In ovo vaccination and chick quality

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In ovo vaccination is currently the standard procedure for hatchery applied vaccines for Marek's disease and infectious bursal disease in the USA.

Additionally, the state of the art technology is rapidly expanding globally with the approval of in ovo vaccines for fowl pox and Newcastle disease.

The laboratory concept of 'in the egg' vaccination has been expanded and developed into a commercially applied technology platform (Inovoject system) that is capable of placing several antigens simultaneously into over 50,000 eggs per hour.

Accordingly, in ovo application principles exist that are pivotal to understanding the physiology of the immune response, as well as how tolerant the embryo is to this relatively invasive technology.

It is of interest to understand the factors that influence in ovo application in order to better understand their link to chick quality.

This article looks at the links between chick quality and in ovo vaccination with respect to the interactions of embryonic age at injection, hatchability, compartmentalised delivery, and immune response.

Furthermore, the physiological challenges of injection techniques, aseptic vaccine preparation, hatchery hygiene, and in ovo equipment sanitation are considered.

Flock age (weeks)	Day of transfer	No. eggs	No. sellable chicks	Hatch (%)	Cull (%)	Net (%)
<31	17	422,676	342,784	82.82	1.75	81.10
	18	421,668	351,546	84.64	1.27	83.37
31-49	17	2,276,424	1,923,148	85.79	1.31	84.48
	18	2,454,048	2,112,552	87.17	1.08	86.08
>49	17	1,270,116	903,824	72.63	1.47	71.16
	18	1,227,708	907,155	75.09	1.20	73.89

Table 1. Evaluation of embryonic age at the time of Inovoject system vaccination based on data collected from a commercial hatchery during a four month period.

Embryonic age and hatchability

Understanding the embryonic stage of development and therefore the timing of egg injection reveals several incubation specific issues that can be evaluated to maximise chick quality as measured by hatchability following in ovo application.

Correct timing of injection in ovo will maximise not only hatchability but chick performance and immune status during the grow out period.

To target preferred locations within the embryonated egg, one must understand the different physiological characteristics associated with late stage embryonic development. Egg injection too early will reduce hatchability by increasing late dead and cull birds, as well as increase the incidence of missed vaccination.

Vaccination too late will create problems for vacuum transfer and increased egg breakage.

In general, the preferred time or 'window' for safely injecting the egg is from day 17 and 12-14 hours of incubation to day 19 and 2-4 hours of incubation, with time 'zero' being normal egg set time.

Egg injection on early day 17 has been shown to reduce hatchability approximately 1-2% when compared to injection on day 18 (Table 1). It should be noted that differences in day of transfer without in ovo vaccination (day 17 versus day 18) have also been shown to reduce hatchability (Table 2).

Differences in hatchability are due to different environmental conditions between the incubators and the hatchers. There exist marked differences in air flow characteristics across the eggs themselves. Eggs in the incubator are situated small end down, allowing for laminar flow of air across the blunt or large end of the egg. In the hatcher, the eggs are lying on their side.

The environmental differences between these equipment types can be evaluated in terms of water loss, humidity, heat, carbon dioxide and available oxygen.

Higher heat, carbon dioxide, and humidity can be found adjacent to the embryo in the hatcher versus the incubator during day 18 of incubation.

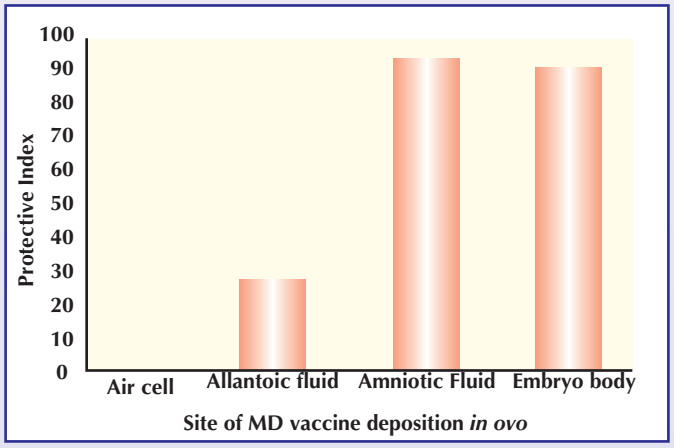
Similarly, slower water loss and reduced oxygen availability will be observed in the hatcher. A positive effect on hatchability is observed when the embryo remains longer in the environment of the incubator.

Hatchability of embryonated eggs is negatively affected by placement in the hatcher earlier, whether they are injected or not.

