Review

In Ovo Vaccination Technology: An Alternative Approach to Post-Hatch Vaccination in Modern Poultry Operations

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Abstract: Poultry production systems are usually exposed to important infections that could be prevented by vaccination programs. Conventional methods of vaccination such as drinking water; spray, eye, or nose inoculation; and injection are usually given after hatching and have many disadvantages. Therefore, there is a great need for searching of alternative ways for vaccination process. In ovo vaccination technology is now regarded as an alternative approach to post-hatch vaccination in modern poultry operations. This technique is effective, fast, provides uniform vaccine dosing and delivery, is suitable for massive production, and reduces labor costs. Routine in ovo vaccination is applied during the late stage of embryonic development between days 17.5 and 19.25 of egg incubation. The best route of inoculation of the vaccine is in the amniotic fluid or in the embryo's muscles, without causing any hatchability or chick quality losses. Accordingly, the inoculation site, the age of the embryos and breeders, presence of maternal antibodies, and the sanitation of equipment's and the environment during the vaccination process affect the efficiency of the in ovo vaccination technique. In ovo vaccination technology is currently applied for vaccination against several economically important viral diseases such as Newcastle, infectious bursal disease, Marek's disease, infectious laryngotracheitis, infectious bronchitis, avian influenza, and avian metapneumovirus. Moreover, vaccines used for prevention of mycoplasmosis and coccidiosis could be applied in ovo instead of in post-hatching application. It can be concluded that in ovo vaccination is a rapidly growing trend of vaccine technology, and it can replace post-hatching vaccination conventional methods.

Keywords: bacterial; in ovo vaccination; parasitic; poultry; technique; viral

1. Introduction

Poultry industry is exposed to variety of stressors and disease conditions that drastically affect production. Therefore, the vaccination of birds is considered a way for reducing disease severity, enhancing bird health, protecting the poultry industry, and providing the consumers with safe meat and eggs [1]. Conventional post-hatch vaccination techniques, including drinking water, spray, injection, or feed, may result in vaccine delivery failure and poor vaccine efficacy [2]. Thus, these techniques, where possible, are being replaced by in ovo vaccination technology, which is more effective and faster and provides a more uniform virus vaccine dose and delivery [3]. Besides, this technique is suitable for a large-scale or massive poultry production system, with a reduction in labor costs and worker's involvement [4].

In ovo vaccination technology was introduced over three decades ago; however, it is currently used in more than 90% of the commercial hatcheries worldwide, including the United States of America, Europe, and Asia [5]. The first description of this technology

was in 1982 as an efficient method of vaccination against Marek's disease (MD) [6]. The efficiency and success of this form of vaccination may be influenced by many factors, including the timing of the inoculation relative to the stage of embryonic development and the precise site of vaccine inoculation in the developing embryo. Routine in ovo vaccination is usually applied between days 17.5 and 19.25 of egg incubation via a direct inoculation in the amniotic fluid or in the embryo's body and without causing any hatchability or chick quality losses [7]. In contrast to other post-hatch vaccination techniques, vaccination at day 18 of embryogenesis reduces the time between early exposure to infectious agents and vaccination [4], which helps in the stimulation of an early chick's resistance and with good protection [8]. The in ovo vaccination technique, particularly for living poultry vaccines, has proven successful for preventing different economically significant viral [9–17], bacterial [18–20], and parasitic [21,22] diseases.

In this context, this review article was designed to put a spotlight on in ovo vaccination technology regarding the mechanism, affecting factors, advantages, and the role of this trend in controlling some important viral, bacterial, and parasitic diseases of poultry.

2. In Ovo Inoculation Technology (Figure 1)

2.1. In Ovo Vaccination Technique

The technique used for the administration of in ovo vaccines is critical. Delivering the vaccine in an incorrect location may result in ineffective vaccination, thus reducing its benefits and entailing severe losses. In ovo vaccination is applied when the incubated eggs are transformed from the setter to the hatchery between 17 and 19 days of incubation. At the top of the blunt end of the eggshell, a hole is pierced into the eggshell, and then the egg membrane is punctured using the inoculated needle [23,24]. The vaccine is injected slightly beneath the membranes at the bottom of the air cell [25,26]. Vaccines should be deposited into the extra-embryonic sacs, mainly into the amniotic sac; however, they could also be inoculated in the breast, neck, or shoulder of the embryo [26]. The deposition of the inoculated vaccine in the embryonic tissues or in the extra-embryonic compartment depends on the duration of egg incubation and the needle size.
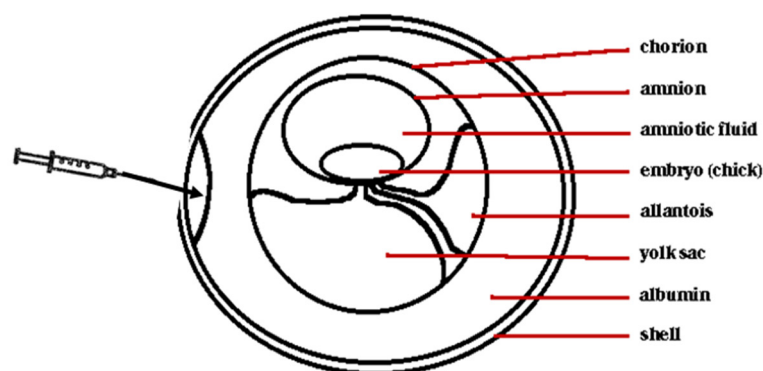

Figure 1. Mechanism of in ovo inoculation technology.

