves as an ideal growth and support media for many viruses based upon the embryo itself as well as the extra-embryonic structures found within the developing chicken egg. For example, human influenza vaccines are produced commercially in embryonated chicken eggs (day 9–11 of incubation) via inoculation and subsequent growth/collection of vaccine virus. Many vaccines utilized in animal health are grown in embryonic tissue in vivo as well as chicken embryo fibroblasts (CEFs) in culture. Virus isolation and pathogenicity determinations utilizing viral and bacterial applications to the developing embryo have long been a standard practice in disease control and epidemiology. In addition to these 'diagnostic' uses of egg injection, embryonic gender determination has been accomplished via sampling of embryonic waste and/or tissue late in development.

Vaccination of the chicken egg as a method of disease control is a different perspective, where the integrity of the vaccine and the safety of the embryo are critical to the application technology to ensure the efficacy of the vaccine against subsequent disease challenge. We shall review the application and understandings with respect to both safety and efficacy, but also the challenges of maintaining these two important factors as part of a veterinary medical device process. For additional information on inoculation of eggs, an excellent overview and inoculation site studies via compartmental delivery have been provided by Manders et al. (2021). Another perspective of use of in ovo delivery to improve poultry health has been investigated by many in academia via injection of nutrients, competitive exclusion bacteria and other select compounds. While the injection of nutrients and other entities are not available or successful in commercial use, a good review of egg injection and in ovo applications of various products in poultry was completed by E. D. Peebles (2018), and a review by P. Ferket is included in a separate chapter of this publication. What differs from all the described in ovo applications and that of the information presented herein is the focus of this review specifically upon egg injection as an application method for vaccination of the chicken embryo.

The in ovo vaccination technology can be traced back to discoveries in the laboratories of the US Department of Agriculture in the early 1980s. The initial work encompassed in ovo application of the herpes virus of turkeys (HVT) as a live viral antigen given to late development stage chicken embryos to protect the hatched chick against subsequent Marek's disease challenge. (Sharma and Burmester, 1982; Sharma and Graham, 1982). The discovery initiated a group focus by Marek's disease researchers to understand key scientific and immunological properties of embryonic vaccination in poultry for protection against the disease (Sharma and Witter, 1983; Sharma et al., 1984; Witter and Lee, 1984). While many research groups have continued to develop the scientific 'library of knowledge' and expand the vaccine platform to other

disease challenges regarding in ovo vaccination, the possibility of the transfer of this technology to the poultry industry spawned the formation of a start-up company focused solely on bringing new technologies to the poultry industry (Embrex®). The foundation and focus of the company were secured in 1985 via a long-term sole-lease agreement of the Sharma/Burmester patent (US Patent #4,458,630, 10 July 1984; Disease control in avian species by embryonal vaccination) between the company and the US government. Extensive research and collaborations between the company and poultry integrators in the late 1980s and early 1990s were completed and involved several different prototypical egg injection devices, extensive laboratory evaluations and a multitude of field-based trials before commercial success was made possible (Miles et. al., 1992; Gildersleeve, et. al., 1993).

Today successful in ovo vaccination involves many options for disease prevention with over 35 different licensed vaccine products (USDA, Table 1). Academic and pharmacological research continues to expand the family of in ovo approved vaccines as the technology represents a robust commercial methodology with accurate and safe access to each bird for individual dosage. The challenging environments and working conditions of the commercial hatchery have demanded robust equipment and supportive processes as well as a greater focus on sanitation and environmental control at the hatchery and breeder farm level. The focus of quality control has improved the overall conditions and reduced the microbial challenges at facilities successfully incorporating the technology. Additionally, more and more reliance upon process automation has become a part of standard operations of the veterinary medical devices used for in ovo vaccination as securing skilled and qualified labor continues to be a challenge globally in agriculture and food animal production.

3 Interacting factors of in ovo vaccination success

3.1 Device adaptation to commercial egg incubation: five critical points

A basic understanding of the commercial incubation process complexity is paramount to the comprehension of the challenges and associated variables of applying vaccines into the developing embryos as part of their everyday routines. A short overview of commercial incubation is provided herein; however, further details can be found in poultry management textbooks and hatchery management guidelines, including *Poultry Meat and Egg Production* by Parkhurst and Mountney (1995) or similar.

Hatching eggs, unlike the more familiar table eggs for human consumption, are produced on breeder farms and are by design mostly fertile eggs. The

farms typically have 2–6 houses or barns of hens and roosters with 8000 to 12 000 birds in each house with approximately 8–10 hens per rooster. These birds have been selected over many generations to produce offspring that are grown for meat production. These birds grow and fulfill market protein needs as to size- and product-specific targets of chicken meat for consumption. Specifically, the target weight and size may define the required breed cross, and these target markets fall into three general categories: small (4.0 lb or 2.5 kg), medium (6.0 lb or 3.75 kg) and large (8.0 lb or 5.0 kg). The hens supply fertile eggs to the hatchery for approximately 40 weeks of production (age 25–65 weeks), and each house will produce approximately 1.5 million eggs over the 40-week production cycle. So, if we imagine a hatchery that incubates one million eggs per week, breeder farm support will require approximately 27–30 houses or barns of laying hens in production at any given time, each averaging 37 500–40 000 hatching eggs per week supplying the hatchery. The integrated production would also include a continuous growth cycle of young hens (pullets) and roosters to supply the ongoing needs of egg production as the older birds go out of production. The young pullets and roosters are typically placed every 2–4 weeks and are grown under strict guidelines and target weights in preparation for egg production at 25 weeks of age. This large-capacity production environment is typical to commercial broiler meat production globally with hatcheries producing 0.5–4 million chicks per week from single facilities. On the average, broiler hatcheries produce between 1.0 million and 1.8 million birds per week with the largest complexes producing close to 4 million broilers per week. In the United States alone, approximately 200 commercial hatcheries produce 9 billion broiler chickens annually. It is important to realize the multitude of variables presented by the large scope of farms, breeds, hatcheries and environmental conditions.

Understanding the production scope of commercial hatcheries is the beginning of the understanding of how the embryonic vaccination can be applied in the industry. We must consider the volume and hatchery processes on a large scale to understand how the ergonomics and production must match the science and biology of vaccination in the egg. Key to hatchery operations is the production of a consistent supply of quality hatching eggs. After the eggs are laid at the farm, they are graded as part of collection process from nests, typically three to four times daily. The acceptable hatching eggs are placed individually with pointed or small end down in plastic incubation egg trays or flats with 25–150 eggs per tray (incubator type specific) and then placed in cool storage on the farm. Hatching eggs require cool temperature storage after being laid prior to incubation. The storage period enables accumulation of eggs from many sources and therefore can provide routine scheduling of a large volume of eggs to set (or begin incubation) at one time. Eggs are typically set four times per week (Monday, Tuesday, Thursday, Friday). The hatching

eggs are usually transported to the hatchery from the farms two or three times weekly. The amount of storage time prior to set can affect incubation length, and this represents just one example of the many variables that impact incubation and normal population characteristics of embryonic development in the production environment.

The incubator egg tray design conforms to the type and manufacture source of the incubation equipment found at the hatchery. Currently, there are over 25 different incubation egg tray designs in use globally, and the tray types are supported by different incubation companies with approximately 20–25 different incubator designs. Incubator designs fall into two general categories: multi-stage or single-stage incubation. Multi-stage incubators have multiple aged embryos at various stages of development found in one machine. The eggs are set in a coordinated repeating schedule to utilize embryonic heat of older embryos and therefore run more efficiently than single-stage or single-age incubators. Both types of incubators have specific beneficial traits; however, single-stage incubation provides better biosecurity and more targeted programable control for the interior environments of the machines (specific temperature and ventilation profiles are different for eggs from different hen ages, storage time, breeds, etc.).

Regardless of incubator type, all chicken eggs require 21 days of incubation. Eggs remain for 18–19 days in the incubator flat (in the incubator or setters) and then subsequently are transferred to hatching baskets where they spend the last 2–3 days in specialized hatching compartments or hatchers. The transfer process enables the division of incubation (clean) and hatch (dirty). Transfer of eggs out of the incubator flats into the hatch basket maintains a relatively clean and stable environment (intact eggs) for incubation. This is also important as transfer to the hatching cabinets provides an enclosed controlled environment for the final challenges of production including disinfection and rigorous cleaning of equipment after hatch (shell debris, hatch debris, unhatched egg waste, etc.). If we consider the calendar of events, eggs set on a Monday will hatch 3 weeks later, also on a Monday. They require removal/transfer on Friday or Saturday preceding the hatch. After 18/19 days of incubation in the egg tray, the eggs are removed from the incubator and 'transferred' into a hatching basket for final development and hatching. The incubator trays are then cleaned and returned to the breeder farm for use again. The 3-week incubation cycle allows for a repeating program of egg set, egg transfer, incubator cleaning, chick hatch, hatcher cleaning, etc. for each hatch day as well as 'recycling' of equipment such as the hatch basket, hatcher compartment, incubation flat, etc. The opportunity presented by the removal of the eggs from incubation and transfer out of the incubation tray into the hatch basket and hatch compartments is the moment that egg vaccination for disease prevention takes place.

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The window of opportunity for egg vaccination exists at day 18–19 of incubation in normal production, but exact timing for optimal performance can be incubation equipment-type dependent. Specific challenges and critical factors are present that can enable or effectively destroy the vaccination process. We must consider that many factors are interactive and revolve around the device interface with the developing embryo in the egg. The factors fall into five general categories: injection device location and positioning on the egg, accurate shell penetration, site of vaccine delivery, sanitation, and vaccine specifications, including handling, safety and efficacy. Each of these five critical factors have unique hurdles to address, including different flat/incubator type as it relates to injection device design, embryonic developmental stages (injection timing) that affect vaccine delivery site, embryo size and safety, population dynamics (multi-stage vs single-stage incubation, relative 'window' of development), hatchery environmental and injection device sanitation and the differences in egg size and microbial challenges due to breeder hen age and management.

