Back to the past: do vector vaccines represent the future?

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Summary

The intensive production of poultry meat and eggs depends to a significant degree on the use of vaccines. Until the development of recombinant DNA technology, these vaccines consisted of inactivated pathogens or attenuated live pathogens. In general, the traditional, live vaccines have provided, and continue to provide, poultry with good to excellent protection. However, the use of live attenuated vaccines can have some negative consequences such as vaccine-induced mild reactions [e.g., Newcastle disease virus (NDV) vaccines] or even cause disease outbreaks [e.g., vaccinal laryngotracheitis after vaccination with chicken embryo origin vaccines for infectious laryngotracheitis (ILT)]. Another concern is the possible reversal to pathogenicity by mutations or recombination between field and vaccine strains. The advance of recombinant DNA technology raised great expectations for the development of vaccines lacking these potential problems. The United States Department of Agriculture (USDA) recognizes three categories of recombinant vaccines. The first one consists of inactivated products such as virus-like particles (VLP). The USDA is currently evaluating the licensing request for a VLP vaccine against infectious bursal disease. The second category consists of generating deletions in the genome of pathogens rendering these pathogens apathogenic while maintaining the ability to infect the host resulting in immunity. Current efforts are focused on generating ILT vaccines. The third category consists of vectored vaccines, in which one or more genes of a pathogen are inserted in a vector such as herpesvirus of turkeys (HVT), fowlpox virus (FPV) and the LaSota strain of NDV. Depending on the pathogen protection by the vectored vaccine will be strong (e.g., vectored vaccines against infectious bursal disease), while other vectored vaccines have not lived up to the expectations. In this paper I will provide a brief overview of the development of vaccine-induced immunity by inactivated and live vaccines, current traditional vaccines and the current state-of-the-art of recombinant vaccines.

Introduction

The production of poultry meat and eggs is forecast to increase between 2011 and 2050 from 1.81x10^6 to 3.72x10^6 tons of meat and from 6.5x10^6 to 1.02x10^7 tons of eggs, which represent increases of 77 and 65%, respectively (43). This increase will only be feasible if exposure to pathogens is successfully controlled by a combination of strict biosecurity, improved genetic resistance to pathogens and the use of appropriate vaccines. Although this review will focus on the development and use of traditional vaccines and the different types of recombinant vaccines, the importance of strict biosecurity needs to be strongly emphasized. The best vaccine will not provide optimal protection when chicks are exposed to severe challenge with many pathogens from the moment they are placed in the chicken house or if the newly hatched chicks are already exposed to pathogens through vertical transmission in the absence of maternal antibody protection.

Traditionally, vaccines consist of inactivated products or modified live pathogens or related nonpathogenic agents. Examples of the latter are serotype 2 and 3 Marek's disease (MD) vaccines. Since the development of recombinant DNA technology in the early 1980s it was clear that recombinant vaccines could become an important new tool to protect animals including poultry against infectious diseases (10). Anticipating this development the Center for Veterinary Biologics (CVB) of the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) developed guidelines for the evaluation of biotechnology products in 2003 (48). Shortly afterwards the first recombinant vaccine was licensed in the USA against pseudorabies virus (suid herpesvirus 1) in which two genes, Tk
and glycoprotein III, were deleted. This vaccine was successfully used to eradicate Aujeszky’s disease in the USA (reviewed in 43).

This review will focus mostly on viral vaccines with the exception of Salmonella typhimurium vaccines based on deletion of genes for two pathogenicity islands adenylate cyclase and cAMP receptor protein (7). I will discuss 1) the use of poultry vaccines and vaccine-induced immune responses, 2) the development, use, and problems of traditional live and inactivated vaccines, 3) the development, use, and problems of current vectored vaccines and 4) expected future developments of recombinant vaccines. Finally, I will give my opinion on the future of recombinant versus traditional vaccines comparing the current use of traditional and genetically engineered vaccines with a conference paper I published in 2001 (39).

General aspects for the use of poultry vaccines
The first requirement for poultry vaccines is that they are cheap. For the average company in the USA, the total cost/lb meat for feed and chick cost was 30.5 cents in January 2015 while the cost for all vaccines and medications was 0.07 cents (49). Based on the very small profit range/lb of meat, a producer will often base the choice for a specific vaccine on the cost-benefit analysis comparing the costs of a possible increase in condemnations and disease versus the increase in vaccination costs.