Differences between in ovo injected and non-injected eggs may be due to the injection hole in the shell of the egg. A 16-gauge needle hole in the shell (punch hole) represents an additional 25-30% increase in the relative pore volume of the egg.

The punch hole does not greatly increase water loss prior to pipping (<0.5%); however, other physiological parameters may be affected. There may be an advantage, physiologically, to respiration and gas exchange of the embryo during late term development, especially in those eggs from older breeder flocks.

Fig. 1. Protective indices for different in ovo sites of MD vaccine HVT/SB1 administration.



Embryo damage

However, there may also be a disadvantage if injection is done too early (prior to day 17.5) because it may cause damage to the embryo or support structures within the egg (too invasive), or it may be overwhelmed by an excessive microbial challenge during hatch.

Different incubator types show different water loss and temperature profiles, resulting in different stages of embryonic development at the same chronological age.

Optimal timing for egg injection is much more dependent on the physiological characteristics of the embryo than on actual incubation time.

The optimum time to inject eggs, developmentally, is the period between when the stalk of the yolk sac is beginning its ascent into the abdomen and the head is tucked under the wing up until external pipping is initiated.

In general, multi-stage tunnel incubators generate greater water

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loss and relative heat during incubation than the multi-stage, walk-in incubators.

This means that the embryos are better suited for egg injection earlier when they are incubated in commercial tunnel type multi-stage incubators versus walk-in type multi-stage incubators.

Minor adjustments in injection timing can be made to compensate for these differences in order to accommodate commercial hatchery schedules.

When conducting laboratory trials, in which eggs are injected by hand, it is important to evaluate a representative sample of eggs prior to injecting in order to ascertain physiological age of the embryo.

Incubation in laboratory and small scale incubation systems most often exhibit delayed embryonic development.

Similar developmental/chronological issues are present in eggs containing embryos that have pipped through the shell.

Although the injection per se is controlled, there is a greater chance of eggs cracking during transfer if there are multiple holes in the shell.

Cracking above the air cell membrane during late stage (day 19) transfer/injection does not necessarily affect the ability of the embryo to hatch. However, eggs cracked below the air cell membrane, on the small end or sides of the egg, will generally trap the embryo in the shell, lowering hatchability.

Eggs that are injected during late stage incubation (day 19) should contain less than 1% pipped eggs.

Flock age (weeks)	Day of transfer	Treatment	No. chicks	Hatch of total (%)	Hatch of live (%)
< 31	17	Control	3,056	81.62	98.04
		Injected	3,101	82.83	97.52
	18	Control	5,849	84.62	98.30
		Injected	5,957	86.18	98.51
31-49	17	Control	30,240	88.24	98.56
		Injected	30,056	87.70	98.45
	18	Control	20,069	89.34	98.62
		Injected	19,956	88.84	98.61
>50	17	Control	5,243	79.15	97.91
		Injected	5,243	79.15	97.82
	18	Control	8,697	83.88	98.23
		Injected	8,701	83.92	98.51
All flocks	17	Control	38,539	86.33	98.43
		Injected	38,400	86.02	98.29
	18	Control	34,615	87.09	98.47
		Injected	34,614	87.09	98.56

Table 2. Comparison of non-injected controls and injected eggs from commercial trials with respect to different breeder flock ages and day of transfer/injection.

Delivery and immune response

There are five separate compartments of the egg during late stage incubation that can be accessed by in ovo injection.

In order to maximise the immune response, and therefore chick quality, in ovo vaccination must access the correct compartment.

The compartments of late stage development include the air cell, the allantoic sac (waste), the amnion, the embryo proper, and the yolk sac.

Each compartment represents a distinct route of vaccine administration to the embryo, and this in turn represents distinct antigenic routes of presentation to the immune system.

Additionally, these compartments are changing dramatically as they are utilised by the embryo during the 'safe' window of injection timing, day 17.5 to 19 of incubation. Segregation of waste and nutrients is critical to the survival of the embryo during incubation.

The separate compartments within the egg perform different embryonic support functions during development, and the respective fluid dynamics and composition in the compartments differ.

Placement of antigenic and therapeutic compounds into the individual compartments within the egg may enhance or limit their uptake by the embryo.

Furthermore, the specific route of uptake and the type or size (molecular weight) of the compound itself may alter the subsequent effect on the embryo.

Research completed by the University of California at Davis in collaboration with Embrex scientists showed that Marek's disease (MD) vaccine efficacy was not achieved by placement of MD vaccine on the air cell membrane (Fig. 1).