2.2. Risks and Challenges

The needles used should be sterilized after the completion of inoculation and before the following inoculation to avoid cross-infection of the inoculated embryos [19]. Due to congenital biological differences of individual eggs, the vaccine cannot be accurately injected; there is lack of control regarding the needle-hit position. This may affect the growth of the chicken embryo [27,28]. During vaccination, the embryo should be in the hatching position where the head under the right wing and the yolk sac would be completely absorbed. Injections after day 19 of incubation may injure the embryo, resulting

in mortality. However, vaccinations at an earlier age may increase the risk of contamination, compromising the livability and quality of the chicks at hatch. Also, during the direct puncturing of egg membranes, the embryo may be injured, causing reduced hatchability, survival rate, and chick health. Leaving a hole in the egg for few days before hatching, along with poor sanitized hatcheries, enhances poor viability of the hatched chicks due to bacterial or fungal contamination [29].

2.3. Advancements in In Ovo Vaccination Systems

The electromagnetic needle-free in ovo injection automation system has been successfully used for accurate inoculation of embryos in eggs [24]. An automatic multiple-head egg injection vaccination system has been distributed in industries worldwide and replaced the conventional method of post-hatch injections at the hatchery [29]. In this technique, liquid jet pressure can be adjusted to the target embryonic tissue; thus, it replaces the traditional needle-type approach and, in consequence, reduces embryo injury and cross-infection, as well as prevents replacing needles and needle-cleaning steps. The egg injection system permits the inoculation of 20,000–30,000 eggs per hour [25], with inoculation doses of 50–200 microliters of injected vaccine per egg [29].

2.4. Effects on the Embryos

Following vaccination, the embryo swallows the dissolved vaccine in the surrounding fluids, and the gut associated lymphoid tissues begin to react with the antigen of the vaccine [30,31]. Moreover, the embryo's choanal cleft can aspirate the vaccine into the nasal cavity and the respiratory tract [28], where the vaccine antigen can stimulate the nasal and bronchial associated lymphoid tissues. Cloacal sucking of the inoculated substances has also been detected [5].

3. Factors Affecting In Ovo Vaccination Technology

3.1. The Inoculation Site

The inoculation site of the egg differs according to the type of vaccine [27,32]. A precise and accurate inoculation site of the egg is required to obtain an optimal response by the embryo [33].

The air cell, amnion, allantois, yolk sac, and embryo's body are the sites where the vaccine could be injected. The selection of the inoculation site may be affected by the survival of the embryos and their response to the vaccine [3]. It has been reported that vaccine inoculation in the amnion could be absorbed by the embryo prior to hatching [5]. For instance, vaccine deposition into the air cell or allantois did not provide adequate protection of chicks against subsequent MD virus (MDV) challenge [33], while amnion inoculation provided the highest vaccine effectiveness [34]. Moreover, the injection might be made directly into the right breast area of the embryo when embryo around 19 days of incubation. Breast inoculation may be considered usual and acceptable; however, injection into another site of the embryo's body can result in either a lack of vaccine efficacy or damage to the embryo [7]. The protection rates against MDV vaccines were 0%, 28.3%, 94.4%, and 93.9% following inoculation in the air cell, allantoic fluid, amniotic fluid, and embryo's body, respectively [11]. Furthermore, Alqhtani et al. [20] demonstrated that the ts-11 *Mycoplasma gallisepticum* (MG) vaccine strain yielded a better protection rate after injection in the amnion (97.2%) when compared to intramuscular injection (2.8%) of the embryos. Intracranial, intraorbital, or intraabdominal inoculations of embryos are regarded as unsatisfactory and ineffective methods that perhaps cause embryonic death [11,35]. In addition, it has been found that the location of vaccine deposition in eggs can have potential consequences on the immune response of the hatched chick [11,36].

3.2. The Age of the Inoculated Embryos

The age of the embryos at the vaccination time plays an important role in in ovo vaccination success. In other words, the timing of in ovo vaccine inoculation should be in synchrony with the stage of embryonic development to achieve the required vaccine outcome [2]. Moreover, an appropriate embryonic development stage allows for selection of the optimum size and position of the embryos for exact and correct vaccine inoculation. Therefore, the late stage of embryogenesis, at approximately 18 days of chickens age, is appropriate due to maturation of the systems, including the immune organs [37] and the best immune response [22,38,39]. No detectable lesions could be observed when inoculating the vaccine to 17- to 19-day-old embryos or older due to the development of protective immunity and resistance, as well as the support of virus replication [28]. Additionally, vaccination at this time has a minimum impact on the hatching rate and it maintains the stable physiological status of embryos. Rapid replication of the infectious bursal disease virus (IBDV) and infectious bronchitis virus (IBV) has been detected in embryos tissues when inoculated on day 18 of their development [40–42].