A second important factor is the ease of application of a vaccine. The advance of automated in ovo vaccination had a major impact in the broiler industry. In 2007, 90% of the broilers in the USA were vaccinated in ovo and in ovo vaccination was used in 26 of the top 30 poultry-producing countries (2). I expect that the use of in ovo vaccination has further increased since 2007. Thus any new traditional or recombinant vaccine for broilers needs to be comparable or superior in inducing protective immunity, comparable in price to existing vaccines, and preferably be approved for in ovo vaccination.

Important general aspects of vaccine-induced immune responses
The choice of a vaccine is not only based on the price and delivery method but also on the type of immune responses that are required, the age of the bird at vaccination in relation to the presence of maternally-derived IgY antibodies (MDA) and the complexity and pathogenesis of the pathogen for which protection is required.

It is well known that immune responses to pathogens are complex consisting of early activation of innate immunity followed by the development of specific humoral and cell-mediated immune responses. The innate immune responses form not only the first line of defense to the invading pathogen, but are also of critical importance for the initiation of the acquired immune responses. The development of antibodies requires the presentation of the relevant antigenic epitopes to B lymphocytes by antigen-presenting cells (APC) in the context of class II major histocompatibility antigens (MHC-II). This process does not require the generation of de novo antigens, in contrast to the generation of cell-mediated immune (CMI) responses, which require de novo synthesis of antigens for the presentation to T cells in the context of MHC-class I antigens. The consequence of this dichotomy is that the generation of strong CMI responses requires the use live vaccine in contrast with inactivated vaccines which generate only strong antibody responses. This difference applies to both traditional and recombinant vaccines. The practical aspects of poultry vaccinations including the generation of acquired immunity were recently reviewed by Schijns et al. (44).

The presence of MDA is an important part of the protective immunity of chicks up to 3 weeks of age when the levels of MDA are becoming too low to protect against infection. For example, the generation of high levels of MDA is essential for the protection of newborn chicks to clinical chicken infectious anemia (42). However, the presence of maternal antibodies can also interfere with proper vaccination especially if life vaccines need to replicate systemically such as infectious bursal disease (IBD) virus (IBDV) vaccines. On the other hand, MDA may have relatively little impact if life vaccines are administered to and replicate in the upper respiratory tract, where there is little or no MDA present.
A third important aspect in the selection of a specific vaccine relates to the nature of the pathogen. Protection against some pathogens (e.g., IBDV) is achieved by the induction of a strong IgY response against viral protein (VP)2. More complex pathogens such as Marek’s disease (MD) virus (MDV) require vaccine-induced cytotoxic T lymphocyte (CTL) responses, which can be directed to a relatively large number of viral proteins and glycoproteins (30, 34). The consequences of these differences are two-fold. If protective immunity is based on immune responses to a single antigen, recombinant vectored vaccines are relatively easy to develop. On the other hand, if multiple antigens are needed to generate a strong protective immunity vectored vaccines may be more difficult to develop. Understanding the basic pathogenesis of the pathogens is also important in the selection of a vaccine and vaccination program. Protection against a pathogen replicating in the epithelium of the respiratory or intestinal tract may depend to a significant degree on the development of an IgA response. In general vaccines that are injected generate an IgY response rather than an IgA response while vaccines given by spray, eye drop, or drinking water may generate better IgA responses especially if the vaccine replicates in the epithelium.

Traditional vaccines
Traditionally, live virus vaccines are typically developed by isolation of a virus in embryonated chicken eggs or cell cultures followed by passages in either substrate to further adapt the virus to the substrate and to attenuate the pathogenicity (reviewed in 9). This approach has served the poultry industry well and continues to serve the needs of the industry. However, there are some problems associated with this empirical approach. First of all, the process of attenuation is poorly understood at the molecular level and residual pathogenicity can cause some vaccination reactions. There is also the concern that vaccines can revert back to pathogenicity. Infectious laryngotracheitis (ILT) chicken embryo-origin (CEO) vaccines are a classic example of causing problems in the field (12). In the USA the vaccine is typically administered by spray or drinking water. The former can cause vaccination reactions especially if the virus is not fully attenuated. Moreover, the vaccine virus can go latent and subsequently recirculate, increase in pathogenicity and may cause vaccinal laryngotracheitis. In addition, recent evidence suggests that recombination of ILT vaccine strains with field strains can occur causing additional problems (27). Recombination events have also been reported for vaccine strains as well as field strains of infectious bronchitis virus (IBV) (18, 23). Finally, inter- and intra-segment homologous recombination events have been reported for IBDV (reviewed in 11). These publications do point to the potential problem of generating reassortments between vaccine and fields strains for at least three different pathogens, but it is not clear how important these risks are for the poultry industry.