Furthermore, a significant reduction in efficacy resulted when vaccine was placed into the allantoic sac. Wakenell et al. (2002) found that MD vaccine injected in the air cell provided no protection; in the allantoic fluid, 28.3% protection; in the amniotic fluid, 94.4% protection; in the embryo body, 93.9% protection. The yolk sac was not evaluated in these studies.

In support of the Inovoject system, many individual MD challenge studies have been completed and reported.

The system has been validated across many breed types, as well as eggs from different ages of breeders (egg size).

Additionally, site of injection studies have revealed that nearly 100% of the embryos vaccinated with the Inovoject system receive vaccine either in the amnion or the right breast area.

The developmental age of the embryo affects the site of injection. Most embryos vaccinated commercially between day 18 and day 18 and 10-12 hours of incubation receive inoculation in the amniotic fluid, while those vaccinated later (early day 19) receive a higher proportion into the embryo proper.

In a series of well designed studies, Phelps (1995) delineated the effects of the site of delivery in ovo of an antibiotic injection upon subsequent absorption within the embryo as measured by plasma blood levels (Fig. 2).

Results of antibiotic administration to the various compartments within the egg at day 18 of incubation show interesting contrast when compared to the administration of MD vaccine.

Air cell administration of MD vaccine results in no efficacy, while air cell administration of antibiotics showed very rapid absorption and sustained blood levels for 24 hours.

Administration of either antibiotics or MD vaccine into the allantoic fluid shows low blood levels of antibiotics or reduced efficacy against MD challenge.

Embryonic support function

The major embryonic support function of the allantois is water conservation and electrolyte reabsorption.

The passage of compounds and antigens from the allantois to the embryo is limited by the chorio-allantoic blood flow as well as the membrane itself.

In contrast, amniotic fluid is imbibed by the embryo prior to hatching. Placement of antibiotics or MD vaccine into the amnion results in oral uptake, and internal absorption by the mucosal surfaces of the respiratory and digestive tract of the embryo.

Vaccine preparation

Field observations have shown that microbial contamination of vaccine may produce a small to large loss in hatchability with a subsequent increase in early mortality.

Obviously, vaccine contamination will negatively impact chick quality, as it involves the introduction of one or more pathogenic bacterial organisms or inappropriate vaccine virus in ovo. Vaccines for in ovo use must be sterile. Vaccines for in ovo use are typically stored and prepared in a separate area than vaccines applied by spray application at day of hatch (Newcastle disease, infectious bronchitis).

Misapplication of a vaccine not safe for in ovo administration can severely compromise chick health and hatchability. It is important to establish separate storage and preparation areas for in ovo and day of hatch vaccines so as to avoid such costly mistakes.

In cases of bacterial contamination during the mixing process, the causative bacterial organism can usually be isolated from the unhatched embryos or clear eggs that have been injected as well as the environment of the vaccine preparation room.

Most often the source of the contamination is the MD vaccine thaw water. Standard vaccine mixing procedures require the use of distilled water (not tap water) in the thaw bath.

A contaminated thaw bath can result from introduction of bacteria from the mixer's hands, a com-

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promised thaw bath container, or from the use of contaminated tap water.

Water droplets on the exterior of the ampule may mix with the vaccine if contact is made between the contaminated water and the ampule opening during the mixing procedure. No process is more critical to control with in ovo vaccination than correct aseptic vaccine preparation.

Removal of antibiotics

Antibiotics are not required for in ovo administration although they have been used in conjunction with MD vaccination to maintain the sterility of the vaccine through the mixing and application processes. More and more producers are removing antibiotics from their broiler vaccination programmes.

This has highlighted the need for stricter aseptic technique and critical control points in the preparation steps of MD vaccine mixing.

Thaw bath water is always compromised with bacteria unless one practices strict asepsis, rigorous hand sanitation, and biosecure water supplies.

We support the use of mild disinfectants, specifically chlorine, in the MD thaw water to prevent bacterial contamination of the vaccine destined for in ovo use.

Studies have been completed at Embrex to determine if thaw water containing chlorine could affect MD vaccine titers. MD vaccine was thawed and opened using standard procedures.

Thaw bath water containing approximately 200ppm chlorine or 500ppm chlorine at volumes of 10, 25, 50 or 100 microlitres was added directly into the MD vaccine vial and allowed to react for 30 seconds.

Vaccine was then introduced to MD vaccine diluent, and the level of plaque forming units (pfu) was determined. These data suggest that using 200ppm chlorine in the thaw bath poses no great threat to MD vaccine titers.