3.2 Device and eggshell interface

Due to the somewhat fragile nature of the eggshell, egg handling for injection has unique challenges. In design of a device to inject an egg, several important functions are quickly realized. Conveyance of the incubator egg tray must be gentle and uniquely adapted to the specific tray characteristics. Some incubation trays have a 'flat' bottom edge, while others are supported by internal posts that act as 'feet' to raise or suspend the egg as part of the characteristic features of the tray for that incubator type. Some incubator trays have a surrounding rigid 'frame' that suspend the grid of plastic that supports the egg off the horizontal surface below the tray, exposing the small end or point of the egg. All the different tray configurations require uniquely designed conveyors to move the eggs and allow for sequential processes of loading, injection and transfer/unloading of hatching baskets and empty trays. It is important to realize that every time the egg is handled, some will inevitably be broken. Acceptance levels for cracked eggs in any commercial production process is very low, with a general target of 0.25–0.50% as a maximum total loss. The less the start/stop motions during incubator tray conveyance, the lower the damage risk to the eggshell. It is also important to consider the differences in shell strength and size as the hen goes through her 40 weeks of production. In general, the smaller eggs produced by younger flocks have stronger thicker shells, and as the hen ages and the eggs increase in size, the shell becomes thinner and more fragile. This is important to consider as the incubation tray remains the same, and the egg will sit differently due to the changing egg size. Eggs from younger hens (smaller) tend to roll or tip off center more often, especially with abrupt

start/stop motions. Eggs from older breeder hens are larger in both length and circumference, and therefore egg breakage can occur more readily on both the bottom of the shell at the tray interface and along the sides due to egg-to-egg contact.

The device that injects the egg must be able to locate correctly and repeatably upon the blunt or large end of the egg as it sits upon presentation in the incubation tray. While at first consideration, this action seems simple; however, characteristics such as of angle of needle entry, different egg height, egg orientation (leaning), tray rigidity and surface friction dynamics (shell, device) make egg location by the injection device a very critical 'first step'. Without proper egg location by the injection device, the vaccination process is compromised. Acceptable levels of 'miss-location' as a quality control point should be extremely low in design and application (0.003%). Desired angle of entry for shell piercing is perpendicular to the shell surface with the egg presented as it has been incubated and nestled in the incubation tray.

3.3 Embryo orientation and late-stage embryo development

We must consider some of the characteristics and physiology of the developing embryo to better understand the angle of entry and therefore the target delivery site for in ovo vaccination. The embryo is orienting for hatch at day 18/19 by positioning its head under the right wing with the upper body positioned toward the blunt end of the egg. The lower body is crouching in the small end of the egg with the feet positioned toward the sides of the egg (Fig. 1). The embryonic positioning for hatch begins on day 17 of incubation

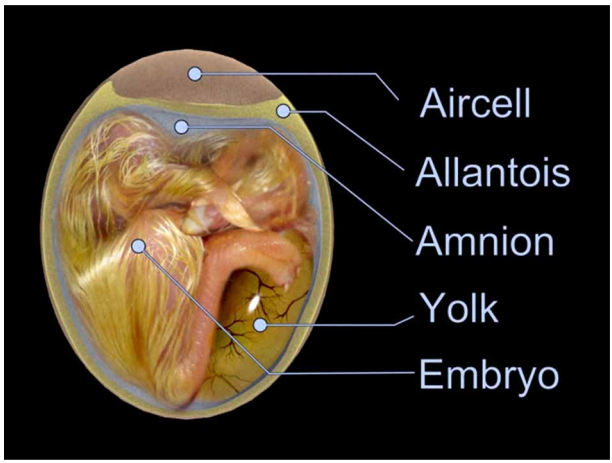

Figure 1 Embryo compartments and positioning for hatch at day 18 of incubation.

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and is influenced by gravity, as eggs set upside down will complete similar orientation; however, the egg itself is misaligned and positioned small end up. Thus, the head will be found in the small end of the egg (embryo malposition). These 'upside down' embryos will not have developed normally due to many reasons primarily associated with compartmental management of nutrients and waste. Many upside down 'malpositioned' embryos do not hatch or, if they hatch, are of poor quality.

It is important to understand the developmental stage and progression during the day 18/19 time frame. Chronological time of development and developmental stage are not necessarily the same between incubation type, breed type, hen age and storage time. Small differences in average heat during incubation (hot spots or cold spots) and egg storage time before incubation can significantly affect embryonic growth rate. This will be discussed further in later section regarding vaccine delivery site in ovo. It is important to consider that the earliest moment for in ovo vaccination is when the embryo is in the normal position for hatching with the head under the right wing and the stalk of yolk has begun to ascend into the abdomen. Intestinal loops should not be visible in the yolk and the yolk has a bi-lobed appearance upon examination. The latest moment of development for successful in ovo vaccination is just prior to external pip. The corresponding chronological time in incubation can be as broad as from day 17 plus 14–16 h to day 19 plus 2–4 h of the normal 21 day (504 h) of incubation.

Figure 1 shows the five major compartments in the egg at day 18 of embryonic development. It is important to understand that the internal structures are changing as the embryo develops. The amnion and allantois will be disappearing, the yolk sac will be pulled into the body cavity and the embryo's body will be growing significantly larger. The air cell chamber grows slightly larger from day 17.5 to day 18.5 as the amnion and allantois regress. Then the chamber becomes slightly smaller due to the embryo's final growth surge just prior to hatch. The 'pip-line' becomes evident as the chick breaks through the shell during hatch. It is defined by the bottom edge of the air cell chamber, where the inner shell/outer shell membrane separate. Here we find a 'weakening' of the shell. Calcium has been removed from the shell from below the base of the air cell membrane by the chorioallantoic (CAM) blood supply. The calcium from the shell is required for bone growth. The shell area above the air cell membrane retains the original calcium level deposited by the hen, thus forming a thick/thin demarcation forming the 'pip-line' around the egg. The air cell structure in the blunt end of the egg also serves as an important 'staging' area and function to the hatching chick. Prior to externally pipping the shell, the embryo's beak breaks through or 'pips' internally through the inner shell membrane early on day 19 of incubation and initiates pneumatic respiration. Once the lungs and air sacs are clear of fluids and functioning (typically 6–18 h)

and the yolk sac is withdrawn into the abdomen, the embryo will externally pip through the shell and begin the final stages of hatch.

Basic knowledge of embryo positioning at injection time is important as changes in egg orientation from an exact vertical stance will affect where the embryo eventually resides inside the egg. An egg tilted one direction will result in embryo positioning with the head pointing away from the angle of tilt. To vaccinate correctly, the site of vaccine placement during injection must target the embryo body or amnion (more detail in subsequent text). The 'target' inside the egg is the area identified by the small bullet point showing 'Amnion' in Fig. 1. This is a central location in upper portion of the blunt end of the egg and can be visualized as a small pocket or cup-shaped area formed by the curled head and neck as it rests beneath the right wing. When the egg is vertically tilted, the angle of entry needs to be opposite the angle of tilt to reach the central 'pocket' or target point. The device location must therefore rotate axis (not just straight down) and create an angle entry toward the direction of the egg tilt while maintaining a 'flat' adjusted approach, directing shell punch and needle trajectory perpendicular to the shell surface. Without rotational freedom and translational movement to locate on the eggshell surface, the angle of entry may not allow the needle to permeate the shell (deflect) at all or may incorrectly direct the shell punch and injection needle toward an improper site such as the head of the embryo. So not only does the device require the freedom of movement to physically locate correctly on the egg, the material and design of the device interface (locator) should permit a 'sliding' or free movement along the shell surface. Light dense polymers of plastics (such as Hydex®[2], Rynite®[3], Ertalyte®[4], Delrin® or polyethylene terephthalate [PET]) provide superior characteristics and performance as part of material and design of egg locator fixtures of injection devices.

3.4 Shell penetration and the injection needle

Force of pressure required to cleanly permeate the shell with a needle stylus (shell punch) depend primarily upon speed (acceleration) and needle tip (punch) design. The greater the speed, the lower the required pressure. In current designs utilizing needle of 16 gauge or 18 gauge, the force to permeate an average individual shell average between 3 and 4 pounds of pressure (1.4–1.8 kg) at a speed of 8–14 in./s. The punch tip itself should be designed to repeatably produce entry (eggshell punch) without radiating cracks at entry point in the shell. It is also important to consider the possible transfer of energy that may break the shell in another area such as the small end that is supported

[2] Hydex® is a registered trademark of AL Hyde Company (Ensinger), Grenlock, NJ, USA.
[3] Rynite® and Delrin® are registered trademarks of DuPont de Nemours, Inc., Wilmington, DE, USA.
[4] Ertalyte® is a registered trademark of Quadrant EPP Europe N.V., New York, NY, USA.

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by the tray or the sides that are in contact with adjacent eggs. Due to the inconsistencies in egg support as provided by the incubation tray, the injection device may require an individualized egg support mechanism that lifts the egg slightly out of the incubation tray (from below). The injection device also requires a mechanism to secure positioning for the entire injection sequence once the device is located upon the eggshell surface. It is important to note that during egg support there should be minimal change in orientation of the egg along its vertical axis. The goal during egg support for injection is to keep the original angle of entry secure and minimize 'rolling' of the egg. Robustness of the needle tip should also be considered as the process requires repeated entry in production flow with minimal need for replacement. Custom tip design of straight cut 30° bevel has shown superior performance when compared to standard B bevel (subcutaneous injection needle, 28° tip) as measured over time with minimal burring or bending. As the punch stylus must also undergo repeated sanitation (between injections), the material of choice is high-quality 316 stainless steel.