Early in ovo vaccination may increase the risk of contamination, which compromises the livability and quality of the chicks post-hatching [43]. When the herpesvirus of turkeys (HVT) vaccine was administered in 10- to 12-day-old embryos, the virus was rapidly replicated and caused severe lesions and deaths of the embryos [44]. Similarly, the in ovo vaccination of 13-day-old embryos resulted in higher embryonic mortality compared to vaccination of 18-day-old embryos [8]. Reduced hatchability around an average 1% was observed in hatcheries when in ovo vaccination was applied on day 17 of incubation [29]. In a similar study [7], the results indicated that the in ovo vaccination on day 16 of incubation reduced the hatchability in 4.68% in comparison to the other vaccination days. Furthermore, Williams [45] demonstrated that too-early inoculation of eggs (before day 17.5) might be very invasive, causing embryonic damage, and the vaccination process could be overwhelmed by the high microbial challenge during hatching. However, the ideal time for safe inoculation of the eggs could be from day 17 and 12–14 h of incubation to day 19 and 2–4 h of incubation [45]. On the other hand, the inoculation of eggs after day 19 of age may induce embryo injury and mortality.

3.3. The Age of Breeders

The breeder's age may also influence the determination of in ovo vaccination timing [46]. Day-old chicks of old breeders, which were usually subjected to a vigorous vaccination program, presented a lower immunity when compared to chicks of young breeders. However, other studies showed that the antibody titers against IBDV and fowlpox virus (FPV) in day-old chicks and their performance at slaughtering were not affected by the in ovo vaccination time or by the breeder's age [7].

3.4. The Maternal Immunity

Maternal immunity may show some adverse effects on the in ovo vaccination technology. In the presence of high titers of maternal antibodies in vaccinated chicks, the vaccine was not able to override immunity, yielding a low vaccination efficiency. For example, maternal immunity can affect the efficiency of in ovo vaccination against certain diseases such as MD. Therefore, developing insensitive vaccines to maternal antibodies or the vaccination of chick embryos on day 18 of development is important to overcome this problem [7].

3.5. Other Factors

Other factors, including sanitization of the equipment, aseptic vaccine preparation, and hatchery hygiene, may affect the efficiency of in ovo vaccination technique [35,47].

4. Advantages of In Ovo Vaccination Technology

The in ovo vaccination technique should be easily applied, achieve vaccine efficiency, enhance embryos health, and ensure chick quality [35]. Furthermore, the approach should allow for an even and uniform mass vaccination with a fast vaccine delivery, provide precise inoculation, reduce stressful conditions on chicks, enhance bird welfare, decrease the contamination and the time between hatching and placement on the farm, reduce labor costs, and allow for the application of other biological materials that may influence broiler performance [29,43].

In ovo embryonic vaccination has a positive effect on the chick's immune response. It has been supposed that this technique contributes to reducing the period between early exposure to infectious agents and vaccination [4], which helps to avoid maternal immunity interference and stimulates earlier embryonic immunity [7,33]. By the time of hatching, vaccinated chicks develop a more significant degree of protection in the form of innate and adaptive immunity when compared to vaccinated chicks after hatching [4,35].

5. Application of In Ovo Vaccination Technology Against Some Important Diseases of Poultry (Figure 2)

5.1. Viral Diseases

5.1.1. Newcastle Disease

Newcastle disease is a devastating viral infection that affects most avian species, and it is caused by avian Orthoavulavirus 1 (previously paramyxovirus 1) [48]. Biosecurity measures and widespread vaccination programs have been unable to prevent outbreaks of NDV [49]. Despite the application of extensive NDV vaccination protocols with more virulent vaccines, particularly in endemic countries, outbreaks continue to occur in many areas all over the world [50–52].

The ideal vaccine against NDV should be able to fill the gap and protect chicks in the first weeks of life. Moreover, it should stimulate both cell-mediated and humoral immunity to reduce the replication of virulent field viruses, the shedding rate, and possible horizontal transmission. Living and inactivated vaccines against NDV are commercially available worldwide [53]. Oral or intraocular application of living NDV vaccines provides neutralizing immunity and induces mucosal immunity [54], but some of them may cause some respiratory manifestations and reduce productivity [55]. Though inactivated vaccines induce long-lasting immunity [56], they neither replicate in the host nor elicit strong cell-mediated immunity [54].
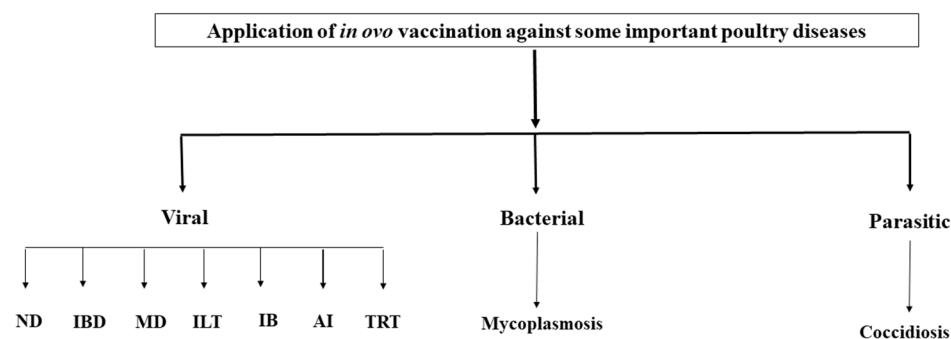
Figure 2. Application of in ovo vaccination technology against some important diseases of poultry.

