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# Newcastle disease vaccines—A solved problem or a continuous challenge?

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ABSTRACT

Newcastle disease (ND) has been defined by the World Organisation for Animal Health as infection of poultry with virulent strains of Newcastle disease virus (NDV). Lesions affecting the neurological, gastrointestinal, respiratory, and reproductive systems are most often observed. The control of ND must include strict biosecurity that prevents virulent NDV from contacting poultry, and also proper administration of efficacious vaccines. When administered correctly to healthy birds, ND vaccines formulated with NDV of low virulence or viral-vectored vaccines that express the NDV fusion protein are able to prevent clinical disease and mortality in chickens upon infection with virulent NDV. Live and inactivated vaccines have been widely used since the 1950's. Recombinant and antigenically matched vaccines have been adopted recently in some countries, and many other vaccine approaches have been only evaluated experimentally. Despite decades of research and development towards formulation of an optimal ND vaccine, improvements are still needed. Impediments to prevent outbreaks include uneven vaccine application when using mass administration techniques in larger commercial settings, the difficulties associated with vaccinating free-roaming, multi-age birds of village flocks, and difficulties maintaining the cold chain to preserve the thermo-labile antigens in the vaccines. Incomplete or improper immunization often results in the disease and death of poultry after infection with virulent NDV. Another cause of decreased vaccine efficacy is the existence of antibodies (including maternal) in birds, which can neutralize the vaccine and thereby reduce the effectiveness of ND vaccines. In this review, a historical perspective, summary of the current situation for ND and NDV strains, and a review of traditional and experimental ND vaccines are presented.



## 1. Historical perspective

Newcastle disease (ND) was first recognized ninety years ago and continues to be a problem for poultry producers. At least four defined panzootics have been recognized (Miller and Koch, 2013); negatively impacting not only economic livelihoods, but also human welfare by decreasing food supplies (Alders, 2014). After the initial, almost simultaneous, identification of Newcastle disease in 1926 in Indonesia, England, and possibly Korea, ND was identified to the Philippines, India, Japan, Australia, and Kenya. By 1952 it was also reported in Palestine, Syria, French Congo (present day Gabon, Republic of Congo, and Central African Republic), the island of Sicily, Europe, and the United States. In the 1960's as part of the 2nd and 3rd panzootics, ND was reported in Hawaii, Canada, Mexico, Central and South America, China and throughout Europe. These panzootics were aided both by the trade and movement of exotic psittacine birds without strict quarantine guidelines, the ubiquitous and synanthropic nature of pigeons, and the industrialization of the poultry industry (Alexander, 1988).

Newcastle disease viruses are single stranded, non-segmented, negative sense RNA viruses encoding for at least six structural proteins and comprising one of three genome sizes: 15,186, 15,192, and 15,198 nucleotides (Miller and Koch, 2013). The six proteins are the nucleocapsid, phosphoprotein, matrix, fusion, hemagglutinin-neuraminidase and the polymerase. Since the nucleotide sequences for the fusion (F) and hemagglutinin-neuraminidase (HN) genes of Newcastle disease viruses (NDV) were published in the 1980's, 1925 fusion (F), and 1094 hemagglutinin-neuraminidase

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(HN) full gene sequences have been placed into GenBank (26th August 2016). The sequence motifs of the F protein cleavage sites have been proven to be reliable indicators of virulence (Toyoda et al., 1987), which has helped to develop more efficient molecular diagnostic assays (Rue et al., 2010).

Clinical disease affecting the neurological, gastrointestinal, reproductive, and respiratory systems (Miller and Koch, 2013) are most often observed in naïve, unvaccinated, or poorly vaccinated birds. Clinical signs vary depending on the species of bird, the strain and challenge dose of the virus, and the immunity of the host (Miller and Koch, 2013). Virus isolation, either in specific pathogen free (SPF) or NDV antibody-free embryonating chicken eggs or cell cultures, coupled with hemagglutination inhibition (HI) with NDV specific antiserum remain the definitive diagnostic assay for ND (OIE, 2012). The mean death time from the minimum lethal dose (MDT/MLD) assay performed on SPF embryonating chicken eggs continues to provide information on virulence for experimental purposes (Miller and Koch, 2013) and the intracerebral pathogenicity index (ICPI) is required by the World Organisation for Animal Health (OIE) due to the assay's ability to discern mixed infections that may be otherwise missed using molecular techniques (OIE, 2012).

Like all other RNA viruses, NDV is constantly evolving. As of 2013, Newcastle disease viruses have been grouped genetically into two classes with only one genotype of class I and 18 genotypes of class II. Historically, grouping NDV strains into genotypes based on the similarities of the genomes began as a way to provide epidemiological information (Lomniczi et al., 1998). The ability to explore the virus repositories of laboratories that had collected NDV strains over the years and to use new sequencing technologies provided previously unknown information as to how the various strains from different outbreaks related to one another. A better understanding on the epidemiological relations among the circulating NDV, their genetic diversity and characteristics, and global distribution is crucial for developing new vaccines and vaccination strategies. Genetic evaluation of the oldest NDV strains grouped them into what is now known as genotypes I, II, III, IV, and IX (Dimitrov et al., 2016b). While genotype I includes predominantly NDV of low virulence, genotype II strains may be virulent or of low virulence, and all of the characterized strains from III, IV, and IX contain cleavage site motifs of virulent strains. Excluding one virus from the 1940s, most genotype IX NDV strains were isolated after the 1980's in China (Dimitrov et al., 2016a).

Genotypes V, VI and VIII were regularly isolated prior to 1990 (Dimitrov et al., 2016b). Genotype VII were first isolated in the early 1990's in Italy, Spain, the Netherlands, Belgium, and Germany, and those strains were genetically most similar to NDV strains from Indonesia in the late 1980's (Lomniczi et al., 1998). The newly classified genotype X strains consisting of strains of low virulence were identified as early as 1986 in the USA (Diel et al., 2012a).

Along with the F and HN sequences, the addition of 372 NDV complete genome sequences into GenBank has assisted in the phylogenetic characterization of additional virulent genotypes from 1990 through 2016 (Dimitrov et al., 2016b). Viruses of genotype XI have been exclusively isolated from chickens in Madagascar between 2008 and 2011 and seem to have common ancestry with viruses of genotype IV. Viruses of genotypes XIV, XVII and XVIII appear to have limited geographic distribution and have been isolated predominantly from domestic gallinaceous birds in West and Central Africa during 2006–2011 (Dimitrov et al., 2016b; Samuel et al., 2013). While genotype XII was first reported in Peru in 2004 and China in 2011, it has been also found in Colombia in 2009 (Dr. Claudio Afonso, unpublished data).