The perspective of egg injection for embryonic vaccination as a two-step process (permeate shell, insert needle to inject vaccine to the embryo/amnion) lends itself to the design of a 'needle inside a needle'. Separate needles allow for separate process priorities for each operation as well as specific needle tip design. Shell penetration exposes the entry needle to both a physical (shell) and microbiological (bacteria/fungus) challenge. Fecal organisms found within the shell can be transferred more easily and directly to the embryo or amnion if the same stylus or needle is utilized for both shell entry and injection. A 'needle inside a needle' approach to egg injection for embryo vaccination significantly reduces the possibility that the needle serves as a vector of transmission and creates a microbial challenge at vaccine delivery point (the embryo or amnion). A short stroke of the outer needle or eggshell punch is possible, and the larger-shell punch needle stops before touching the embryo or chorioallantoic membrane (CAM). The air cell chamber is present directly below entry site on the blunt end of the egg, and the shell punch entry is only into this space. The shell punch needle should enter the egg 0.635 cm (0.25 in.) as measured from the shell surface. Injection needle penetration should target 2.54 cm or 1.0 in. injection depth. The segregated two-step action separates the required force and speed to permeate the shell from those required for a softer slower needle penetration of the CAM and embryo/amnion. The needle for vaccine delivery can also be designed specifically for that purpose (injection proper) and be of small diameter to minimize trauma to the embryo. A smaller needle and small needle tip design will additionally minimize needle lumen interface and exposure of the vaccine to microbial challenge and sanitation fluids as well as improves sanitation efficacy by limiting total surface area to disinfect. The injection needle should have a minimal effect on the embryo

and support structures such as the chorioallantoic blood supply, the inner shell membranes and allantoic and amniotic membranes. The needle should be a small size (gauge) and the correct tip design for piercing the membranes or the embryo. Additionally, the needle should be large enough to deliver the vaccine without causing physical damage to the vaccine. Consideration must be given to the total surface area of the needle that is exposed to the internal contents of the egg and therefore the ease of sanitation. The surface area created by large needle sizes, extended canula openings or side port openings restrict the ability to sanitize the needle. An additional benefit to a small-tip angle is a more precise delivery point. Comparisons between a customized 20 gauge 45° straight cut needle tip and standard 28° B bevel (subcutaneous) tip show both a more consistent delivery site and a greater sanitation efficacy for the customized tip design. The 45° needle tip also minimizes membrane and embryo trauma in ovo as compared to the 28° needle tip, presumably due to cutting action presented by the design for subcutaneous injection.

3.5 Vaccine specifications and delivery in ovo

Vaccines licensed for use in ovo include several cell-associated vaccines comprised of CEFs infected with herpes viruses for Marek's disease (Table 1). These vaccines have been shown to be safe for the developing embryo as well as efficacious against subsequent disease challenge later in life. The vaccines include not only all three serotypes of Marek's disease but also several recombinant vaccines utilizing the serotype 3 herpes virus of turkeys (HVT) as the vector to express immunogenic proteins of other diseases (Newcastle's disease, infectious laryngotracheitis, infectious bursal disease). The Marek's disease vaccines are cell cultures (CEF) with approximately 20% of the cultured cells infected with the herpes virus. The vaccine is stored frozen prior to use in sealed ampules submerged in liquid nitrogen. Once thawed, the vaccine is diluted in special nutritive diluent that not only supports the viability of the cell culture but also provides a pH-sensitive medium (color change) to visually ensure osmotic stability of the vaccine mixture. The mixed vaccine has a moderate cell concentration rarely exceeding ~1.0×10^7 per milliliter of total cells, of which ~2.0×10^6 are infected with vaccine virus. In contrast, human red blood cell concentration is approximately 5.3×10^7 per milliliter. As the Marek's disease-based vaccines are a live cell culture, and the cells are somewhat swollen due to the viral infection, dispensing or pumping of the mixture must be gentle and cause little or no further damage. Successful designs include peristaltic and diaphragm-type pumping systems that simulate kidney dialysis or heart bypass pumps that handle blood. It is also important to consider the required time and pressure created by the pump to complete the injection process if the needle penetrates the embryo

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proper (versus delivery into the fluids of the amniotic sac). Dispense timing or time required for fluid pumping to initiate and complete dispense out of the needle tip must be coordinated with device programing such that needles are in the egg correctly before dispensing initiates. Similarly, coordination must occur such that needle retraction happens after dispensing is complete. The displacement-type pumping presented by peristaltic devices easily meets pressure requirements for placement of vaccine into embryonic tissue; however, diaphragm pumps and their control mechanisms require unique pressure assurance by design. Normally the diaphragm is driven by a spring-load electronic solenoid valve. The strength of the spring, and therefore the voltage required to open and set the spring tension, is paramount to the ability of the pump to dispense into tissue and against a 'head pressure'. These pressures and therefore the whole pumping mechanism design require thorough intralumenal pressure analysis and testing to ensure no cavitation (formation of bubbles) occurs during fluid movement to remove any risk of damage to the vaccine. Other vaccines licensed for use in ovo include live viral antigens that are not cell-associated (fowl pox, fowl pox recombinants [infectious laryngotracheitis, Newcastle's disease], infectious bursal disease), antigen–antibody complex viruses (IgG plus infectious bursal disease) and protozoal vaccines of *Eimeria* (coccidia) oocysts of combined species (*E. maxima*, *E. acervulina*, *E. tenella*). While not as sensitive to pressure and handling, the live viral and protozoal antigens also require safe and aseptic handling during preparation and administration. It is important to consider the diluent not only as a supportive media, but also the key constituent that must be routinely removed and cleaned effectively from the system to prevent inadvertent contamination.

Important features of the vaccine delivery systems include smooth polished bores and manifolding, high-density plastics (such as Hydex®, Ertalyte® Rynite®, Delrin®, PET), bottom-sealing barb-type fittings (for tubing connections), pharmaceutical grade tubing and robust, accurate, low maintenance operation. Equally important to the design of the pumping system are the material and internal surface characteristics to enhance and support clean-in-place sterilization. Commercial application of vaccine in ovo in a practical sense requires that the system remain closed by design to maintain a sterile internal environment in storage and impervious to environmental contamination during use. Key components of the vaccine delivery system need to remain in place and subjected to 'clean-in-place' sanitation on a daily use basis. Disposable entities such as tubing and needles must also maintain a relatively long duration of use and be easily replaced during planned maintenance during off use period. In considerations for programs of cleaning and sanitation, two key entities exist. One entity is the complete cleaning and disinfection of the vaccine pumping system (before and after use) and the other is the sanitation

required between injection sequencing (from egg to egg) as part of the in ovo vaccination process.

3.6 Sanitation for in ovo vaccination

It is imperative that the process of egg to egg or needle/punch sanitation between injections be robust and effectively sterilize the key delivery components of the injection device that interface with each egg/embryo. The sanitation process requires a very short time frame, and the disinfecting agent must have a broad microbial kill spectrum. Successful systems utilize a high concentration of chlorine with buffered pH to maintain a high level of hypochlorous acid. In direct 'contaminated egg to live egg' injection studies (bacterial challenge modeling), needle/punch disinfection with 2500–5000 ppm chlorine (with pH of 7.0–8.0) utilizing a ~6 s injection interval can successfully address a bacterial challenge of $1 \times 10^{5-7}$ CFU per milliliter of egg content. The cycle time requirement (~6 s) between injection is the primary restriction of the process and limits the possible candidates for needle/punch disinfection. Kill time for many disinfectants with or without additional organic challenge normally takes minutes, much less seconds. Another important aspect is addressing and minimizing organic buildup. Without addressing the entire lower portion of the injection device, calcium 'dust' or organic debris from 'exploding' rotten eggs created or liberated during egg punch can build up and deposit/contaminate the exterior of the punch and inner areas of the location device. Targeted dispense of the needle sanitation fluid to only the injection needle is not adequate. The sanitation process should permit cyclic rinsing and cleansing/disinfection of these adjacent areas. Small holes in the upper regions of the punch within the needle sanitation fluid pathway or cyclic rinsing of the complete lower portions of the devices can accomplish this additional sanitation requirement.

The cleaning and sterilization of the vaccine delivery system of in ovo vaccination devices present a unique challenge (Denoya et al., 2010). When clean and sterile, the vaccine system cannot contain any residual chemicals or biofilm formation which may negatively affect the vaccine. The cleaning process must focus detergent activity upon the removal of primarily carbohydrates (sugars) that make up the diluent but must also address certain levels of proteins (albumen, chick embryo fibroblasts) and inadvertent contaminants (bacteria, fungi, virus) that may have been introduced by mistake. The disinfection steps cannot utilize any residual chemicals, and storage of the equipment must consider probable microbial challenges in the harsh environment of the hatchery. Catastrophic results have been observed when pathogenic bacteria or viruses have been inadvertently introduced into vaccine or vaccine pathway utilized for in ovo application. It is important to

consider that these accidents, while rare, do occur. The vaccine clean-in-place system must be designed and be able to overcome the mistake during every cleaning cycle. A vaccine contamination is not apparent until 2–3 days after application, so the clean-in-place system must completely sterilize the vaccine delivery system before and after every use. Any level of biological or chemical 'carryover' from day-to-day production process is not acceptable for successful in ovo veterinary medical devices used for vaccination of embryos. Chemicals typically utilized by clean-in-place in ovo vaccination devices are chlorine-based detergents and sanitizers that leave no residual chemical. Volumes and exposure times must be adequate to remove not only the residual vaccine and diluent but also all chemicals used in the cleaning cycles. Different pumping systems require different cleaning programs, as the pumps themselves may be included in the vaccine pathway or the pump may be mechanically separated (peristaltic). Regardless of pumping type for vaccine dispense through the needles, clean-in-place systems require control by a separate dedicated pump system to adequately address pressures, pathways and possible low flow areas of dispense systems. During each step of the cleaning cycle (each chemical), the solutions should replace the dead volume of the total vaccine pathway at least ten times. It is important to note that sterile saline is utilized to remove storage chemicals (isopropyl alcohol) just prior to the addition of vaccine. Dead volume of delivery system should be replaced at least ten times as target volume for sterile saline to retain sterile and chemical free pathway. Overnight and short-term storage should include system fill and fluid exposures to the entire internal surface via isopropyl alcohol. Isopropyl alcohol is preferred as it leaves no residual upon evaporation, is easily removed with sterile saline, scavenges free chlorine, has minimal effect on most plastics and stainless steel and presents an effective bacteriostatic hurdle within the clean interior of the delivery systems.