There have been some attempts to develop other methods for NDV vaccination. Vaccination against NDV using a living vaccine in ovo faces many limitations and challenges such as high embryo mortality, the presence of maternal-derived antibodies, and incomplete protection during the early weeks of life [8,57–61]. Low geometric mean titers against

the NDV vaccine have been observed in specific pathogen-free (SPF) chickens vaccinated against IBDV and NDV using in ovo techniques [62]. The best timing of in ovo vaccination against NDV is on day 18 of embryogenesis when the last and third wave of thymus colonization occurs [63]. The study of Ohta et al. [64] showed that in ovo administration of an aluminum hydroxide-adsorbed attenuated NDV vaccine resulted in improved hatchability and survival rate, as well as better antibody responses of protection-level immunity compared to the administration of NDV alone.

There are about 13 recombinant viral vector vaccines against NDV. The first generation consisted of two FPV vectors, followed by six second-generation and five third-generation vaccines incorporating an HVT vector with the fusion (F) protein or the hemagglutinin (HA)-neuraminidase (NA) protein of NDV. Most of these vaccines are administered using an in ovo route [65,66]. A recombinant (rFPV) expressing the F and HA-NA glycoproteins of NDV and chicken type I or type II interferon (IFN) were applied in the eggs of turkeys [67]. They did not affect the hatchability or survival rates, the performance, the weight gain, or the immune response of hatched chicks. A recombinant HVT expressing NDV F protein (rHVT-ND) (Meleagrid alpha herpesvirus 1) has been successfully used for in ovo vaccination against NDV infection in chickens. Despite the fact that a single application of rHVT-ND vaccine provided long-lasting protection against the virulent strain of NDV with no obvious clinical manifestations, it took up to 4 weeks to mount a protective immune response, meaning it cannot be used as a primary vaccine with other rHVT-vectored vaccines and would be expensive to implement [68–70]. Recently, Marcano et al. [17] reported that in ovo vaccination of 19-day-old embryos with a recombinant, live, attenuated NDV vaccine containing the antisense sequence of chicken interleukin 4 (IL-4) (rZJ1*L-IL4R) induced the highest degree of protection against NDV challenge without adverse effect on the survival rate of the hatched chicks. However, the antisense RNA may cause transcriptional interference via different mechanisms, so modulating gene expression by reducing or completely silencing a gene is important [71,72]. A recombinant FPV co-expressing type I IFN, as well as the NDV HA-NA and F proteins, enhanced the protective efficacy of the vaccine against NDV challenge when given in ovo and at hatching [73]. Moreover, a recombinant virus vaccine containing rNDV, rNDV49, and rNDVGu was compared with a living NDV Clone 30 vaccine following in ovo injection [74]. The findings indicated that only one bird from the NDV Clone 30 vaccine group survived for 3 weeks, whereas the survival rate of hatched chicks from other groups receiving recombinant NDV vaccine was between 40% and 80%. Moreover, in ovo administration of an antigen–antibody complexed live B1-La Sota NDV vaccine provided full immunity against phylogenetically distant virulent viruses at an early age in the presence of maternal immunity [75].

5.1.2. Infectious Bursal Disease

Infectious bursal disease is regarded as an important disease of broiler chickens that is caused by the Avibirnavirus genus. Due to the resistant nature of the virus, biosecurity measures and vaccination protocols are routinely used for the prevention of IBDV infection.

The classical live attenuated IBDV vaccines are usually applied in the drinking water, while the vector-based and immune complex vaccines are applied either in ovo or subcutaneously. The immune complex vaccines containing live intermediate plus IBDV strains have been mixed with hyper-immune sera to produce virus–antibody complex vaccines. Application of this type of vaccine in ovo provided good protection against the virulent IBDV challenge even in the presence of high levels of maternal-derived antibodies to the virus [76]. In Spain, a single in ovo immunization of broiler chickens with an immune complex IBDV vaccine induced high and uniform protective immunity between 35 and 51 days of age, with mean antibody titers values ranging between 6331 and 7426 [16].

Combining a live IBDV vaccine virus with the immune complex vaccine could be used in ovo or at hatching [77,78], as it can be replicated at the most suitable time and provide active immunity [43,79].