In recent years the number of reported viruses has increased (Dimitrov et al., 2016b). An average of 60 countries reporting ND outbreaks yearly from 2013 to 2015 and the increasing number of genotypes demonstrate the broadening of virulent NDV genetic diversity, suggesting that perhaps vaccination may have contributed to this effect. Furthermore, the currently used vaccine strains (mainly genotypes I and II) are three to seven decades old and are genetically distant (18.3–26.6% nucleotide distance) from the currently circulating virulent NDV (Dimitrov et al., 2016b). Such high genetic distance between the vaccine and the contemporary NDV strains prevents effective reduction of shedding of the virulent virus from vaccinated birds, as discussed later in this review (Miller et al., 2009, 2007).

To complement vaccination, control of ND has been facilitated by strict biosecurity, which prevents the virus from contacting poultry (Miller and Koch, 2013). This included implementing quarantine stations for imported birds, controlling the movement of birds and eggs inside the areas of concern, and the stringent and proper administration of vaccines. Currently, the effective containment of ND outbreaks is normally achieved with the utilization of a combination of vaccinations, rapid diagnostic assays, and culling of infected flocks. From the early 1950's to the late 1990's live and inactivated ND vaccines were the only vaccine platforms available and were used to decrease economic losses resulting from morbidity and mortality (Gallili and Ben-Nathan, 1998).

Inactivated vaccines became commercially available in the USA in 1945, but were not adopted by the poultry industry at that time as they were comparatively expensive, and were unable to prevent clinical disease to a sufficient level to merit wide spread use. The first live vaccines licensed in 1948 were formulated with strains now designated as virulent that produced disease in younger birds and were only applicable for use in chickens at least four-week old and needed to be applied with a wing-web application (Goldhaft, 1980). During this time several laboratories were investigating NDV strains that could be used as a live vaccine with acceptable levels of post-vaccinal clinical disease symptoms. Within two years, two NDV strains of low virulence (B1 and LaSota) isolated from chickens from the USA were also licensed for use (Goldhaft, 1980; Hitchner, 1975). Shortly after these products were available for individual bird administration, mass application techniques deemed necessary for larger commercial settings were investigated and implemented despite varying responses, because of uneven coverage in flocks and less than an optimal percentage of seroconverting birds (Lancaster, 1966). Since the earliest implementation of live ND vaccination, the transfer of antibodies to offspring that can even partially neutralize the live ND vaccines, was known to be a complication. It was also evident that even the vaccines formulated with the more virulent vaccine strains would not provide lifelong immunity and that additional vaccinations would be necessary in layers and breeders. Unfortunately, many of the problems in controlling ND that were clear from the first few decades of ND vaccine use, continue in 2016.

## 2. Current situation

When an outbreak of ND occurs from infections of poultry with the virulent forms of the virus, referred to as mesogenic or velogenic NDV, the country is obligated to report it to the OIE, and trading partners may suspend imports of poultry or poultry products from that country. The World Livestock Disease Atlas surveyed 176 countries included in the OIE Animal Health Yearbooks from 2006 to 2009 and concluded that Newcastle disease is the fourth most problematic disease of poultry, behind highly pathogenic avian influenza, avian infectious bronchitis, and low pathogenic influenza (Anonymous, 2011). When evaluating the number of wild animals lost through destruction, disease or slaughter, ND ranked 8th out of the 71 diseases evaluated (Anonymous, 2011).

The widespread distribution of ND and the high number of annual outbreaks demonstrate that although globally used, current ND vaccines and vaccination practices alone cannot control the disease. Countries most affected during 2006–2009 in descending order were Iran, South Africa, Israel, China, Vietnam, Columbia, Romania, South Korea, Kuwait, and Sweden (Anonymous, 2011). With 56 countries reporting ND outbreaks on average per year from 2006 to 2009, ND ranked 2nd only behind rabies in the reported disease outbreaks (Anonymous, 2011). From 2008 to 2010, 77 countries confirmed ND outbreaks in domestic poultry with 68, 61 and 56 countries reporting ND outbreaks in 2013, 2014, and 2015, respectively (www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statuslist). Underreporting of ND, especially in areas where virulent NDV is endemic in poultry, may mean that these numbers are underestimating an already bad situation. Many of the countries affected by ND also lack good biosecurity practices. Thus, ND will likely persist in these areas of the world until both vaccines and biosecurity practices are improved.

Vaccination efforts against ND are focused in the poultry sector. However, it is likely that all bird species are susceptible to infection with NDV strains, and to date, more than 236 avian species have been documented with NDV infections (Kaleta and Baldauf, 1988). Besides chickens, other birds (pigeons, cormorants, psittacines, pheasants, peafowl, wild waterfowl and shorebirds) are often reported with infections of virulent NDV (Cardenas Garcia et al., 2013; Diel et al., 2012a; Pearson and McCann, 1975) and NDV of low virulence (Kim et al., 2007). In general, turkeys are slightly more resistant to NDV than chickens; however, some wild-bird NDV strains that have adapted to turkeys are able to infect this species more readily (Dr. Patti Miller, unpublished data). Geese and ducks also show some resistance to infection, with ducks rarely presenting signs of clinical disease and geese showing slightly higher susceptibility, depending on the strain of NDV.

When genotype V NDV strains began to be isolated in the USA, in addition to chickens, peafowl were among the birds noted to present clinical disease (Pearson and McCann, 1975). During 2012–2015 virulent NDV strains from captive, non-poultry species (peafowl, pigeons, pheasants, and parakeets) in Pakistan were nearly identical to those isolated from chickens in the same geographical locations, suggesting the existence of an epidemiological link between the two groups of birds (*submitted for publication*). The roles of the different bird species reported with infections of NDV in the maintenance of the disease are still unclear. Nevertheless, their importance should not be underestimated as sometimes they are reared in large flocks in close proximity to poultry, and their vaccination (when appropriate and feasible) could contribute to the control of ND. Vaccination protocols for these species are dependent on national and international regulations and the specific epidemiological situation (OIE, 2012).