However, if amounts of 500ppm or higher are used there is the potential for MD vaccine titre reduction.

Physical challenges

It is important to understand the physical process of shell penetration and needle penetration through the chorio-allantoic membrane into the embryo or amnion to understand the impact on chick quality. The requirements of the tip of the injection needle and the shell punch differ, as well as their relative size or gauge. These are important considerations when evaluating successful in ovo inoculation, both commercially and under laboratory conditions, because microbial and physiological challenges must be addressed.

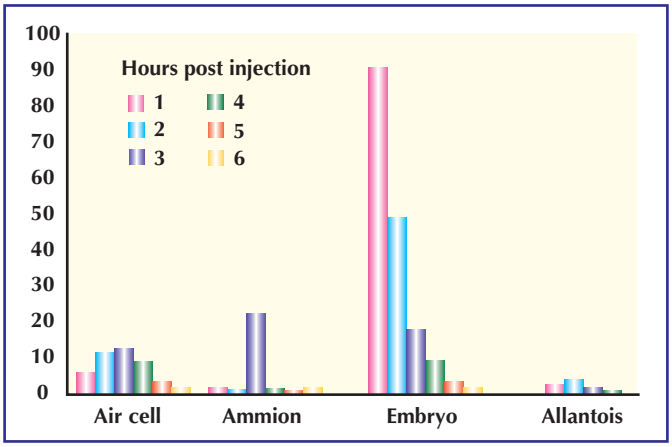

Fig. 2. Plasma gentamicin levels (ug/ml) following in ovo administration of 1mg gentamicin into compartments of day 18 embryonated broiler eggs.

The physical challenge of piercing the egg shell is the accurate creation of a hole through the shell without breaking the shell, hence the need for the punch to have a specialised bevelled tip.

The shell itself also represents a significant microbial challenge to the injection process.

The punch device should not breach the embryonic cavity (inner shell membrane and the chorio-allantoic or air cell membrane), as the likelihood that it will transmit microbes from the shell to the embryo is high.

Additionally, the punch should be sanitised thoroughly between injections. Under extreme microbial challenges, thorough sanitation of the egg shell surface may be required prior to breaching the shell.

The injection needle itself should have no negative effect on the embryo. The needle should be a small size (20 gauge or smaller) and the tip correctly designed 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. This is important with respect to the ease of sanitation. The surface area created by large needle sizes, extended canula openings (bevel), or side port needles restrict the ability to sanitise the needle.

Additionally, the larger the injection needle, the greater the incidence of embryonic trauma.

Minimal exposure to organic material on the surface of the needle and punch tip is critical to thorough and rapid sanitation of injection needles.

The 'needle inside a needle' design found on the Inovoject system incorporates a specially designed outer needle to pierce the shell and a smaller gauge needle designed to inject into either the amnion or embryo.

The use of two needles is designed to minimise the negative impact on the shell and embryo, to maximise the sanitation efficacy (smaller area to sanitise), and to target the critical areas of the needle and punch to sanitise.

Hatchery hygiene

It is well documented that poor hatchery hygiene will compromise the quality of the day old chick, and in turn lead to poor performance later in life.

These issues exist, regardless of preference for in ovo or day of age vaccine application. Egg injection may exacerbate certain issues of poor hatchery sanitation, thus a risk analysis is normally conducted prior to incorporation of the technology into any specific hatchery. The risk analysis includes both a physical and microbial site survey.

The surveys are used as a tool to outline problem areas of the hatchery environment that may affect the injection process.

Corrective measures may then be imposed prior to initiation of egg injection.

The microbial risk analysis is based on the amount of fungi found during the survey of the various environments within the hatchery. Generally, high levels of fungi indicate a problem with the sanitation, maintenance, and/or design of the ventilation systems of the hatchery. High levels of fungi have been found in approximately one out of five hatcheries worldwide. Of primary concern is aspergillus, identified in approximately 55% of the hatcher rooms and incubators globally.

When present in high enough levels, aspergillus can cause mycotic infections in the young chick. This is true for all hatcheries, whether they inject eggs or not. Non-viable eggs that have been injected are sensitive to aspergillus challenge, as the aspergillus utilises the yolk as a nutrient source.

The level of fungal growth found in these infertile and early dead eggs after hatch can serve as an indicator of the level of challenge that the hatchery environment is imposing on the hatching chick. The growth inside the clear eggs may further increase the aspergillus levels in the hatchery, primarily during chick processing.

Aspergillus levels in the hatchery should be minimised in order to maximise chick quality after egg injection.