Despite the fact that the presence of high persistent levels of maternal antibodies can protect chicks during the first weeks of life, they can prevent the classical living IBDV vaccines from providing active protection following single or repeated applications. Live IBDV strains might be neutralized or break down through maternal-derived immunity and induce constant damage to the chick's immune response [80]. In four field trials of Ashash et al. [81], a conventional live intermediate plus IBDV vaccine strain and the IBDV MB-1 were applied in maternally immunized embryos and in one-day-old chicks using a subcutaneous route. The findings confirmed that the in ovo application of live IBDV MB-1 vaccine induced full protection of broilers against a very virulent virus challenge at 22 and 36 days of age as indicated by the bursa lesion score, the q polymerase chain reaction, and virus genotyping. Furthermore, in ovo vaccination with an intermediate live IBDV vaccine was found to be able to evoke active immune response and protection of 21-day-old SPF chickens as testified by bursal lesions, the bursal index results after challenge, and vaccine immuno-response [62]. In addition, the study of Lara et al. [82] proved that the inoculation of an intermediate live IBDV vaccine strain in 18-day-old embryos or in day-old hatched chicks derived from vaccinated breeders induced no effect on the feed intake, feed conversion ratio, and weight gain till 40 days of age. Hence, it could be concluded that in ovo vaccination against IBDV did not adversely affect the hatchability or survival of hatched chicks [83]. The immuno-pathogenesis of IBDV following in ovo vaccination using an intermediate live IBDV vaccine strain has been described [84]. The results indicated that inoculation of the living IBDV vaccine in ovo induced faster recovery from bursal lesions and similar protection against virus challenge when compared to post-hatch vaccinated chickens. Recently, Zaheer et al. [85] compared the immuno-pathological effects of in ovo vaccination with a post-hatch vaccination in white Leghorn chicks, and they concluded that in ovo vaccination with live IBDV vaccines provoked protective immunity even in the presence of IBD-specific maternal immunity.

Some previous studies showed the presence of IBDV in the bursa of Fabricius, with bursal lesions after in ovo vaccination with a modified live IBDV [86,87]. In ovo vaccination with a classic or Delaware E variant strains of IBDV produced a 6-day delay in the onset of IBDV infection [88]. However, McCarty et al. [89] revealed that in ovo inoculation of modified live IBDV vaccine infected the bursa of Fabricius in maternal-antibody-negative and maternal-antibody-positive eggs. In addition, in ovo vaccination of antibody-free broiler chickens with modified live IBDV vaccine induced immunity to post-hatching IBDV exposure [62,90].

The efficacy of the in ovo prime-boost vaccination against IBDV using a DNA vaccine followed by a killed-vaccine boost post-hatching in addition to the adjuvant effects of plasmid-encoded chicken IL-2 (ChIL-2) and chicken IFN-γ (ChIFN-γ) in conjunction with the vaccine were tested [91]. The findings revealed that the in ovo DNA vaccination followed by a killed vaccine could completely protect chickens after challenge with very virulent IBDV in terms of the absence of mortality and reduction in both the bursal damage and lesion score. In addition, the addition of ChIL-2 or ChIFN-γ did not enhance the protective immunity. Both IBDV and MDV vaccines could be jointly administered in ovo without interference with each other [29]. The study of Gagic et al. [92] showed that a single in ovo inoculation of a vaccine containing serotypes 1, 2, and 3 of MDV, serotype 1 of IBDV, and a recombinant FPV vaccine with HA-NA and F genes of NDV (rFP-NDV) produced protection against all these viruses. Furthermore, the inoculation of a vaccine containing

serotypes 1, 2, and 3 of MDV and IBDV did not affect hatchability, although the addition of rFP-NDV to the mixture reduced hatchability by 23% to 26%.

It was summarized that vaccination against IBDV using in ovo inoculation technology decreased the stress of bird handling, induced better immune response, and minimized the risks of contamination and disease spreading [93].

5.1.3. Marek's Disease

It has been shown that in ovo vaccination could give greater protection against an early MDV challenge than subcutaneous vaccination at hatching [6]. Since the success of this initial study, the in ovo vaccination technology was developed and distributed worldwide to combat MDV infections [25,29]. However, the exact successful mechanism of in ovo vaccination with HVT has not been fully determined [94].

Early infection with MDV and consequent early immune response and protection against infection were obtained after in ovo vaccination [40]. It has been reported that vaccination against MDV on days 17 and 18 of incubation did not interfere with the immune response, decreased the susceptibility of chicks to the onset of disease lesions, and supported virus replication by the embryos [28]. Similarly, in ovo vaccination against MDV after 18 days of chicken's eggs incubation provoked earlier protection than vaccinations on the first day of life, because the vaccine took up to 8 days to become effective [95]. Chickens given serotype 1 of MDV (CVI988) during embryogenesis showed better protection against virulent MDV challenge than those given MDV CVI988 at hatching, as the virus could be expressed in the embryo to initiate the pre-hatch immunologic response [96]. Regarding other research, Schat [70] reported on immune interference following vaccination with an HVT vaccine in the embryo, wherein antibodies could neutralize a second HVT vector vaccine after hatching. The site of MDV vaccine inoculation in ovo may also affect the protection rate of embryos. In the study of Wakenell et al. [11], the findings showed that when the HVT/SB-1 vaccine was injected in the air cell, allantoic fluid, amniotic fluid, and embryo's body, the protection percentages were 0%, 28.3%, 94.4%, and 93.9%, respectively.