3.7 Hatchery hygiene and biosecurity

Additional sanitation requirements to support the vaccination process include heightened awareness and cleanliness of the hatchery, and more specifically the hatch baskets and hatcher compartments. The hole in the egg from vaccination procedure is not sealed and represents a possible entry point for microbial contamination after the process. This is most apparent with fungi, specifically *Aspergillus* species. Other areas of focus for fungal control in the hatchery include the large ventilation equipment (HVAC, heating units, evaporative cooling units) that supply air to the entire facility as well as the systems utilized to control pressure of the exhaust systems (plenums). Priority can be placed upon the ventilation for the hatcher and incubators (hallways), the egg storage area and the dirty exhaust from processing and hatcher compartments. The

control of hatch dander or fluff in exhaust systems is a priority such that minimal recirculation to fresh air inlets is accomplished. Plenums and misting exhaust ducts are usually required to capture and collect the airborne contamination within the building for routine waste handling and cleaning. It is important to understand the risks of poor-quality hatching eggs being introduced into incubation and the production environment. These eggs represent a significant long-term negative influence and microbiological burden due to high probability and incidence of horizontal transmission of bacteria and fungus throughout the various environments of the hatchery. There exists a threshold of microbial challenge which can influence the quality of all production parameters, including in ovo vaccination.

Biosecurity of the hatchery work areas as well as product (egg/chick/equipment) flow through the hatcher is integrated into the hatchery ventilation systems and programs. Clean areas such as clean basket storage, egg storage and incubation hallways should maintain positive air pressure environments and designated dirty areas should consistently have negative pressures that supplement 'clean to dirty' biosecurity of the facility. Limited compromises of counterflow (dirty to clean) may happen as well as minimizing shared work areas function to reduce environmental risks of the hatchery operation. Additional attention to detail includes the water quality that supplies various functions in the hatchery. Humidification units are of primary importance as they can serve as dispersion source for high levels of bacteria through contaminated water supply. The use of steam humidification for humidity in air supplied to incubator and hatcher hallways greatly improves the quality of the humidity, it also serves to eliminate bacteria from the water source. For equipment that utilizes spray for cooling incubation and hatching compartments, it is recommended that the water be treated with reverse osmosis (RO) to greatly reduce the mineral content and to chlorinate the RO water prior to use to prevent subsequent contamination downstream of the treatment (water storage accumulation tank). It is recommended to evaluate main water supply and treat accordingly. In the hatchery, many end-use applications (water hoses, pressure washers, utility sink, laboratory faucet, etc.) are contaminated with environmental and fecal organisms. Routine chlorine 'shock' or drain/dry programs need to be enforced along with automatic pH and disinfectant application to incoming water supplies.

Qualitative and quantitative microbial sampling should be completed prior to the initiation of in ovo vaccination at any given facility (hatchery). Additionally, routine microbial monitoring should be completed (typically on a semiannual basis) to assess ongoing risks associated with quality of the air, water and hatchery environment. Table 2 is a summary of environmental assessments completed in commercial hatcheries during the late 1990s prior to the incorporation of in ovo vaccination. Approximately 20% of the hatcheries

Table 2 Percentage of 250 commercial hatcheries with *Aspergillus* contamination[a]

Level of *Aspergillus*[b]	Hatchers	Hatcher ventilation	Hatcher hall	General ventilation	Incubators (setters)	Miscellaneous[c]
	(%)					
None	63	56	46	68	45	53
Low	23	18	27	15	30	28
Moderate	6	10	8	7	10	10
High	8	16	19	10	15	9

[a] Data from Williams et al. (1994) and King (1995).
[b] Low levels = 1–3 colonies per plate; moderate levels = 4–10 colonies per plate; high levels = >10 colonies per plate.
[c] Egg room, vaccine prep, roof, etc.

evaluated required remediated cleaning and disinfection prior to incorporation of in ovo vaccination technology due to high level of fungal challenge found in the hatcher hall and ventilation systems.

3.8 Importance of compartmental delivery for in ovo vaccination

Detailed evaluations were conducted to better understand in ovo vaccine delivery site and subsequent efficacy to Marek's disease vaccination (Wakenell et al., 2002). The studies clearly showed preferred compartments or sites of vaccine delivery within the egg and subsequent efficacy against disease challenge. Marek's disease vaccine requires placement into the amnion or the embryo body during the in ovo vaccination process. Cell-associated vaccines placed into the waste compartment (allantois) or directly onto the air cell membrane showed very reduced or no efficacy against subsequent disease challenge. Research conducted by Jochemsen and Jeurissen (2002) investigated and determined that localization and uptake of substances injected in ovo and particularly to the amnion were characterized by the delivery site as well as the substance to determine its final localization. The amniotic delivery of particulate colloidal carbon and microspheres resulted in the substances entering the embryo by the mouth (imbibed) and ingested in the intestinal and respiratory tract. These substances reached the lungs via the trachea and the bronchi and were absorbed by the air capillaries. Substances were also absorbed by the bursa of Fabricius (retrograde absorption). Viral substances (infectious bursal disease vaccine) were also found in many different organs after delivery into the amnion.

In contrast to the amnion, the allantois supports embryonic development primarily as a waste reservoir and the very important function of water balance

and control of water loss. The chorioallantoic blood supply supporting the allantois serves a respiratory function (CO_2, O_2) across the shell and air cell membrane, but also works in conjunction with water balance and waste compartmentalization. The resulting allantoic fluid is acidic and thus will negatively affect cell cultures such as Marek's disease vaccine. The allantoic fluid environment serves an important antimicrobial barrier as it is found along the exterior of the inner egg along the eggshell surface. Similarly, the air cell membrane is a significant physical barrier, and the cell-associate (MD) vaccine cannot cross directly into the vascular bed or access the amnion or embryo. Molecules with a size below ~10 000 Å and presented in liquid solutions can pass directly into the chorioallantoic blood supply when placed upon the air cell membrane (examples include many antibiotics). It is important to note that in the past, antibiotics were routinely incorporated into in ovo vaccination. While not necessarily required, the use of antibiotics with in ovo vaccination represented a very controlled and efficacious delivery route. In the last 4–5 years, we have seen the elimination of all antibiotics important to human medicine used in animal production, including the use in ovo. Work by Phelps (1995) showed compartmental absorption levels of gentamicin following delivery to the air cell, amnion, embryo and allantois (Fig. 2). These data highlight the differences and importance of delivery to the individual compartments found in the egg in late-stage embryonic development.

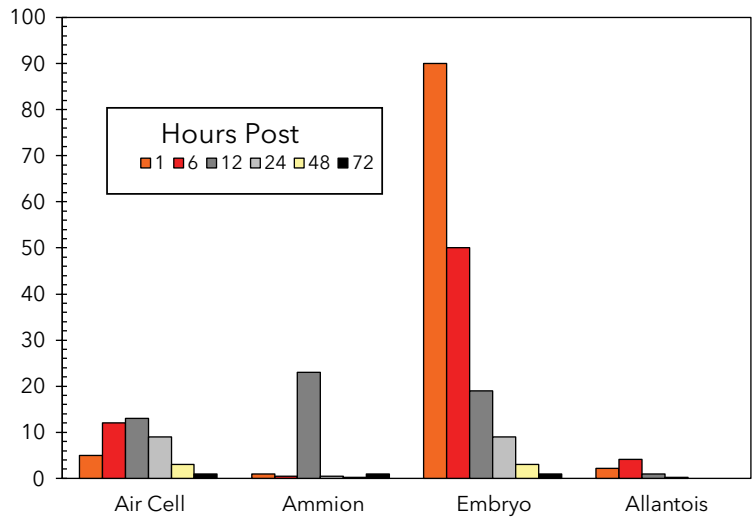

Figure 2 Plasma gentamicin levels (μg) following in ovo administration of 1 mg gentamicin into specific compartments of day 18 embryonated broiler eggs. Source: Phelps, P., 'The In Ovo Administration of Antibiotics into Broiler Eggs at Transfer', PhD dissertation, North Carolina State University, 1995.