The small size of the NDV genome, along with the low likelihood of genetic recombination, facilitates the use of NDV as a

**Table 1**
Properties of live, inactivated and vectored Newcastle disease vaccines.[a]

| | Live | Inactivated | Vectored[b] |
|---|---|---|---|
| Storage and constitution | frozen, freeze-dried; chilled, liquid | chilled, suspension, emulsion | frozen, cryo-frozen (liquid nitrogen) |
| Adjuvants | no | yes | no |
| Administration route | mass (spray, aerosol, drinking water) or individual (eye drop, injection) | injection | *in ovo*, individual (eye drop, injection-subcutaneous or wing-web) or mass (spray, aerosol) depending on the vector |
| Duration of immunity | short | long | long |
| Response to the vaccine | systemic and local | systemic | systemic and local |
| Antibody immune response | IgY, IgM, IgA | IgY, IgM | IgY, IgM, IgA (depending on the vector and route of administration) |
| Cell-mediated immune response | strong | weak | strong, for Newcastle disease virus (NDV)-vectored |
| Affected by maternal antibodies | yes, depending on the level of antibodies | yes, depending on the level of antibodies | yes, depending on the vector, can be overcome by route of inoculation and by vaccine dose; affected particularly by antibodies against the FPV vector |
| Affected by pre-existing antibodies from previous vaccinations | yes, if induced by live vaccines | depending on the level of antibodies | yes, if induced by live vaccines; HVT-vectored vaccine significantly affected by pre-existing anti-HVT antibodies |
| Protection onset | 2–3 weeks | 3–4 weeks | 4–5 weeks |
| Clinical signs after vaccination | possible mild respiratory signs depending on many factors (age, immunity, etc.) | no | NDV-vectored – not studied; NDV-inserted – no |
| Thermostability | no (studies made with strains that show some thermotolerance) | no | no |
| Cost | less expensive | more expensive | Variable |
| Genotype | I II | Any | Any |
| Vaccine strain | I-2 LaSota<br>V4 B1<br>PHY-LMV42 VG/GA<br>Ulster Clone 30 | Any | Any |

[a] Additional information regarding ND vaccines produced throughout the world could be found at http://www.poultrymed.com/Vaccines.
[b] For the purpose of this table the term vectored vaccines is used for the rNDV with non-NDV inserts and for rFPV and rHVT with NDV inserts.

vaccine vector. Information can be found in other reviews that summarize the body of knowledge for each gene individually, which has facilitated the genetic manipulation that has furthered recombinant NDV (rNDV) vaccine development (Ganar et al., 2014). The virus can also replicate in many mammalian species, allowing NDV to be used as a vector for development of veterinary and human vaccines against other diseases such as rabies, West Nile disease, infectious bursal disease, canine distemper, influenza, Ebola, severe acute respiratory syndrome, human immunodeficiency syndrome, respiratory syncytial virus syndrome, among others (Kim and Samal, 2016). However, there is no evidence of mammals serving as a biological vector for the spread of ND to poultry, although mammals, including humans, often serve as mechanical vectors for the virus.

## 3. Newcastle disease vaccines

There are three main goals when using vaccination to help control ND: i) decrease or eliminate clinical disease; ii) decrease the amount of virulent virus shed; and iii) increase the infectious dose of the challenge virus (Kapczynski et al., 2013). Unfortunately, only the first goal is considered to be an objective of current control strategies, as field veterinarians do not have the tools to assess the effectiveness of vaccination on the accomplishment of the second and third objectives. Biosecurity is a critical component of keeping the challenge virus away from the flock before they achieve a protective level of immunity, or ideally preventing any exposure. The success of any ND vaccination program also may depend on a minimum of 85% of the flock receiving a proper dose and responding to vaccination to achieve herd immunity (van Boven et al., 2008). Those studies were performed under optimal conditions and remain to be validated in the field, under sub-optimal conditions that may include deficient nutrition, stress, immune suppression, and repeated challenges. Exemplary current vaccination strategies for different poultry sectors have been provided in the Merck Veterinary Manual (www.merckvetmanual.com), however, these vary depending on the specific ND epidemiologic situation. Table 1 summarizes the main properties of live, inactivated and vectored vaccines, the most widely used ND vaccines.

### 3.1. Traditional vaccines

Worldwide, the most commonly used ND vaccines are live vaccine viruses formulated with strains isolated in the 1940's and 1960's. Viruses circulating in poultry were the source of the LaSota, B1, and VG/GA vaccines. All of those viruses belong to genotype II and are genetically and antigenically highly related among themselves (>98% nucleotide identity). The main differences among those vaccines are the tropism and the capacity to replicate in naïve chickens, which is highest in LaSota and results in higher levels of neutralizing antibodies compared to other strains (Meulemans, 1988). Thus, the LaSota strain is nearly always used in countries where virulent NDV is endemic (Diel et al., 2012b). The VG/GA strain is normally sold as an enterotropic vaccine, and the B1 strain as the most attenuated vaccine to be used in cases of low challenges or in very young birds. While live vaccines provide both mucosal and humoral immunity and can be administered using mass application techniques, they may cause clinical respiratory disease, drop in egg production, and are easily inactivated when not kept at the required temperature (commonly 4 °C) (Winterfield and Dhillon, 1981).

The effectiveness of live ND vaccines is directly correlated with the dose of the administered vaccine, and under experimental conditions the mean embryo infectious vaccine dose ($EID_{50}$) of $10^4$–$10^5$ reliably achieves 100% protection against mortality in adult SPF chickens, but did not prevent challenge virus infection and replication (Cardenas Garcia et al., 2015; Cornax et al., 2012; Miller et al., 2013). Doses of the LaSota vaccine of $10^6$ $EID_{50}$ or higher produced strong humoral immune responses and no increase in titers was observed after challenge, suggesting little to no replication of the challenge virus (Cornax et al., 2012). Similar survival rates and viral shedding amounts after challenge with virulent NDV strains from genotype VII have been observed with the LaSota vaccines in SPF chickens (Dr. Cardenas Garcia, University of Georgia, USA, personal communication) (Samuel et al., 2013). Cornax et al. results suggested that it may be possible to achieve the three objectives of vaccination when a very high dose of vaccine is used (Cornax et al., 2012). Those results appear to be confirmed by results in the field in which a more aggressive vaccination strategy often results in improved ND control. Unfortunately, the administration of high doses of classical ND vaccines significantly increases the cost of vaccination, thus this practice is not widely utilized.