Ricks et al. [29] reported that both IBDV and MDV vaccines could be administered together in ovo with no interference with each other. The in ovo vaccination with a vaccine containing both MDV and IBDV vaccine viruses did not enhance the inhibitory effect of individual viral agents on the humoral and cellular immune competence [92]. In addition, an HVT vector vaccine expressing IL-2 enhanced the protection induced by IBDV and IBV live attenuated vaccines when given via an in ovo route [97]. Moreover, the protective efficacy of in ovo vaccination with rHVT against NDV and MDV in chickens was evaluated with successful results [98].

5.1.4. Infectious Laryngotracheitis

Infectious laryngotracheitis virus (ILTV) is taxonomically classified as Gallid herpesvirus 1 [99], and it is associated with expectoration of bloody exudate, gasping, and asphyxia in chickens [100]. The disease usually generates severe production losses as a result of increased mortality, decreased egg production, loss of body weight, and predisposition to other respiratory pathogens [101].

The prevention of ILTV infection is based on biosecurity measures and vaccination using living attenuated virus vaccines produced in embryonated chicken eggs (ECEs) or tissue culture [102–105]. However, new generations of recombinant viral vector ILTV vaccines containing FPV and HVT as vectors have been developed [106]. Vector vaccines reduce the possibility of viral transmission from one bird to another, in addition to the absence of latent infection after vaccination or reversion of virulence. There are different immunization routes for ILTV vaccines, but the most practical route is in ovo vaccination

with HVT, MDV, or via a GaHV-1 'meq' gene-deleted vector. Vectored ILTV vaccines are now being applied in ovo [107]. It has been documented that in ovo inoculation of recombinant HVT-ILTV was ineffective to break the chain of viral transmission [108]. Vaccinated chickens showed reductions in clinical signs, maintained body weight gain after challenge, and lessened the challenge virus replication in their tracheas at a rate of 52–65%. However, in spite of this reduction, the transmission of challenge virus from rHVT-LT-vaccinated to contact-naive chickens was evident. This study is the first to support that rHVT-LT vaccination did not prevent the spread of the challenge virus to contact birds. Though the study of Johnson et al. [109] indicated that in ovo vaccination with FPV-ILT and HVT-ILT vector vaccines slightly reduced the clinical signs of ILTV, the vaccine did not significantly reduce the replication of the virus in the trachea. In ovo vaccination with either an HVT-ILTV or FPV-ILTV vaccine did not provide as much protection as ECEs-derived vaccines given at 2 weeks of age. In addition, Vagnozzi et al. [107] found that inoculation of the recombinant ILTV vectored vaccines in eggs or subcutaneously at hatching provided more protection and reduction in the severity of clinical signs and virus replication in the trachea when compared to eye drop vaccination of broiler chickens with a living attenuated ILTV vaccine at 14 days of age. Moreover, inoculation of the HVT-ILTV vaccine in ovo resulted in inhalation or swallowing the vaccine from the amniotic fluid of the embryo, and the vaccine could reach the trachea [11], while injection of the vaccine in day-old chicks resulted in deposition and absorption of the vaccine by the tissues and systematic distribution in the blood. The HVT-ILTV vaccine was more efficient than the FPV-ILTV vaccine in alleviating the disease and reducing the challenge virus level when applied either in ovo or subcutaneously in chicks. Moreover, it has been found that reactions to the FPV-ILTV vector vaccine can be obtained when administered at 17.5 to 18 days of embryonic age rather than at 19 days [110].

The safety and efficacy of a novel "glycoprotein J gene-deleted strain of ILTV" were evaluated in broiler chickens following in ovo vaccination at 18 days of embryo age [111], and the results indicated that this method of vaccination was safe and did not affect the hatchability or survivability of the hatched chicks. Moreover, the vaccination of broilers using either in ovo alone or in ovo combined with eye drop routes revealed significant reduction in the signs and the ILTV load post-challenge [111].

The study of Thapa et al. [112] showed that the CpG DNA ILTV vaccine delivered in ovo reduced the pre-hatch viral infection in correlation with the expression of IL-1β, increased macrophages in lungs, and decreased the mortality and morbidity resulting from ILTV infection post-hatch. Gimeno et al. [113] found that inoculation of the rHVT-ILTV vaccine via inoculation of the amnion at day 18 and day 19 of egg incubation resulted in more virus replication in the spleen, with more detectable glycoprotein I gene transcripts than in subcutaneous inoculation of day-old chicks.

5.1.5. Infectious Bronchitis

Infectious bronchitis is a highly contagious and an acute upper respiratory tract disease in chickens. The disease is caused by a virus that belongs to the genus corona virus and family Coronaviridae [114]. Infection of chickens with IBV induces severe economic losses in terms of drops in egg production and reduction in egg quality layers, as well as poor weight gain and increased mortalities and condemnation rates of broilers [115]. Prevention of the disease depends mainly on the application of both live and inactivated IBV vaccines. However, the emergence of new IBV variant strains [116], the labor, and the costs of vaccine production increase the need for other types of vaccines such as the recombinant DNA types [117].