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Compartmental delivery of Marek's disease vaccine in ovo has been thoroughly evaluated; however, the application of other vaccine viruses to various compartments of the egg is not as well understood. Evaluations with infectious bursal disease vaccines have shown that while many strains are safe and licensed for in ovo use, certain intermediate plus and 'hot' strains of the vaccine virus are not safe when given in ovo unless combined into an antibody–antigen complex vaccine (Bursaplex®, Bursammune®[5], Magniplex®). Without the antibody–antigen complex, the more aggressive bursal disease vaccine viruses, when given in the egg, invade the bursal tissue and replicate to the degree that the tissue is functionally destroyed, severely affecting the humoral arm of immunity for the life of the animal. The bursal tissue of the embryo is still immature and maternal antibody levels are lower than those found at hatch. In addition, bursal-derived immunocompetent cell migration to secondary tissue (bone marrow, spleen) is not fully complete until post-hatch. We know immune development of chickens is not complete at hatch, but many of the necessary tools are in place for a protective immune response to certain vaccines on day 18 of incubation. During late-stage embryo development at injection/transfer (day 18/19), the bone marrow, spleen, bursa of Fabricius, liver and thymus are populated with functioning immune cells, although less than the full complement of T and B cells. B cells are still undergoing extensive growth and development in the bursa. T-cell migration from the thymus occurs in three waves, with the first two waves occurring prior to hatch and the third wave occurring during the first week after hatch. Destruction of the bursa prior to these migrations can be seen with certain IBD vaccines and viruses.

Similarly, certain fowl pox vaccines are safe and efficacious (licensed), but others can cause issues when given in ovo (Williams et al., 2010). The problems are exacerbated with the use of chick embryo origin pox viruses as well as in ovo vaccine delivery timing that is early on day 18 of incubation, presumably applying most of the vaccine into the amnion rather than the embryo body. Efficacy and safety studies show that dosage control (viral units) as well as virus type (origin, culture media) and injection timing should be considered in design and use of fowl pox and pigeon pox vaccine viruses for in ovo use (Avakian et al., 1999). Application should be limited to tissue culture origin pox viruses at log 2–3 concentration given late on day 18 or early day 19 of incubation. Avian reoviruses (tenosynovitis) have been shown to be efficacious against subsequent disease challenge; however, the vaccines are immunosuppressive when given in ovo to chickens with low or no maternal immunity (Guo et.al., 2004). The safety of the reovirus vaccines can be enhanced using an antibody

[5] Bursamune® is registered trademark of Boehringer Ingelheim, Ingelheim am Rhein, Germany.

complex; however, no commercial vaccines are available in this design or for use in ovo.

3.9 Immunity as a function of vaccine specifications

Without vaccination in ovo, the immune system of the chicken develops only after hatch (Lehtonen et al., 1989; Mast and Goddeeris, 1999; Schierman and Ordskog, 1961; Seto, 1990) and only transient T cell responsiveness occurs during the first few weeks of life (Lowenthal et al., 1994). Expression of MHC-1, activation of T cells and humoral immune responses are low or nonexistent at hatch and develop within the first weeks of life. The poor immune response is not necessarily due to the lack of T cells but rather due to the cells inability to complete the steps necessary for cell activation (cytokine secretion, receptor expression) (Lowenthal et al., 1994). More specifically, receptors that mediate interferon types 1 and 3 transcripts are delayed and develop gradually (Karpala et al., 2012). It is also important to consider the role of maternal antibodies during the critical first weeks of life, as they enable growth and maturity of immunocompetent organs and spread of immunocompetent cells to secondary sites within the chicken without overt compromise of the tissues due to viral or infective challenge. One of immunology's central tenets is the concept of neonatal tolerance. Immunization of neonates may result in clonal deletion with subsequent tolerance. It has been shown that animals in the prenatal state are able to mount a protective, lasting immune response to antigens or pathogens administered during neonatal life (Bot et al., 1997; Ridge et al., 1996; Sarzotti et al., 1996; Sharma, 1987a).

Work done at the College of Veterinary Medicine in Raleigh, NC (Gimeno et al., 2015a,b, 2018) showed that the vaccination of chicken embryos at day 18 of incubation with herpesvirus of turkeys (HVT) rendered chickens more immunocompetent at hatch as measured by an increase in activation of cell phenotypes (MHC-I+, CD3 plus MHC-II+) to levels comparable to non-vaccinates at 10–14 days of age. Additionally, the lymphocytes of the chickens vaccinated in ovo responded better to Con A in vitro as well as improved response against antigens unrelated to the HVT antigen (phyto-hemagglutinin subunit L (PHA-L) and Keyhole limpet hemocyanin (KLH)). It is important to note that administration of other Marek's disease vaccines in ovo (SB-1, CVI-988) did not have the same effect on cell phenotypes of the spleen (Gimeno and Cortes, 2011; Gimeno et al., 2015b). The mechanisms through which the herpesvirus of turkeys enhances and matures the immune competence of the chicken embryo are not completely understood; however, more recent work suggests that increased transcripts of interferon gamma (IFN-ɣ) and toll-like receptor 3 (TLR-3) in the lung and spleen may be the mechanism that accelerates immune maturation.

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Viral replication after in ovo vaccination is evident with all live viruses whether delivered to the embryo or the amnion and viral replication contributes not only to the subsequent immune response but also to the safety of vaccine when delivered to the embryo. Differences exist with regard to viral replication within vaccine types and these differences are related to the pathogenesis of the individual strains. However, even with limited viral replication after vaccination in ovo, CVI-988 elicits a very good immune response, especially against highly virulent Marek's disease isolates (Zhang and Sharma, 2001). The type of virus, the concentration and the delivery site as a function of embryo compartmentalization and maturity contribute to replication rate but more importantly to both safety and efficacy of the vaccine and is unique for any given virus. Many vaccine viruses are not safe given in ovo, for example the respiratory viruses of Newcastle's disease, infectious bronchitis and infectious laryngotracheitis. Their replication rates and invasive pathogenesis cause embryo morbidity and mortality even with repeated passages and isolations designed to reduce virulence. As noted earlier, the safety of the specific virus utilized for in ovo vaccination is paramount and cannot be understated. HVT is unique in that it accelerates immune maturation, but also replicates at a high rate when administered to the embryo (Abdul-Carrem et al., 2008; Sharma, 1985, 1987b, 1989), it is safe at excessively high doses (25X) and it is safe and efficacious across a large delivery window of development (day 14 to day 19 of incubation). The use of HVT as the viral backbone for recombinant or vector-based vaccines presents a unique vaccine construct, as the HVT virus is a large virus suitable for gene insertion, the virus replicates at a high rate, the insertion does not significantly affect replication and expression, the protein expression resulting from the insertion are intact and exact, and most importantly from a regulatory and ethical stance, the virus is not readily shed from the bird in feather dander to the environment. While the vaccine virus can be found in the feather pulp of the developing feather of the young chicken after vaccination, the vaccine virus for HVT in both recombinant and non-recombinant form does not shed horizontally via follicular dander release. This characteristic of the virus in vivo improves the safe application and acceptance of these genetically modified viruses for vaccine use.

When we consider the overall enhancement of immune competence and earlier protection against Marek's disease afforded by in ovo vaccination of chickens, we can see why the vaccination process in commercial industry applications results in improvements to parameters such as feed conversion, mortality and production efficiencies (Gildersleeve et al., 1993; Sarma et al., 1995; Ricks et al., 1998; Williams and Villalobos-Chaves, 2000; Bruzual et al., 2005). Feed efficiencies during the growing period can be directly related to better immune status at any given time, but we should also consider the

energy requirements utilized during maturation. 'In the egg' maturation of immune competence does not involve feed consumption; however, maturation and immunocompetence developed during the first weeks of life for birds vaccinated at hatch will have a feed cost variable.

3.10 Asepsis of vaccine mixing and handling

While strictly defined with standard procedures and basic in concept, mixing and handling of vaccine for in ovo use is challenged primarily by repeated execution and shear volume requirements. It is also a challenge due to the frozen glass ampule design for Marek's disease vaccines and the limits and challenges that the procedure to thaw and open the ampules present to aseptic technique. The glass ampules require rapid thaw process, typically in a temperature-controlled water bath. After thawing, the ampules are dried and the top is snapped or broken off, opening the contents to atmosphere. The concentrated vaccine is then removed from the ampule with a needle and syringe and transferred to an intravenous-type bag of diluent. It is important to note the utilization of the IV bag. Not only is sterility easily maintained through mixing procedures via injection ports, the collapse of the bag as the vaccine is utilized during injection requires no venting or air introduction. Glass bottles require ventilation (due to negative back pressure) and should not be utilized for in ovo vaccination due to contamination risks. The diluent also contains pH-sensitive dye (phenylalanine red) that remains red at the desired neutral pH = 7.0. If diluent contamination occurs during storage prior to use, the waste products and enzymes produced by the contaminating bacteria will change the pH of the fluid and the color of the diluent will change (yellow = acidic pH <6.5, maroon = basic pH > 7.5).

Many procedures outlined from vaccine manufacturers recommend rinsing of the vials, thus further increasing the risk of contamination of the open glass ampules utilized for Marek's disease vaccines. It is advised to review these recommendations in light of contamination risk versus reward of recovering less than 2% of vaccine during nominal ampule rinse. Several critical control points exist during the mixing process and involve limiting inadvertent environmental contamination. The vaccine preparation area or laboratory should be a strictly controlled environment with high-quality sanitation and hygiene. In many high-quality incubation facilities, the mixing procedures are completed in a laboratory area supplemented with a work area beneath a laminar flow hood to improve biosecurity. Daily, weekly and monthly programs of sanitation should be enforced for the laboratory environment as well as limiting entry to only designated and trained personnel.

Inadvertent bacterial contamination of vaccines for in ovo use has been traced primarily to the thaw bath water and subsequent poor handling

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technique in drying and opening the ampules. Capillary action of thaw bath water on the exterior of the ampule at opening can result in transmission of the thaw bath water into the interior of the ampule and bacterial contamination of the vaccine at that point. It is a routine recommendation and practice to use a low level of chlorine (100–150 ppm Cl) in the thaw bath water. It is also advised to eliminate direct handling of the ampules while they are in the bath (utilize use of spaghetti strainer or perforated ladle). Additionally, a new clean paper towel (not cloth) should be utilized with every mixing session as well as fresh needle and syringe. It is important to consider other types of contaminations such as respiratory vaccines (viral or bacterial). Segregated work areas for preparation of vaccines for in ovo and spray vaccines applied at day of hatch are required to ensure no sharing of thaw bath containers or needle/syringes. Inadvertent contamination of vaccine for in ovo use can result in catastrophic morbidity and mortality for the developing embryos such that prevention is a requirement.