A second group of traditional vaccines that is widely used are vaccine strains from class II genotype I (i.e. I2, V4, and PHY-LMV42), which are avirulent and safely used in chickens of all ages (Cardenas Garcia et al., 2013). Strains of NDV that have increased stability to heat are especially advantageous in rural areas of the world with limited refrigeration. The I-2 strain has improved thermostability in comparison to the V4 ND vaccine strain it was derived from, and is mainly used in areas with higher ambient temperatures (Alders, 2014). The I-2 seed strain is produced by the Australian Centre for International Agricultural Research and provided to countries for the production of ND vaccines for village poultry flocks (Copland and Alders, 2005). Progress in identifying and characterizing other thermostable and immunogenic NDV strains continues (Jeong et al., 2013). These vaccines are also effective in preventing clinical signs upon infection with virulent NDV, but like the others, do not prevent viral replication (Susta et al., 2015b).

An additional consideration for village poultry is the number of doses able to be purchased and the ease at which the vaccine can be reconstituted. Using a freeze-dried pelleted LaSota NDV strain, researchers were able to provide a consumer friendly and cost-effective vaccine for smaller flocks (50 doses) that had more heat resistance than the usual lyophilized product (Lal et al., 2014). A similar pelleted commercial product formulated with the VG/GA strain, in an effervescent tablet can be reconstituted in drinking water and administered within two hours by mass application methods.

Inactivated ND vaccines have the disadvantage of requiring a withdrawal period before vaccinated birds can be processed for human consumption, and each vaccine requires individual administration by a subcutaneous or intramuscular injection. Even though birds vaccinated with inactivated vaccines tend to have higher humoral antibody levels, they do not develop a strong cell mediated response (Schijns et al., 2013), and shed larger amounts of virulent challenge virus compared to birds vaccinated with live ND vaccines (Miller et al., 2013, 2009). Although live and inactivated vaccines protect against clinical disease in SPF chickens, there are continuous reports of vaccine failures under field conditions (Perozo et al., 2012; Rehmani et al., 2015). One of the possible reasons for these failures may be poor vaccination response that is also dependent on field-associated factors unrelated to the vaccines, such as immunosuppression (Meulemans, 1988) from infections prior to ND vaccination. Different avian pathogens such as *Infectious bronchitis virus*, *Gallid alpha-herpesvirus 1*, *Infectious bursal disease virus*, and *Mycoplasma* spp. have been associated with immunosuppressive effects. Understanding the role of field factors and field immunosuppressing agents during and after ND vaccination may lead to the

development of more effective vaccines or vaccination strategies. Vaccines that are co-expressing antigens of different pathogens and are simultaneously inducing immunity against several avian diseases would be of great value. Presence of maternal antibodies interferes with the development of active immunity when live vaccines are administered via intramuscular, subcutaneous, intranasal route, in drinking water, and through aerosol. In chickens with maternal immunity, the best response to live ND vaccine is achieved through conjunctival and intranasal routes of administration, perhaps due to the development of local immunity induced by these vaccines. However, immunity induced by inactivated vaccines was less affected by the presence of maternal antibodies (Lancaster, 1966).

### 3.2. Vectored vaccines

#### 3.2.1. Fowlpox virus *and herpesvirus of turkeys used as vectors for ND vaccines*

For more than 20 years, efforts have been directed towards the development of recombinant vaccines against ND, using other avian viruses as vectors. In 1990, the *Fowlpox virus* (FPV) vector-based vaccines expressing the NDV F or HN protein were proven to protect chickens from a challenge with virulent NDV (Boursnell et al., 1990). Later, multiple studies were conducted, employing both genes (alone or in combination, also with other viral genes), to investigate the protective efficacy of the recombinant vaccines (Karaca et al., 1998; Taylor et al., 1996). While some have shown that maternal antibodies to the influenza A virus hemagglutinin (HA) protein can interfere with recombinant FPV (rFPV) vaccines expressing HA (Faulkner et al., 2013), others have shown that immunity to FPV from previous FPV vaccinations, not maternal antibodies, are the problem (Bublot et al., 2006). At least two commercial rFPV-ND vaccines have been registered and are sold commercially. However, the rFPV-ND vaccines are not widely used because they cannot be applied through mass methods. Furthermore, previous exposure to FPV, which is commonly present in the environment, decreases efficacy of the rFPV vaccines.

The *Meleagrid alphaherpesvirus 1*, commonly known as herpesvirus of turkeys (HVT) or a serotype 3 Marek's disease virus, is one of the most widely used vectors in recombinant vaccine production. In the early 1990's, Morgan et al. and Reynolds et al. first showed the protective efficacy of HVT vector-based vaccines to protect chickens from ND and Marek's disease (Morgan et al., 1992; Reynolds et al., 1993). These vaccines are made by inserting the coding region of the fusion protein of NDV into the thymidine kinase site of the viral genome, and are capable of expressing the protein encoded by the gene during replication. Currently, two bivalent commercial recombinant HVT (rHVT) vaccines have been registered and are used internationally.

While the replication of rHVT-ND vaccines appears to be mildly hindered by the presence of maternal antibodies (Le Gros et al., 2009), they are able to prevent clinical disease and mortality when challenged with a virulent NDV six weeks after vaccination (Sonoda et al., 2000). The antibodies induced after the administration of the rHVT-ND vaccines occur at the same time in which maternal antibodies are waning. The rHVT-ND vaccines have many benefits, among these is that they can be administered *in ovo* at the hatchery or subcutaneously after hatch, and produce long-term immunity (Armour and García, 2014; Esaki et al., 2013). However, the rHVT-ND vaccines are cell associated, so like Marek's disease vaccines, they are required to be kept in liquid nitrogen, and to be administered within an hour of being thawed. Unfortunately, rHVT-ND require four weeks before full immunity will be reached (Palya et al., 2012), which would require the strictest level of biosecurity to prevent infection during that period. This may be impossible in countries where ND is endemic. Recombinant HVT vaccines have been widely used in countries where minimum viral challenges exist; however, in endemic countries, these vaccines may need to be used in combination with other ND vaccines to confer acceptable protection. After hatch, the administration of a killed or live ND vaccine to birds that were vaccinated *in ovo* with rHVT-ND vaccine, increases the level of immunity to facilitate more complete protection and helps decrease the amount of virulent NDV shed after challenge (Palya et al., 2014). This approach is commonly referred to as a Prime-Boost strategy. Because of the lack of simple serologic tests to measure the immune response to rHVT-ND vaccines expressing the NDV fusion protein, a quantitative real-time polymerase chain reaction assay to evaluate the rHVT-ND vaccine load from feather follicles has been developed (Rauw et al., 2015). This approach may be helpful in evaluating if rHVT vaccine administration was successful. A commercial ELISA kit has been recently advertised that can detect anti-NDV antibodies induced by rHVT-ND vaccines.