In ovo vaccination with IBV vaccines has been successfully applied [42,118–121]. The early study of Wakenell et al. [118] revealed that embryos or day-old chicks vaccinated with tissue culture serially passaged IBV vaccines had similar lesions in the trachea, primary and secondary immune responses, and absence of the effector cytotoxic cells to IBV. In addition, Chew et al. [122] studied the effect of the inoculation of live IBV vaccine and attenuated kidney cell culture IBV in 18-day-old embryos, and the results showed that the hatchability rate was significantly lower in the group vaccinated in ovo (27%) than those who received the attenuated virus (45–58%). Moreover, inoculation of the vaccine and the attenuated IBV in day-old chicks with maternal antibodies induced no pathological lesions in the oviduct, and the respiratory signs were seen in 61% and 5% of chicks inoculated with the vaccine in the eggs and at hatching, respectively. In another study [123], hatched chicks from inoculated 17-days-old embryos with eight different strains of IBV that represented seven different serotypes showed strict epitheliotropic nature and wide tissue tropism of the IBV strains in the chicken embryos and the universality of riboprobe.

Broilers that received HVT/SB-1 and HVT plus CVI-988 MDV vaccines either in ovo or at hatching showed better protection rates to IBV challenge than those that received IBV vaccines (Ark and Mass serotypes) at hatching [124]. In addition, MDV vaccines that were inoculated either in ovo or at hatching did not affect the efficiency of the IBV vaccine given by eye drop at hatching. It has been noticed that in ovo vaccination at 18 days of age with the IBV M-41 vaccine could affect the hatchability; therefore, using an automatic injective system capable of handling 60.000 eggs at a time could improve the hatching rate [125].

Vaccination of chickens with the DNA vaccine containing the S1 glycoprotein gene from IBV induced protective immunity against infection [126,127]. Inoculation of this vaccine in ovo or intramuscularly after hatching provided chickens with some protection against clinical signs following homologous virus challenge [120]. Babapoor et al. [117] studied the effect of the in ovo inoculation of a plasmid DNA pTracer-CVM2-IBVS (pCMV-S) vaccine that expresses the immunogenic S glycoprotein genes of the IBV serotype Massachusetts 41 and a recombinant chicken interferon type I alpha (rChIFN) as an adjuvant. The results showed that chicks that received the pCMV-S vaccine had mild signs and were protected at the level of 66%, but those that received a combination of the pCMV-S with 2000 IU or 500 IU of rChIFN revealed protection at levels of 83% and 89% respectively. Furthermore, bolstering the pCMV-S and 500 IU rChIFN inoculated chickens with the live attenuated IBV vaccine indicated significant protection (more than 92%) against IBV challenge. Resiquimod and toll-like receptor 7 ligand agonist delivered in ovo enhanced host responses and produced secretory immunoglobulin, as well as antigen-specific humoral responses against the IBV vaccine [128].

5.1.6. Avian Influenza

Avian influenza virus (AIV) is an enveloped, negative-sense single-stranded segmented RNA virus in the family Orthomyxoviridae [129]. Despite immunization against AIV having been established as one of the most significant control strategies in the poultry production system all over the world, AIV has gone through antigenic drift due to the presence of immune pressure [130]. Therefore, it is difficult to select a vaccine strain that has the potency to face the widely circulating mutants of AIV.

In ovo inoculation of 18-day-old white rock and white Leghorn chicken embryos with oil-emulsion-inactivated NDV or AIV H5N9 vaccines induced acceptable hatchability, seroconversion rates, and protective immunity when prepared with sufficient antigen amounts and proper administration [131]. Many types of AIV vaccines have been studied for in ovo administration, including non-replicating adenovirus vectors expressing hemagglutinin proteins [132–134], attenuated vaccines [135], and recombinant attenuated vaccines [136].

Chicken embryos that received 10^9 infectious units of an adenovirus vector AIV vaccine showed that less than 1% of hatched chickens displayed serum antibody titers by 10 days post-hatching, while the percentage rose to 65% and 85% by 20 and 40 days post-hatching, respectively [133]. A previous study showed that in ovo vaccinated chicken embryos with a recombinant attenuated H5N1 AIV vaccine induced humoral immune response to the H5 protein in 30% of the hatched chickens, and 80% of vaccinated chickens survived post-challenge [136]. Moreover, vaccination of 18-days-old embryos with a live AIV H9N2 vaccine produced higher antibody response at young age and showed better protection upon challenge than eggs inoculated with an inactivated oil emulsion AIV vaccine [137].

It has been reported that the in ovo administration of CpG ODN decreased the replication of H4N6 AIV and IBV in the inoculated eggs [138,139]. Recently, the immune responses in chickens against a beta-propiolactone inactivated H9N2 AIV vaccine after a primary vaccination on embryonic day 18 and a secondary intramuscular vaccination on day 14 post-hatching were detected [15]. Also, the toll-like receptor 21 ligand, CpG ODN 2007, and an oil emulsion adjuvant were included [15]. The secondary vaccine induced antibody-mediated immune responses, and a beta-propiolactone inactivated virus combined with adjuvant provoked cell-mediated immune responses.

5.1.7. Avian Metapneumovirus Infection

Metapneumovirus infection in turkeys is termed as turkey rhinotracheitis in turkeys and swollen head syndrome in chickens.