Primary source of aspergillus

One primary source of aspergillus in the hatchery is the breeder farm, via the eggshell surface. Dirty nest box materials, dirty automatic nest belting, including floor eggs, and humidification systems in on-the-farm egg storage have been identified as primary sources.

Another primary source of aspergillus is the immediate environment outside the hatchery.

Obvious sources include feed mills, wood and paper mills, various agricultural plots including oats, rice, corn, and wheat fields, primarily during harvest season, and hardwood forests, especially in the autumn.

Seasonal variation in airborne aspergillus conidia occurs with the change of seasons from spring to summer and summer to autumn.

The hatchery itself may be a source, via poorly maintained gutters and organic debris decomposing on the roof or immediate environment.

The hatchery, once aspergillus enters the internal environment, may serve as a further source for proliferation, especially in the air system filtering, evaporative cooling pads, air conditioning units, duct work, and insulation.

Additional challenge can be present during the winter as more

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air is recirculated to conserve energy and this air can be exposed to highly contaminated concentration points within the ventilation system.

Critical areas of the hatchery may continually become recontaminated from internal sources by airflow from dirty to clean areas. In addition, recontamination from the external environment via exhaust to intake airflow can occur. Therefore, the hatchery is never truly aspergillus free and this requires constant management and sanitation of the various systems.

Control of aspergillus begins with design and maintenance of the air handling systems within the hatchery. No amount of cleaning and disinfection can eliminate the continual challenge due to improper ventilation.

Basic flaws in design need to be corrected. Once in place, a biosecure ventilation system needs routine maintenance, cleaning, and disinfection.

The increased seasonal challenges of aspergillus should be addressed with heightened focus on sanitation or replacement of filters and evaporative cooling pads prior to seasonal change.

Breeder farm management should focus control measures on nest box, egg collection (belting), and litter management, as well as ventilation systems in the farm egg room.

The air handling systems of egg transportation vehicles, as well as the storage areas of the vehicles should be on routine cleaning and sanitation schedules.

In ovo equipment sanitation

The process of egg injection must be supported by sound sanitation programmes in the hatchery, but just as critical is the sanitation of the vaccination equipment itself.

Chick quality may be severely compromised by direct contact with contaminated vaccination equipment.

Daily cleaning and disinfection of the equipment after use is imperative, and similarly, equipment storage during non-use periods must minimise organic and microbial challenge to the system.

The design of egg injection equipment should be conducive to cleaning. The injectors themselves, as well as the needles and transfer components should be routinely cleaned and disinfected.

Automated disinfection and sterilisation of the vaccine delivery components are required to maintain the process over time.

Egg to egg carryover of pathogens by the needles must be addressed with controlled sanitation of each component that touches the egg between every injection.

Chick quality can be compromised by a breakdown in any of these sanitation requirements, such that redundancy and ease of use become critical design elements of the system.

Chlorine based sanitisers are used during the process of egg injection. They are tolerated by the embryo, leave no residual chemical on the shell, and possess a rapid bacterial and viral kill rate.

The Inovoject sanitation fluid, which sanitises the needles and egg punches between each injection, is the foundation for egg injection sanitation and was designed to prevent egg to egg transmission of microbes.

The sanitiser is composed of a buffered chlorine solution. The chlorine concentration of the sanitiser during the injection process has a required standard of 5000ppm of active chlorine (0.5%) at mixing, and should never drop below 3500ppm during the process.

It is important that hatchery personnel follow the mixing instructions as outlined in the 'Inovoject User's Manual'.

Just as important is the required managerial input in stressing these procedures and supplying the correct products.

The equipment itself is also cleaned with chlorine based detergents and sanitisers, and supported during storage with isopropyl alcohol.

Conclusions

In ovo vaccination must be quality controlled to achieve maximum chick quality. Egg injection technology is not simply a matter of delivering biologically active compounds to the egg.

The technique must be sterile and controlled; the delivery must be made to precise locations within the egg.

Incubation conditions must be coordinated with the timing of egg injection in order to maximise hatchability and vaccine efficacy.

Each hatchery and incubator type may have specific incubation and environmental criteria that need to be managed in order to maximise the potential benefits from the technology.

Detailed embryonic evaluations should be made when conducting in ovo application studies to assure optimal embryonic age and site of injection.

The measure of in ovo vaccination quality depends on not only the manner in which the vaccine is applied, but the timing of the injection in relation to the stage of development, the sterility of the vaccine, and the exact site of injection in the developing egg.

Chick quality is a relative measurement of the success in optimising the application of these principles.

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