It is important to note that the use of one rHVT vaccine *in ovo* prevents the use of other rHVT-vectored vaccines subcutaneously in the same birds after hatch, as the immunity that is induced from the first vaccine will neutralize the viruses from the second application after it is administered (Schat, 2015). However, the simultaneous subcutaneous administration of a recombinant Marek's disease virus vaccine of serotype 1 (Rispens strain) expressing the protein encoded by the VP2 gene of IBDV with a rHVT-ND vaccine resulted in 94%, 100%, and 94% survival after challenge (five weeks after vaccination) with Marek's disease virus, IBDV, and NDV, respectively (Ishihara et al., 2016). A recombinant infectious bursal disease virus (IBDV) containing the HN of NDV has also been created, but it only provided 50–60% protection to SPF birds following a virulent NDV challenge (Li et al., 2014b).

#### 3.2.2. NDV-vectored vaccines

During the late 1990's, reverse genetics technology was developed to rescue infectious NDV from assembled sub-genomic overlapping cDNA fragments under control of a T7 RNA polymerase promoter (Peeters et al., 1999; Romer-Oberdorfer et al., 1999). This technology allows researchers to genetically manipulate the genome of NDV and insert non-NDV genes in it. This lead to the development of genetically engineered vaccines while retaining the replication competency of the original virus. Since then, using the reverse genetics technology, many strains of NDV have been developed as vectors to express proteins encoded by the inserted foreign gene for the purposes of developing avian vaccines and for human cancer therapy. A comprehensive review lists the main features of NDVs that allows them to be promising vaccine vector: they replicate well in vivo, induce a robust mucosal and systemic immune response, allow easy genetic manipulation, and avirulent strains usually used for vaccine vectors are safe, do not recombine, and do not incorporate into the DNA genome during replication (Kim and Samal, 2016).

Many recombinant ND vaccines have been created by the insertion of a foreign gene into an intergenic region of the NDV genome for the expression and dual use as a vaccine against both the NDV and the second agent. The evaluation of these vaccine candidates in clinical trials revealed different levels of protection against targeted pathogen challenge (DiNapoli et al., 2007; Huang et al., 2004; Nakaya et al., 2001; Park et al., 2006; Yu et al., 2013). Although most of them are not yet commercially available, numerous experimental vaccines using NDV of low virulence as vectors have been developed and investigated for protection against different avian diseases. Huang et al. reported the generation of LaSota vector-based vaccine expressing the VP2 protein of a variant IBDV in 2004 (Huang et al., 2004). A decade later another experimental recombinant NDV (rNDV)-IBDV (VP2) vaccine was evaluated in SPF and commercial broilers after *in ovo*

administration and resulted in 90–96% hatchability and 91–100% survivability after intramuscular challenge with virulent NDV (Ge et al., 2014).

In 2014, two teams reported the successful formulation of NDV (LaSota) vector-based vaccines expressing, the *Gallid alphaherpesvirus 1* (commonly known as Infectious laryngotracheitis virus) surface glycoproteins gB, gC and gD, together or separately (Kanabagatte Basavarajappa et al., 2014; Zhao et al., 2014). With a prime-boost application, 42-day-old SPF birds survived a virulent NDV challenge two weeks after the last vaccine was administered (Kanabagatte Basavarajappa et al., 2014). During the same year, recombinant LaSota virus containing the IBV S2 gene was also generated as a priming vaccine (Toro et al., 2014).

Following the emergence and worldwide spread of the high pathogenicity avian influenza virus (HPAIV) H5N1 in the 1990's, and the increasing need for better protection of the poultry industry against HPAI and low pathogenicity avian influenza, multiple NDV vector-based vaccines containing different avian influenza virus (AIV) hemagglutinin (HA) genes were created after replacing the polybasic cleavage site of HPAIV with a low pathogenicity cleavage site: e.g. H5, H6, H7, H9 (Goff et al., 2013; Lardinois et al., 2012; Park et al., 2006; Romer-Oberdorfer et al., 2008; Schroer et al., 2011). Recently, the experimental use of a modified virulent NDV vector-based vaccine expressing AIV H5 protein was reported and showed the potential of virulent NDV to be used as vectors (Kim et al., 2014). A NDV vector-based vaccine expressing H5 protein was recently commercialized (Sarfati-Mizrahi et al., 2010). These rNDV-AIV vaccines are heavily used in the field in China with roughly 11.7 billion doses having been applied from 2006 through 2012 (Li et al., 2014a). Limited data from Mexico is available, but during a three-month period in 2008, 32 million doses of rNDV-AIV were administered (Villarreal, 2009).

In addition to the "conventional model" for foreign gene expression through an additional independent transcription unit (ITU), different approaches for expression of a foreign gene by NDV have been explored (Wen et al., 2015) (Gao et al., 2008). Some of them increased the capacity of expressing a larger gene or more than one foreign gene. Expression of a foreign protein through an internal ribosomal entry site (IRES) from a second open reading frame in a NDV vector has also been investigated (Zhang et al., 2015), and results suggest that the NP gene downstream non-coding region is the optimal insertion site for a high level of foreign gene protein expression.

Not all rNDV vaccines are equal in their levels of immunogenicity or their ability to replicate in chickens, therefore, each vaccine that is created should be evaluated for its ability to replicate in chickens, and to induce a protective immune response against a virulent NDV challenge. The immune response to vaccination with recombinant vaccines is influenced by many factors, and the expression of the desired level of foreign genes is undoubtedly one of the most important and critical factors for the success of the vaccine. The level of foreign gene expression from a NDV vector can be affected by the rate of replication, the tissue tropism of the viral vector, the size and the sequence of the foreign gene insert, and the genomic location of the foreign gene in the vector. Among these, the genomic location of the foreign gene has been shown to be crucial. To date, most of the foreign genes have been inserted into a non-coding region in the NDV genome as an additional independent transcription unit that consists of NDV gene start (GS), the foreign gene, and NDV gene end (GE) sequences (DiNapoli et al., 2007; Huang et al., 2004; Nakaya et al., 2001; Park et al., 2006; Yu et al., 2013; Zhao et al., 2014). Based on the sequential transcription of negative stranded RNA viruses (Lamb and Parks, 2013), the best position for foreign gene expression is hypothesized to be the closest to the 3'end of NDV genome. However, the insertion of a foreign gene as an ITU into a promoter-proximal position may interfere with NDV replication more seriously than a promoter-distal position, resulting in lower levels of foreign gene expression (Carnero et al., 2009; Zhao and Peeters, 2003; Zhao et al., 2015b). Therefore, a balance in virus replication and the abundance of foreign gene expression must be considered for selection of a foreign gene insertion site. The insertion of a foreign gene more proximal to the 3′ end, between the NP and P gene, expressed a low level of the foreign protein (Carnero et al., 2009). Further studies supported the conclusion that the P and M junction region is the optimal insertion site for an optimal level of foreign gene expression by a NDV vector (Nakaya et al., 2001; Zhao et al., 2015b).