The vaccination of turkey eggs with avian metapneumovirus subtypes A and B vaccine revealed that the hatched turkey poults showed resistance to the virulent virus challenge [13]. It has been also found that hatched chicks from inoculated eggs with metapneumovirus vaccine showed earlier immunity than day-old vaccinated turkey poults [140]. Inoculation of a living attenuated metapneumovirus vaccine subtype C in the amnion of turkey eggs showed no effect on the hatching rate, and the vaccine virus was able to replicate in the upper respiratory tracts and the lungs of vaccinated birds, along with low histopathological lesions seen in vaccinated birds after challenge with a virulent strain of avian metapneumovirus at 3 weeks old [141]. Moreover, high resistance to the virus replication was observed, which was correlated with a low count of the virus in the upper respiratory tract.

5.2. Bacterial Diseases

Mycoplasmosis

Mycoplasma gallisepticum (MG) is the main cause chronic respiratory disease of chickens an infectious sinusitis in turkeys, with subsequent severe adverse impacts on production worldwide [142]. Affected layers show drastic drops in egg production, and broilers show low growth rates [143,144]. Eradication of the MG infection could be achieved through vaccination, isolation, and biosecurity measures [145]. Commercially available live MG vaccines include an F strain of MG (FMG), a 6/85 strain of MG (6/85MG), and a ts-11 strain of MG (ts-11MG), which have different features and might be used to effectively control and eradicate the field strains of MG. Vaccination against MG is usually applied for pullets before the start of egg production at 9 weeks old through spray, eye drop, wing web, or intramuscular/subcutaneous methods [146,147].

The effects of the in ovo vaccination with the FMG vaccine on layer chick hatchability [148] and post-hatch survivability, immunity, and horizontal transmission were studied [144]. The results revealed that injection of the FMG vaccine in 18-day-old embryonated chickens eggs induced higher embryonic [19,148] and post-hatch [144] mortalities in comparison to control non-injected eggs. The bacterium was detected in the trachea,

mouth, esophagus, yolk sac, and duodenum of 22-day-old chick embryos [148]. However, a signification decrease in the growth rate [148] and depression of the humoral immune response [144] were observed in post-hatching chicks till 6 weeks of age. Furthermore, another study showed that the F strain of MG was horizontally transmitted from vaccinated to unvaccinated layer pullets through direct contact [149]. However, Alqhtani et al. [20] demonstrated that hatched chicks from eggs injected with the ts-11 strain of MG vaccine remained MG-free till 21 days post-hatch, and the vaccine had no impact either on the hemagglutination inhibition titers or on the post-hatch serology of the layers. The timing of the humoral immune response in the layer chicks during the first 4 weeks of age following in ovo vaccination with FMG was evaluated [150]. The results of the previous study indicated early detection of MG antibodies at 7 days of age. Moreover, the in ovo FMG vaccination was more effective in male layer chickens than females.

5.3. Parasitic Diseases

Coccidiosis

Avian coccidiosis is a host, tissue, and immune-specific intestinal protozoon parasitic disease caused by the *Eimeria* species. The disease is associated with high economic losses, including reduced performance parameters, increased mortalities, and high medication costs [151,152]. In addition, coccidiosis is regarded as a major predisposing factor to necrotic enteritis that is caused by *Clostridium perfringens* [153]. In the field, the prevention strategy of coccidiosis is based on using anticoccidial drugs in the feed, as well as living vaccines [154,155]. It is important to mention that the vaccine strains of *Eimeria* are sensitive to anticoccidial drugs.

Live *Eimeria* oocysts vaccines are applied in the early life of chicks to help in the development of immunity against the wild-type *Eimeria* species following oocyst cycling [156]. Live vaccines against coccidiosis could be applied through the in ovo injection of embryos during incubation [157,158]. These vaccines were administered in 18-days-old chick's embryos [159,160]. A living oocysts vaccine containing a mixture of *E. acervulina*, *E. tenella*, and two strains of *E. maxima* was used.

Commercially, the ideal time for in ovo vaccination against coccidiosis is during late embryonic development (between 17.5 and 19.0 days of chick embryonation) at +14 h with a maximum amniotic fluid [3,27]. Moreover, the inoculation of embryos with infective *Eimeria* strain vaccines at 18.0, 18.5, or 19.0 days of embryonation did not effect hatchability or chick quality [2,38,159]. Sokale et al. [22] demonstrated no significant difference in the cycling of coccidiosis following vaccination at 18.5 and 19.0 days of incubation.

Following in ovo inoculation of *Eimeria* oocysts, they were absorbed in the amniotic fluid and remained dormant in the embryo's intestine without occurrence during the life cycle until chick hatching [161]. Weber and Evans [159] reported that the in ovo inoculation of *Eimeria* species oocysts resulted in a shedding peak at approximately 7 days post-hatching.

6. Conclusions

Nowadays, in ovo vaccination is regarded as an alternative approach to the post-hatch conventional vaccination of birds, especially in broiler chickens. It is a rapidly growing trend of vaccine technology. Currently, in ovo vaccinations against some avian viral, bacterial, and parasitic diseases are still under investigation, and there is progress regarding the production of safe and efficient vaccines.

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