There exist several types of licensed vaccines for in ovo use that are not frozen in sealed glass ampules. These vaccines are presented in concentrated dehydrated form or as a refrigerated liquid contained in a sealed glass bottle with a rubber septum. These types of vaccines are much more user-friendly container and ensure asepsis due to the sterile entry made possible by the rubber septum.

While the vaccine mixing and handling process requires a certain level of skill and attention to detail, there also exists the challenge of sheer volume of vaccine and harsh environment of application. A hatchery incubating 1.2 million eggs per week will require approximately 20 800-ml bags of vaccine prepared each day (4 days/week). Each bag will require handling four to ten frozen Marek's vaccine ampules through the thawing and diluting process to provide the final solution of vaccine for use. On an annual basis, the procedure demands attention to detail and safe handling up to 50 000 ampules as well as safely attaching over 4000 individual bags of vaccine to injection devices, with no mistakes. Routine training and re-training programs are an important part of success as well as routine evaluations and monitoring of the adherence to protocol. Monitoring and training should include microbial sampling of mixed vaccine during normal run days representative of applications.

3.11 Egg handling during injection/transfer

As aforementioned, the window for timing of vaccination in ovo is chronologically set to occur during the late stages of development between day 17.5 and 19 days of incubation. Considerations include the optimal time for removal of the eggs from the incubator as part of the individual incubator design and operation as well as relative embryo development or stage. Multi-stage incubators can be

divided into two basic types: one whereby the eggs of each age or batch are contained in a trolley, and the second type whereby the eggs are placed by individual incubator flat into fixed turning racks secured inside the incubator. Single-stage incubation utilizes trolleys, so for most practical purposes they can be treated as a 'trolley' system. The trolley system allows for whole racks of eggs (5000–8000) to be removed at one time with the ability to move these eggs directly to an injection/transfer device for in ovo vaccination. While requiring a certain amount of labor to move the trolley, the trolley systems are more readily adapted to further automation for 'de-stacking' these trolleys and 're-stacking' the full hatch baskets during the process (more information later). The fixed rack systems require individual handling of each flat of eggs to remove them from incubation. This is obviously more labor intensive, and these types of incubation may process their eggs by double handing (placing incubated eggs into a 'farm' trolley, moving the trolley to injection/transfer device) or by removing eggs from the fixed rack positions and conveying eggs directly from the incubator to the injection/transfer device. It is important to consider that the removal process must be managed and controlled with attention to detail as to egg cracking and managing embryonic temperature. The embryos are exothermic and individually produce approximately 190 mW (0.65 BTU) of heat at this stage of incubation. Egg shell temperatures should target 100–101°F (not exceed 102.0°F) upon removal from the incubator and caution should be taken to stage the trolleys in horizontal airflow while they await the injection/transfer process. Overheating is the primary concern during the injection/transfer process.

Optimal timing for removal of eggs is different for the two multi-stage incubator designs. It is important to consider that the metabolic mass of eggs that are being removed represent a significant heat supply for that incubator. Removing the oldest eggs are an important step in the continual incubation process for all the remaining eggs and stages still in the incubator. The trolley systems recommend removal and transfer of eggs to the hatching compartments on day 18–18.5 of development. Extending the incubation cycle results in excessive heat management issues as the embryonic mass (same day of development) is localized into the trolley and position in the incubator. Earlier removal will conversely affect the incubation with cooler incubation challenges (Brake and Williams, 2002). Fixed rack incubation results in a broader spread of embryonic heat within the incubator as the six different developmental stages found within the machine are spread uniformly across the entire internal environment. Typically, these incubators perform optimally with transfer occurring after day 18.5 of incubation but before day 19 and 2–4 h. Their embryonic development is slightly behind trolley-based incubators in strict chronological versus developmental stage comparison.

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Embryonic development and population dynamics should be determined for both incubation systems to set final protocol and adjust 'start-to-finish' scheduling for the entire process day at the commercial hatchery. Utilizing embryonic stage of development for optimizing in ovo vaccination should be cognizant of population curves associated with eggs from young, prime and older breeder hens. Minimal development target delineated via egg breakout should be the embryo in the position to hatch with the head under the right wing and the stalk of the yolk physically entering the abdomen (no visible intestines at navel). The maximum development target would be 5–15% internally pipped with less than 1% external pip when injection process is complete. During the final scheduling of injection/transfer timing, considerations include total incubation time (eggs from older hens/younger hens > prime hens), egg set schedule (begin incubation) and general microbial load (eggs from older hens/younger hens > prime hens). For these purposes, eggs are usually processed on any given day in the order of prime, then young and finally eggs from older hens last.

Supportive quality assurance of the injection/transfer process for in ovo vaccination includes coordination of hatcher and hatch basket sanitation such that all baskets and hatchers are clean and dry, preferably pre-warmed to receive the eggs immediately after vaccination. The filters of the ventilation system (HVAC) and handling units also require sanitation and maintenance prior to introduction of vaccinated eggs into the hatching environment. As previously mentioned, fungal challenges to the hatching environment are a primary concern and focus of control in the 'everyday' processes of the hatchery utilizing in ovo vaccination. The vaccination process may take place in the hatcher hallway itself, in a designated injection/transfer room (preferred) or in an adjacent hallway. The environment for the device and process should be clean and dry at start with adequate air exchange to ventilate for embryonic needs (8–12 ft^3/min or 13.6–20.3 m^3/h per 1000 eggs) while maintaining a neutral or slightly positive room pressure. This is especially important to consider if the vaccination process is conducted in the hatcher hallway. Optimal environmental control (temperature, pressure) should be established when the first eggs are vaccinated and placed into the initial hatcher. The hatcher hallways for both single- and multi-stage incubation are typically held at 78–82°F with slightly positive air pressure (1.25–3.74 Pa or 0.005–0.015 in. of water draft) (Barnwell, 1998; Jung and Magrans, 1997; Martin, 2019). These conditions should be established and maintained during the vaccination procedures as the complete process may take several hours and the environment controlling air supplied to the hatchers must be controlled. Upon completion of the vaccination process, the hallway environment is normally cleaned of any organic debris (wash down) and care should be taken to not introduce excess water into the hatcher or adjacent incubator environments. Disinfectant

fogging is also commonly utilized after hallway cleanup to minimize the fungal and bacterial challenge to the hatching environment as supplied by the air of the hallway.

3.12 Automation and in ovo vaccination

The global acceptance and utilization of vaccination in the egg has opened the poultry production environment at the breeder farm and hatchery to further automation and labor efficiencies. Vaccination by individual handling and injection of day-old chicks after hatch created a significant delay and holding period between actual hatch time and transport to the farm. With the incorporation of in ovo vaccination, chick handling after hatch could realize significant improvements through automation. Downstream processing efficiencies include rapid chick separation from unhatched eggs, hatch basket/trolley washing, chick counting, spray vaccination and chick sorting and boxing for transport to farms. The efficiencies in after-hatch processing has significantly reduced the time between removal of the birds from the hatcher environment to transport and placement on the grow-out farm with access to food and water. It is important to realize that the early and uniform access to feed and water for any given flock significantly improves feed efficiencies and time (days) to achieve desired body weights and size (Peebles et al., 2017). It is typical of most production programs at present to require only 2–4 h between removal of chicks from the hatcher to placement on the farm in the United States. The industry has also looked at improved efficiencies at the breeder farm and incubation processes and how they interact. New incubator flat designs are larger and are one- or two-piece design per tray or level in the incubation rack. This makes egg handling more efficient and more easily adapted to robotic handling of the full incubation trays during injection/transfer. Multipurpose trolleys are designed to hold eggs from the initial loading at the breeder farm completely through incubation until removal of the flats for injection/transfer. There has been an increase in the construction and conversion to single-stage incubation due to the inherent efficiencies and technological advances presented by these technologies.

In more direct application to the in ovo vaccination process, automated unloading of incubation flats from incubator trolleys and loading them robotically onto in ovo injection devices has developed primarily over the last 8–10 years. Optically controlled robotic functions have advanced considerably over the last decade and have provided automation equipment specifically designed to handle manipulations required for individual incubator flat and trolley designs as they are utilized with in ovo vaccination. Additionally, robotic devices can repeatedly remove vaccinated eggs from the incubation trays following vaccination and place them accordingly in stacks of hatching

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baskets ready for placement in the hatcher compartments. The automation of these processes is unique to each type of incubation flat and presents unique challenges in their application; however, most are continually quality controlled and produce more predictable and consistent results with the elimination of human error. Automated production can result in up to 60 000–70 000 eggs/h vaccinated and transferred to hatchery baskets with 2–3 operators handling eggs through automation devices and one operator managing vaccine and in ovo device quality control. The labor savings alone between production utilizing hand vaccination after hatch and in ovo vaccination can exceed 500 man-hours/week. Additional benefits to bird health and growth due to lower stress during handling after hatch as well as shorter times to access feed and water combine to improve feed to body weight gain efficiencies.