An important aspect that has to be considered when using NDV as a vaccine vector is the efficacy of the virus in the presence of both vector- and insert-specific maternal antibodies (Armour and García, 2014). It has been previously demonstrated that the efficacy of recombinant NDV vector-based vaccines expressing avian influenza proteins was reduced when the vaccines were administered in birds with pre-existing anti-ND and anti-AIV antibodies (Faulkner et al., 2013; Sarfati-Mizrahi et al., 2010; Schroer et al., 2011). Notably, not all rNDV vector-based vaccines have been evaluated in NDV-challenge experiments.

### 3.3. Antigenically matched engineered vaccines

Autogenous vaccines were the first true antigenically matched vaccines used in poultry (Smith, 2004). However, autogenous vaccines are not defined and are difficult to use, especially in food animals, as they are usually inactivated and have long withdraw periods. It is not uncommon for the virus strains used in vaccines for respiratory diseases caused by paramyxoviruses, such as measles and canine distemper, to be changed over time to improve the efficacy of the vaccine and the achieved immunity (Griffin and Pan, 2009; Martella et al., 2011). These changes are made when the virulent challenge strains accumulate too many genomic mutations over time, and the vaccine virus strains are no longer similar antigenically to the challenge strain. Avian influenza viruses with their multiple serotypes require serotype specific vaccines to prevent morbidity and mortality. In addition, the shedding of virulent HPAI challenge virus after infection was decreased with smaller amounts HPAI shed when the vaccine and challenge virus were more similar (Swayne, 2003). The principle of utilizing a NDV strain closely related or homologous to the challenge strain was tested by our laboratory and produced similar findings (Miller et al., 2013, 2009, 2007).

Live and inactivated antigenically matched ND vaccines have been developed using reverse genetics. One type of such vaccines contain viruses that are identical to the circulating virulent NDV with the exception of the fusion protein cleavage site, which is modified to decrease virulence. Another type of vaccines, developed by our laboratory, is based on the use of a vaccine backbone (e.g. LaSota) with the replacement of the fusion and hemagglutinin neuraminidase genes. These two genes are replaced with ones homologous to currently circulating viruses, with the modification of the cleavage site of the fusion protein, which is normally engineered to be identical to the cleavage site of the LaSota vaccine strain.

When live and inactivated ND vaccine strains were antigenically matched they produced a higher humoral immune response to the challenge viruses than the heterologous vaccine, and the amount of virulent ND challenge virus shed from vaccinated birds was lower than the amount secreted by the heterologous vaccine (Miller et al., 2009, 2007). The efficacy of inactivated vaccines from genotypes I, II, V, VI, VII, and XII against challenge viruses of different genotypes were evaluated and found, under optimal conditions, to prevent 90 to 100% of the birds from having

morbidity and mortality against all the virulent NDV strains used no matter their genotype (Miller et al., 2013, 2009, 2007). However, when heterologous (non-matching genotypes) live or inactivated ND vaccines were administered properly and chickens were given enough time to develop a proper immune response, all the birds lived and showed no sign of disease (Cornax et al., 2013; Miller et al., 2013), supporting the claim that the administration of the proper amount of vaccine is also crucial to ND control (Dortmans et al., 2012).

More recently, recombinant ND vaccines with the F and HN protein genes homologous to the challenge virus in a LaSota backbone have been shown to induce higher levels of antibodies, and reduced viral shedding after challenge in comparison to the commercial LaSota vaccine. Furthermore, when birds were sub-optimally vaccinated with low doses of vaccines given only seven days before challenge with a virulent NDV, a decrease in morbidity and mortality rates was observed with one homologous vaccine compared to a traditional heterologous ND vaccine (Cardenas Garcia et al., 2015). Teams from Korea (Cho et al., 2008), China (Liu et al., 2015), and Indonesia (Xiao et al., 2012) have had similar findings in terms of reduction of viral shedding, while others have reported no improvement with their homologous rNDV vaccines (Dortmans et al., 2012).

The primary benefit of antigenically matched vaccines compared to traditional vaccines is a possible decrease in the amount of challenge virus shed from vaccinated chickens, but this parameter is not one that has been routinely part of evaluating ND vaccine efficacy for commercial production. Our recent finding of improvements in clinical protection in sub-optimally vaccinated chickens suggests that the advantages of antigenically matched vaccines may be even clearer in the field than under laboratory conditions. Vaccines homologous to NDV genotype V are heavily used in Mexico with 1.1 billion doses applied from January 2015 through July 2016 (Dr. Arnulfo Toscano, Investigación Aplicada S.A.de C.V., Mexico, personal communication), suggesting that the market is starting to appreciate the benefits of a reduction in viral shedding. A disadvantage of homologous vaccines is the difficulty of the vaccine company to work with virulent NDV in the laboratory because of the need for higher biosecurity. Most countries do not have vaccine companies with the level of biosecurity to safely make antigenically matched vaccines.

### 3.4. Other experimental vaccines

#### 3.4.1. Antigen-antibody complex vaccine

Chickens are vaccinated conveniently *in ovo* at 18 or 19 days of embryonation when they are moved into hatching trays. This system presents the NDV antigen to both the respiratory and gastrointestinal tracts and allows newly hatched chickens to develop an early immune response (Kapczynski et al., 2012). However, live NDV given *in ovo* can cause decreased hatchability and weak chicks. An innovative approach was developed to use an antigen-antibody complex live ND vaccine for *in ovo* vaccination to slow the replication without adversely affecting the hatchability. The NDV specific antibodies were able to dissociate from the vaccine virus after the birds hatched, resulting in acceptable hatchability, otherwise not achieved with even the least virulent, asymptomatic wild type or recombinant NDV strains administered *in ovo* (Kapczynski et al., 2012).

#### 3.4.2. Vaccines utilizing toll-like receptor ligands as adjuvants

Over the last few decades understanding of the mechanisms of innate immunity increased significantly. Innate immune system specifically recognizes foreign pathogens (or their pathogen-associated molecular patterns) with the help of pattern recognition receptors (PRR). Toll-like receptors (TLR) are a type of PRR and efforts have been made to enhance vaccine potency and to stimulate immune responses by using TLR ligands as adjuvants (Gupta et al., 2014). In a recent review by Gupta et al., chicken TLRs agonists and their use as adjuvants in vaccines against poultry infectious diseases (including ND) have been extensively described (Gupta et al., 2014). Ramakrishnan and colleagues demonstrated significant up-regulation of the transcriptional expression of interferon (IFN)-β, IFN-γ, interleukin (IL)-1β, IL-4, and TLR-7 genes in chicken peripheral blood mononuclear cells after administration of resiquimod (R-848, a TLR-7 agonist) (Ramakrishnan et al., 2015) and the synergetic effect of R-848 and lipopolysaccharide, a TLR-4 agonist (Annamalai et al., 2015). The use of R-848 as an adjuvant for inactivated ND vaccine administered intramuscularly in chickens showed significantly up-regulated expression of IFN-α, IFN-β, IFN-γ, IL-1β, IL-4, inducible nitric oxide synthase and MHC-II genes and increased protection after virulent NDV challenge when compared to two inactivated ND vaccines used alone (Sachan et al., 2015). Lipopolysaccharide used in combination with liposome encapsulated NDV administered to chickens via the intranasal route induced significant tracheal IgA and serum IgG levels, with increased levels of CD4+ and CD8+ cells, and 80% survival after virulent NDV challenge (Tseng et al., 2009). When polyinosinic:polycytidylic acid (poly I: C, TLR-3 ligand) was used in chicken embryo cells and exposed to NDV, it induced an antiviral state and reduced the plaque-forming capacity of the NDV (Gupta et al., 2014).

Another approach utilizing the TLR agonist effects is the use of oligodeoxynucleotides containing unmethylated CpG motifs (CpG ODN), which has also been proven to have immunostimulatory effects. CpG motifs are recognized as pathogen patterns by the innate immune system to activate defensive mechanisms and induce the immune response of immunized chickens (Vleugels et al., 2002). After internalization by target cells, CpG ODNs reach the late endosomal/lysosomal compartment where they signal by interacting with TLR-21 (chicken ortholog of TLR-9). After intramuscular and intranasal administration, CpG ODN enhanced the activity of a ND vaccine in chickens; increasing the systemic antigen-specific IgG levels in serum (T-cell proliferation), and mucosal (IgA) levels when administered intranasally. Specific-pathogen-free chickens co-vaccinated with ND vaccines and at least 100 μg of CpG ODN by either route were protected from challenge with an otherwise lethal dose of virulent NDV (Zhang et al., 2007, 2008). Vaccines containing CpG ODNs can be applied either systemically or via the mucosa, have good safety profiles, increase the immunogenicity of co-administered vaccines by improving the function of professional antigen-presenting cells, and boost the humoral and cellular vaccine-specific immune responses, highlighting their potential to be used as effective adjuvants for NDV and other poultry vaccines (Vleugels et al., 2002).

#### 3.4.3. Cytokine-expressing vaccines

The co-expression of immunostimulatory cytokines by virus vectors has been suggested to improve protective immunity induced by several avian vaccines (Armour and García, 2014). Chicken IFN-γ (chiINF- γ) is a macrophage activation modulator, inhibits viral replication, promotes development of the Th1 response by inhibiting Th2 cytokine production, and enhances antigen presentation and antigen processing and destruction of intracellular pathogens. This cytokine has been shown to improve protection and enhance immune responses in avian species against different avian pathogens; however, no commercial product has ever reached the market, and controversial results highlight the need for further evaluation of the potential of this cytokine (Cardenas Garcia et al., 2016). Cardenas Garcia et al. demonstrated that co-delivering chicken IFN-γ with a ND vaccine using three vaccination systems (DNA-vaccine administered *in ovo*,

recombinant vaccine expressing chiINF-γ used *in ovo*, and inactivated recombinant vaccine expressing chiINF-γ administered subcutaneously in two-week-old SPF chickens) did not improve the immunogenicity or the protective efficacy of the evaluated vaccine candidates (Cardenas Garcia et al., 2016). Expression of chicken IL-2 by a highly virulent strain of NDV led to decreased systemic viral load, but did not significantly affect mortality in chickens (Susta et al., 2015a), while expression of IFN-γ by the same virulent NDV attenuated the virus and decreased morbidity and mortality in SPF chickens (Susta et al., 2013).

Activation of TLRs or over-expressing certain cytokines result in the up- or down-regulation of various interleukins, chemokines and interferons, which in turn directs the immune response towards either a Th1 or Th2, or a mixed immune response. Some TLR combinations can produce a stronger and selective immune response, and others can down-regulate cytokine expression (Gupta et al., 2014). Cytokines are components of a fine-balanced network of immune responses with multiple feedback loops (Schat, 2015); thus, it is possible that inserting cytokine genes or using TLR ligands may deregulate the fine-tuning that exists in the naturally perfectly-timed system that leads to an enhanced immune response (Cardenas Garcia et al., 2016). In addition, the immunomodulatory effect of cytokines and TLR ligands may depend on various factors, such as the type of the pathogen, the amount and type of the co-delivered antigen, the relative time of administration, and the amount of delivered cytokine/ligand. Further studies to evaluate these approaches and the level of protection they induce in comparison to the existing traditional vaccines are necessary.

### 3.4.4. Chitosan as an adjuvant

The use of other adjuvants to enhance the immune responses induced by ND vaccines has also been investigated. Chitosan is a non-toxic, biocompatible, biodegradable polysaccharide derived from the exoskeleton of crustaceans and insects. It has been shown to improve the Th1 pathway of immunity by inducing a stronger and earlier peripheral cellular immune response with no effect on the systemic, lachrymal and digestive antibody-mediated immunity after oculo-nasal co-delivery with live ND vaccine in day-old chickens (Rauw et al., 2010a). The same scheme was also evaluated as a second vaccination after an *in ovo* vaccination with a rHVT-ND vaccine at day 18 of embryonation. The combination of rHVT-ND with mucosal co-delivery live NDV and chitosan demonstrated the best protection against mortality and morbidity, as well as the strongest reduction of virus shedding correlated to higher levels of cellular immunity and gastrointestinal antibody-mediated immunity in comparison to each of the vaccines used alone (Rauw et al., 2010b).