4 Additional interactions and considerations for in ovo vaccination

4.1 Egg candling for viability

In ovo vaccination devices have advanced in their application processes to include candling technologies to determine embryo viability prior to injection. Candling or embryo viability determination has enabled targeted delivery of vaccine only to viable eggs as well as the ability to remove the non-viable eggs prior to injection and transfer. Removal of non-viable eggs reduces the microbial challenge presented by the nutrient potential for bacterial and fungal entities associated with these eggs. Additional benefits from candling include information as to individual flock status at injection/transfer with regard to viability (predictive analytics) and the removal of these eggs also presents an improvement in airflow and environmental conditions of the individual hatch baskets for the chicks during the hatching period. Candling can be accomplished with visible light or infrared light for simple determination of developmental stages or can be accomplished with more elaborate viability detection such as heartbeat or pulse oximeter determinations. The in ovo vaccination devices utilized throughout the industry routinely utilize viability detection for vaccine delivery (>90%) and additional non-viable egg removal is included in approximately 80% of production globally.

4.2 Chick handling and welfare

As aforementioned, in ovo vaccination has eliminated the need for the individual handling of day-old chicks for injectable vaccinations after hatch, and this in itself reduces stress on the hatched chick, thus improving welfare status. Chick handling after hatch involves removal of the stacks of hatching baskets from

the hatcher compartments and manual transport to central area of hatchery for manual or automated chick shell separator (baskets stacked upon dolly with wheels or use of pallet jack). Welfare considerations are primarily chick comfort as to appropriate temperature and fresh air supply (10–25 cfm per 1000) while in the basket stacks and during separation processes. The separation of chicks from unhatched egg and shell residue requires handling the individual hatch baskets. The automated process gradually inverts the basket onto roller or shaker bars that allow the chick to fall through the gaps between the bars while the unhatched eggs and eggshells are carried into an offal vacuum system. Humane handling includes maceration of all unhatched eggs. The live chicks are conveyed into a clean room (chick room) via belted conveyors for subsequent counting and sorting into handling boxes for spray vaccination and accumulation for transport to grow-out farms. To complete these processes in a repeatable rapid process, it is important in the design and execution to have a uniform presentation of the hatch baskets and control of the environmental conditions where the process is completed. The separation process includes automated devices similar in concept to other agricultural food-handling mechanization that sorts vegetables from organic debris during packaging. Air handling and temperature control are important to maintain the animals' body temperature and control organic debris (e.g. chick fluff, unhatched eggs) liberated during the process. Obvious challenges are evident as the safety and welfare of the day-old chicks require attention to their physical well-being and comfort and the fact that we are handling live somewhat fragile animals. Special attention to the mechanics of speeds, conveying belts and surfaces as well as drop heights required for isolating individual animals for counting and quality control. These equipment operate in relatively harsh and organic laden environments and must be robust to withstand rigorous cleaning and disinfection routines performed daily after processing. The devices must also be somewhat impervious to organic buildup as biofilms and microorganisms cannot build up within the animal contact and waste-handling entities of the equipment.

4.3 Labor efficiencies

As discussed earlier in Section 3.12, labor savings in direct comparisons between vaccination of day-old chicks and in ovo vaccination significantly favor the latter. Many individual complexes reduced labor requirements in the hatchery by more than 500 man-hours/week with the incorporation of in ovo vaccination to replace vaccination by hand after hatch. These efficiencies are not however the only efficiencies that can be attributed to the in ovo vaccination processes. With chicks receiving their vaccinations before hatch, chick handling after hatch became a great opportunity to improve efficiencies. Automated shell separators, hatch basket or tray wash equipment and robotic de-stack and

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stacking devices replaced manual labor required to perform the after-hatch processes. Efficiencies include labor reduction, but also eliminated many of the health and injury claims related to repetitive motion disorders associated with hand vaccination and handling the hatch baskets during chick and shell separation. Similarly, the handling efficiencies of hatching eggs and incubator functions became more attuned to the egg-handling possibilities prior to and after incubation. Efficiencies at the breeder farm and hatching egg production facilities include larger single-tray incubation flats more readily adaptable to automation (versus smaller multi-flat designs). Many incubator companies now offer dual-purpose trolleys for hatching eggs that can be loaded on the farm, transported to the hatchery and then utilized in the incubation process. The cycle will repeat once the trolleys are unloaded during injection/transfer, cleaned and sanitized and then returned to the farm. Prior designs required two different sets of trolleys, one for use at the farm and transport to the hatchery and the second set only utilized in incubation. Eggs required transfer from farm rack to incubation rack, thus doubling the amount of egg handling. The double handling not only required more labor but also increases the level of cracked or damaged eggs. Arguably, both improved egg handling and after-hatch automation could have occurred without in ovo vaccination; however, both efficiencies would not be as important and require longer-term return on investment associated with commercial poultry production.

4.4 Quality assurance

In ovo vaccination is not a simple veterinary medical application, and thus requires shared microbial responsibilities in assurance to standards and operating procedures. The quality assurance aspect begins prior to the incorporation of the technology with a basic understanding of the challenges of hatchery ventilation and work areas. As reviewed in Section 3.6, an evaluative risk analysis is recommended prior to incorporation of in ovo vaccination. The analysis is focused upon quality control of the ventilation system and the overall sanitation programs of the hatchery. More detailed quality control programs become part of the hatchery's standard operating procedures once in ovo vaccination is embraced. These include greater attention to detail with cleaning and disinfection programs and routine microbiological evaluations to support the execution of these programs. The mechanical aspects of all in ovo vaccination devices require routine maintenance and functional operations should be ensured by appropriate technically trained personnel. Quality control should include routine and periotic evaluations as to compliance to protocols for setup and shutdown procedures as well as operator functions to be completed during vaccine

application. These include appropriate training and educational exchanges to ensure confident operation of the equipment. Technical evaluations to ensure that biological and mechanical interface is correct include evaluations of embryonic stage of development at injection/transfer time, vaccine site of injection delivery in ovo and determinations to ensure quality of vaccine preparation and application through the device (microbial sampling, live/dead cell counting and vaccine concentration).

5 Conclusion and future trends

The discovery of the virus that causes Marek's disease in 1967 opened the door for the development of a preventative strategy, with the first vaccines available in the early 1970s. Continued research and development with Marek's disease led to the discovery and expansion of the science of in ovo vaccination in the early 1980s, and this continues today. Engagement and collaboration between the poultry industry, academia and biotechnical scientists at Embrex brought in ovo vaccination to commercial reality during the 1990s. Veterinary medical in ovo injection devices now bring the vaccination technology to more than 35 countries with a growing list of more than 35 different approved and licensed vaccines. Globally, more than 24 billion broiler chicken eggs are vaccinated in ovo annually. The technology has enabled adjacent evolutions of candling systems, automation for chick and egg handling as well as a required influence on improved sanitation and environmental controls at the hatchery and breeder farm level.

The application of egg injection technology is not simply a matter of delivering biologically active antigens to the developing embryo. The technique must be sterile and provide precise execution through the process. The details and specific aspects of avian incubation must be coordinated with the timing of egg injection and transfer of eggs from the incubator to the hatching environment to maximize the potential benefits. Each hatchery has specific microbiological criteria that must be controlled and optimized to incorporate in ovo vaccination and maximize the potential benefits from egg injection, while working with a real-world situation of people, programs and costs. With respect to the injectable antigens and vaccines, they must be safe for the embryo, as well as efficacious in establishing a superior immunological response to defend against disease challenge and cannot interfere with each other's efficacy.

The future holds clear challenges to develop more safe and efficacious vaccines for in ovo use; however, the technology has clear advantages as outlined herein. We will see further development of recombinant vaccines for in ovo use utilizing the HVT virus construct, and these families of vaccines will continue to provide tailored programs for disease prevention of the broiler chicken. The adjacent technologies of candling, egg handling and hatchery automation will also advance in their predictive and applied use during the

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vaccination process as well as support the efficiencies required in poultry production. We will see improvements in sanitation and quality control to address bacterial and fungal disease challenges of the environment and within the production of hatching eggs. Vaccines targeting bacterial and protozoal diseases will also be developed and become available as reliance on antibiotic treatment and use will continue to wain in the animal health industry. Prevention of disease requires controlled exposure such as vaccination provides. In ovo vaccination will continue to be the most accurate and uniform application route for cell-associated vaccines in support of broiler production for the global poultry industry.

6 Where to look for further information

As briefly reviewed in the final paragraph of Section 5, there will be future developments in vaccines at commercial level through animal health industry as well as research in many universities globally. Additionally, there are current governmental agencies (e.g. USDA) conducting research involving in ovo vaccine applications both internally and through university settings.

7 References

Abdul-Carrem, M. F., Hunter, D. B., Lambroune, M. D., Read, L. R., Parviszi, P. and Sharif, S. (2008), Expression of cytokine genes following pre- and post-hatch immunization of chickens with herpesvirus of Turkeys, *Vaccine*, 26(19), 2369–2377.

Avakian, A., Singbeil, B., Poston, R., Grosse, D., Klein, D., Whitfill, C. and Tripathy, D. (1999), Safety and efficacy of fowl and pigeon pox vaccines administered in ovo to SPF and broiler embryos, Proceedings of the 48th Western Poultry Disease Conference, Vancouver, CA, 56–60.

Barnwell, R. (1998), Recognizing common hatch ventilation problems, *World Poult.*, 14, 11.

Bot, A., Antohi, S. and Bona, C. (1997), Immune response of neonates elicited by somatic transgene vaccination with naken DNA, *Front. Biosci.*, April, 173–188.

Brake, J. T. and Williams, C. J. (2002), Timing egg transfer to improve hatchability, Proceeding Broiler/Hatchery Conference. Delmarva Poultry Industry.

Bruzual, J. J., Padilla, J. and Campos, R. (2005), Field trial conducted in Peru to evaluate the route of vaccine application against Marek's and infectious bursal disease (Burcell-HVT) in broiler chickens. In Ovo versus Subcutaneous. Proceedings of the For 54th Western Poultry Disease Conference, Vancouver, BC Canada.