### 3.4.5. Nanoparticle vaccines

The rapid development of nanotechnology has provided various biodegradable nanomaterials that have become useful in vaccine research. In particular, nanoparticle systems have long been developed as vaccine delivery vehicles in human vaccines by providing protection from maternal antibodies and nucleases, leading to an interest in their use against animal pathogens (Chahal et al., 2016; Dai et al., 2015; Zhao et al., 2016b). The nanoparticle delivery systems protect the delivered antigen from disruption and have advantages such as higher antigen uptake, controlled release, and increased duration of responses (Dai et al., 2015; Zhao et al., 2016b). Many types of materials, such as polylactic acid, poly (lactide-co-glycolide), calcium phosphate, carboxymethylcellulose, chitosan, and magnesium phosphate among others, can be used as nanoparticle carriers and administered by multiple routes including oral, mucosal, and parenteral (Dai et al., 2015; Zhao et al., 2016b). Two chitosan derivatives, O-2′-hydroxypropyltrimethyl ammonium chloride chitosan and N-2-hydroxypropyl trimethyl ammoniumchloride chitosan, have been utilized to make nanoparticles as a mucosal delivery vehicle for live attenuated ND vaccines (Dai et al., 2015; Zhao et al., 2016b). In both systems the release of NDV was effective and sustainable and resulted in stronger cellular, humoral, and mucosal immune responses as measured by levels of specific humoral and local antibodies, spleen lymphocyte proliferation, and levels of cytokines. Both systems also conferred protection upon challenge with virulent NDV and no clinical signs or microscopic lesions were observed in vaccinated birds, while 20% mortality and some hyperplastic changes were observed in the chickens vaccinated with a traditional commercial ND vaccine.

Silver @$SiO_2$ and double hydroxide @$SiO_2$ nanoparticles have been developed for intranasal delivery of DNA ND vaccines (Zhao et al., 2015a, 2016a). In experiments with SPF chickens these $SiO_2$ nanoparticles showed very low toxicity, sustainable release after initial burst, and induced stronger cellular, humoral, and mucosal immune responses compared to the intramuscular administration of the same vaccine or the naked DNA plasmid. Both systems demonstrated 100% protection in chickens after challenge with the virulent F48 NDV strain.

Great potential lies in nonretroviral mRNA-based vaccines. Recently, a delivery system required to deploy conventional unmodified replicon mRNAs based on the genomes of Venezuelan equine encephalitis virus (VEEV) as a vaccine was developed (Chahal et al., 2016). This system supports self-amplification via a double-stranded RNA intermediate in the cytoplasm to drive efficient expression of the immunogenic antigen. Single dose modified dendrimer nanoparticle (MDNP)-delivered VEEV replicon RNAs encoding single or multiple pathogen proteins were shown to protect mice against lethal infection of H1N1 influenza virus (A/WSN/33), Ebola virus or *Toxoplasma gondii*. This synthetic highly innovative, adjuvant-free system is flexible and can multiplex (co-formulate and co-express) different antigen-expressing replicons (i.e. simultaneously raise immunity against multiple antigens from a single disease and/or multiple antigens from multiple diseases). Moreover, it induces vital antigen-specific CD8+ T-cell and antibody responses without additional adjuvants (Chahal et al., 2016). While the system has not been tested in chickens, some of its main advantages are that the design and production of the vaccines take only 2 weeks, do not require high biosecurity level facilities, and allow for a rapid and on-demand response to currently circulating pathogens, including highly diverse NDV strains.

### 3.4.6. Virus-like particle vaccines

Virus-like particles (VLP), although available for a long time, are increasingly being considered as viral vaccines. VLPs are formed by the assembly of viral structural proteins and lipids, but without the incorporation of the viral genome and may offer significant advantages over many currently used or developing vaccine technologies: i) they are not infectious and cannot spread infection; ii) there is no chance of reversion of virulence or recombination; iii) they mimic the structure of the infectious virus; iv) they are very immunogenic; and v) stimulate both humoral and cellular immune responses (McGinnes et al., 2010; Morrison, 2010). Many different paramyxovirus VLPs can be produced upon expression of the M protein or M protein with various combinations of the glycoproteins and NP after the transfection of cells with vectors containing cDNAs encoding for these proteins, with ND VLPs having high efficiency of release (Morrison, 2010). Newcastle disease VLPs, although not being commercialized, have a potential to incorporate glycoproteins of different strains of NDV, could potentially be used against different ND viruses (McGinnes et al., 2010), and have significant potential applications as they can be designed to express protein sequences from many pathogens (Morrison, 2010).

One aspect of vaccine production that has been addressed in many, but not all areas of the world, is the quality assurance standards for the production and testing of ND vaccines (Gallili and Ben-Nathan, 1998). The Code of Federal Registration, Title 9, from the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) (sections 113.329 and 113.205), the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (sections 1.1.8, and 2.3.14) and Principles of Vaccine Production from the OIE (OIE, 2012, 2015), and Council Directives 92/66/EEC, and 93/152/EEC from the Commission of European Communities regulate the development and testing of ND vaccines to ensure that only reliable, safe, and effective products are available. However, as recent as 2012, ND vaccines illegally transported from Mexico, intended for use in fighting gamecocks in California, USA, were confiscated at the border between the countries and shown to be contaminated with virulent NDV, in addition to the vaccine NDV strain (Pedersen et al., 2013).

## 4. Conclusions

Extensive use of currently available vaccines, strict quarantine combined with rapid diagnostics and biosecurity, and stamping out and other containment measures seem to keep ND under control in developed countries. However, as evident from the multiple outbreaks occurring worldwide, current vaccination strategies are not fully efficacious under different environmental conditions and the development of new concepts for vaccine generation are needed. To enhance the efficacy of vaccines and to improve the immune responses induced by them, investigation of innovative approaches together with the development of safe and novel strong adjuvants are necessary. Future ND vaccine systems that allow rapid development to target emerging NDV strains, and enable design of multiplexed vaccines, will have advantage over currently existing vaccines.

## Conflict of interest

None.

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