Denoya, C., Dawson, E., Sessoms, D., Baumstein, M. and Shabushnig, J. (2010), A rapid microbiological assay to monitor the effectiveness of a vaccine injector sanitization following a microbial challenge procedure. *Am. Pharm. Rev.*, 13, 4.

Gildersleeve, R. P., Hoyle, C. M., Miles, A. M., Murray, D. L., Ricks, C. A., Secrest, M. N., Williams, C. J. and Womack, C. L. (1993), Developmental performance of an egg injection machine for administration of Marek's disease vaccine, *J. Appl. Poult. Res.*, 2(4), 337–346.

Gimeno, I. M. and Cortes, A. L. (2011), Chronological study of cytokine transcription in the spleen and lung of chickens after vaccination with serotype 1 Marek's disease vaccines, *Vaccine*, 29(8), 1583–1594.

Gimeno, I. M., Cortes, A. L., Faiz, N. M., Barbosa, T. and Villalobos, T. (2015a), Evaluation of factors influencing efficacy of vaccine strain CVI988 against Marek's disease in metatype chickens, *Avian Dis.*, 59(3), 400–409.

Gimeno, I. M., Cortes, A. L., Barbosa, T., Villalobos, T. and Pandiri, A. R. (2015b), In ovo vaccination with HVT hasten Maturation of chicken embryos immune responses in specific Pathogen Free Chickens (SPAFAS), *Avian Dis.*, 59, 375–383.

Gimeno, I. M., Glaize, A. and Cortes, A. L. (2018), Effect of Marek's disease vaccines on interferon and toll like receptors when administered in ovo, *Vet. Immunol. Immunopathol.*, 201, 62–66.

Guo, Z. Y., Giambrone, J. J., Liu, Z., Dormitorio, T. V. and Wu, H. (2004), Effect of in ovo administered reovirus vaccines on immune responses of specific-pathogen-free chickens, *Avian Dis.*, 48(1), 224–228.

Jochemsen, P. and Jeurissen, S. H. M. (2002), The localization and uptake of in ovo injected soluble and particulate substances in the chicken, *Poult. Sci.*, 81(12), 1811–1817.

Jung, S. and Magrans, R. (1997), Hatchability depends on adequate ventilation, *Zootecnica Int.*, December 19–21.

Karpala, A. J., Bagnaud-Baule, A., Goossens, K. E., Lownthal, J. W. and Bean, A. G. (2012), Ontogeny of the interferon system in chickens, *J. Reprod. Immunol.*, 94(2), 169–174.

King, S. F. (1995), The basics about *Aspergillus*, *Inovoject Customer Serv. Newsl.*, 3(5), 4–5.

Lehtonen, L., Vainio, O. and Toivanen, P. (1989), Ontogeny of alloreactivity in the chicken as measured by mixed lymphocyte reaction, *Dev. Comp. Immunol.*, 13(2), 187–195.

Lowenthal, J. W., Connick, T. E., McWaters, P. G. and York, J. J. (1994), Development of T cell immune responsiveness in the chicken, *Immunol. Cell Biol.*, 72(2), 115–122.

Manders, T. T. M., Matthijs, M. G. R., Veraa, S., van Eck, J. H. H. and Landman, W. J. M. (2021), Success rates of inoculation of the various compartments of embryonated chicken eggs at different incubation days, *Avian Pathol.*, 50(1), 61–77.

Martin, S. (2019), Hatchery Audits. Webinar Series from Scott Martin, LLC., scottmartinllc @att.net.

Mast, J. and Goddeeris, B. M. (1999), Development of immunocompetence of broiler chickens, *Vet. Immunol. Immunopathol.*, 70(3–4), 245–256.

Miles, A. M., Williams, C. J., Womack, C. L., Murray, D. L. and Gildersleeve, R. P. (1992), Commercial broiler studies of Marek's disease vaccination in ovo. Proceedings of the XIX WPC Amsterdam, the Netherlands (vol. 1), 320–322.

Parkhurst, C. R. and Mountney, G. J. (1995), *Poultry Meat and Egg Production*, Boston, MA: Springer Publishing.

Peebles, E. D. (2018), In ovo applications in poultry: a review, *Poult. Sci.*, 0, 1–17.

Peebles, E. D., Barbosa, T. M., Cummings, T. S., Dickson, J., Womack, S. K. and Gerald, P. D. (2017), Comparative effects of in ovo versus subcutaneous administration of the Marek's disease vaccine and pre-placement holding time on the post-hatch performance of Ross 708 broilers1,2,3, *Poult. Sci.*, 96(5), 1071–1077.

Phelps, P. (1995), The in ovo administration of antibiotics into broiler eggs at transfer, Ph.D. Dissertation, North Carolina State University.

Ricks, C. A., Avakian, A., Bryan, T., Gildersleeve, R. P., Haddad, E., Illich, R., King, S., Murray, L. M., Phelps, P., Poston, R., Whitfill, C. and Williams, C. J. (1998), In ovo vaccination technology, *Adv. Vet. Med*. 41, 495–515.

Ridge, J. P., Fuchs, E. J. and Matzinger, P. (1996), Neonatal tolerance revisited: turning on newborn T cells with dendritic cells, *Science*, 271(5256), 1723–1726.

Sarma, G., Greer, W., Gildersleeve, R. P., Murray, D. L. and Miles, A. M. (1995), Field safety and efficacy of in ovo administration of HVT +SB-1 bivalent Marek's disease vaccine in commercial broilers, *Avian Dis.*, 39(2), 211–217.

Sarzotti, M., Robbins, D. S. and Hoffman, P. M. (1996), Induction of protective CTL responses in newborn mice by a nurine retrovirus, *Science*, 271(5256), 1726–1728.

Schierman, L. W. and Ordskog, A. W. (1961), Relationship of blood type to histocompatibility in chickens, *Science* 134(3484), 1008–1009.

Seto, F. (1990), Immune responsiveness of neonatal chicks and immunocyte precursors from unprimed juvenile chicken donors, *Poult. Sci.*, 69(7), 1103–1109.

Sharma, J. M. (1985), Early pathogenesis of turkey herpesvirus in chickens inoculated as embryos, Proceedings of the International Symposium on Marek's Disease (Calnek and Spencer Editors), AAAP, Kennett Square, PA, 462–468.

Sharma, J. M. (1987a), Delayed replication of Marek's disease virus following in ovo inoculation during late stages of embryonal development, *Avian Dis.*, 31(3), 570–576.

Sharma, J. M. (1987b), Embryo vaccination of chickens with turkey herpesvirus: characteristics of the target cell of early viral replication in embryonic lung, *Avian Pathol.*, 16(4), 567–579.

Sharma, J. M. (1989), In situ production of interferon in tissues of chickens exposed as embryos to turkey herpesvirus and Marek's disease virus, *Amer. Jour. Vet. Res.*, 50, 842–852.

Sharma, J. M. and Burmester, B. R. (1982), Resistance to Marek's disease at hatching in chickens vaccinated as embryos with turkey herpesvirus, *Avian Dis.*, 26(1), 134–149.

Sharma, J. M. and Graham, C. K. (1982), Influence of maternal antibody on efficacy of embryo vaccination with cell-associated and cell-free Marek's disease vaccine, *Avian Dis.*, 26(4), 860–870.

Sharma, J. M. and Burmester, B. R. (1984), Disease control in avian species by embryonal vaccination, US Patent No. 4,458,630, July 10, 1984.

Sharma, J. M., Lee, L. F. and Wakenell, P. S. (1984), Comparative viral, immunologic, and pathologic responses of chickens inoculated with herpesvirus of turkeys as embryos or at hatch, *Am. J. Vet. Res.*, 45(8), 1619–1623.

Sharma, J. M. and Witter, R. L. (1983), Embryo vaccination against Marek's disease with serotypes 1, 2 and 3 vaccines administered singly or in combination, *Avian Dis.*, 27(2), 453–463.

Wakenell, P. S., Bryan, T., Schaeffer, J. L., Avakian, A., Williams, C. J. and Whitfill, C. E. (2002), Effect of in ovo vaccine delivery route on herpesvirus of turkeys/SB-1 efficacy and viremia, *Avian Dis.*, 46(2), 274–280.

Williams, C. J., Griffin, C. L., Klein, D. R., Sorrell, W. R., Secrest, M. N., Miles, A. M., Gildersleeve, R. P. and Brake, J. T. (1994), A microbiological survey of broiler hatcheries in North America – the prevalence of *aspergillus* and other deuteromycetes, *Poult. Sci.*, 73(Suppl. 1), 166.

Williams, C. J. and Villalobos-Chaves, T. (2000), Field testing of the application of Marek's disease vaccination (HVT) to broiler chickens in costa Rica, In: ovo versus

sub-cutaneous. Proceedings of the For XXI World's Poultry Congress, Sixth International Symposium on Marek's disease. Montreal CA, Aug 20-24, 2000.

Williams, S. M., Smith, J. A., Garcia, M. C., Brinson, D., Kiupel, M. and Hofacre, C. (2010), *Severe Histiolymphocytic and Heterophilic Bronchopneumonia as a Reaction to In Ovo Fowlpox Vaccination in Broiler Chicks*, American College of Veterinary Pathologists publication.

Witter, R. L. and Lee, L. F. (1984), Polyvalent Marek's disease vaccines: safety, efficacy and protective synergism in chickens with maternal antibodies, *Avian Pathol.*, 13(1), 75–92.

Zhang, Y. and Sharma, J. M. (2001), Early post hatch protection against Marek's disease in chickens vaccinated in ovo with a CVI988 serotype 1 vaccine, *Avian Dis.*, 45(3), 639–645.

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