The large majority of the live poultry vaccines against viral diseases consists of cell-free virus preparations. The exceptions are the three Marek’s disease vaccine strains CVI-988 (serotype 1 MDV), SB-1 (serotype 2 MDV) and herpesvirus of turkeys (HVT), which are cell-associated vaccines requiring vaccination with live infected cells. Only HVT can also be used as a cell-free vaccine, but the lyophilized version is used mostly to vaccinate back-yard flocks and seldom, if ever, for the vaccination of commercial flocks. The advantage of the cell-associated MD vaccines compared to cell-free vaccines is the resistance to neutralization by MDA (41). This is also one of the reasons why MD vaccines are useful as vectors for recombinant vaccines (see below).

In addition to the live anti-viral vaccines, there are many inactivated vaccines available, which are mostly made in embryonated chicken eggs although some IBD vaccines are made from bursae after infection of young chicks (17). As mentioned before, inactivated vaccines only induce antibody responses and are often given as a booster vaccine after vaccination with a live vaccine. The use of adjuvants is an essential part of inactivated vaccines to increase the antibody responses. The majority of the adjuvants in poultry vaccines are oil-based (44), but with the increased knowledge of the initiation of immune responses new approaches are being developed. Inclusion of Toll-like receptor (TLR)
ligands to stimulate immune responses is actively pursued for poultry vaccines (14, 44) although to date there are no vaccines with TLR-ligands available for commercial use.

Recombinant vaccines

Introduction
The USDA recognizes three categories of recombinant products that can be used for production animals including poultry (48). Category I-A consists of bacterins, killed viruses and subunit vaccines. Only the latter is of importance for this paper. Category I-B-1 and -2 includes monoclonal antibodies and expressed proteins for therapeutic use and diagnostic kits which falls outside the scope of this paper. Category II covers gene-deleted vaccines. Live vectored vaccines, in which gene(s) are inserted in a vector, are covered in category III. I will refer to the inserted gene(s) as transgene(s) in this paper.

DNA vaccines have attracted a lot of attention because plasmids coding for specific parts of a pathogen, and thus not the complete sequence of that pathogen, are able to induce protective immunity. DNA vaccines are currently of little practical value for poultry for two reasons. First and foremost, there is considerable public concern about the safety of DNA vaccines in production animals, which is probably not justified. Thus far, the only DNA vaccine approved for use in production animals is used to vaccinate Salmonids and is allowed only in British Columbia, Canada (reviewed in 43). The second reason is that experimental use of DNA vaccines suggests that more than one injection is needed to generate solid protection against challenge, which is highly impractical in poultry especially in broilers. It is, of course, possible that future developments will show that DNA vaccines can be used successfully in commercial poultry especially if new adjuvants like TLR ligands can be included.

Category 1A vaccines
The subunit vaccines are the only interesting group in this category. Genetically altered killed virus vaccines or bacterins with or without recombinant products are basically the same as traditionally killed virus or bacterial vaccines. Subunit vaccines can consist of proteins or virus-like particles (VLP), which resemble virus particles but lack the DNA or RNA viral genome. To my knowledge, there are currently no recombinant protein vaccines used in poultry or other production animals with the exception of a classical swine fever virus vaccine based on the envelope glycoprotein E2, which is used in Europe (43). One of the problems with the development of recombinant protein vaccines is that proper folding of the proteins to express tridimensional epitopes remains problematic. In contrast, VLP express the relevant epitopes in the proper way. The VLP vaccines are typically made using the baculovirus system and therefore can also contain replication-competent baculovirus. This is most likely not a problem in chickens, but may cause some problems with the standardization of the amount of VLP in the vaccine. Careful titration of VLP probably by using antigen-capture ELISA or other techniques will be essential and feasible.

Currently, a VLP-based vaccine against porcine circovirus (Mreck) is commercially available (reviewed in 43). Jackwood (17) recently showed that multivalent VLP expressing VP2 from classic and variant IBDV strains protected against challenge with both strains. It is expected that this product will be licensed in the near future for use in the USA (D. Jackwood, personal communication 2015; http://laradinc.com/).

Category 2 vaccines
Currently, live Salmonella typhimurium (St) vaccine with deletions are being used as vaccines. St lacking two pathogenicity islands, cAMP receptor protein and adenylate cyclase, was developed by Curtis and Hassan (7) and is produced by Lohman Animal Health (now part of Elanco). Stocker (46) developed aro A-negative St mutants, which have been commercialized by Zoetis. The latter company has also a live bacterial vaccine E. coli with the aro A deletion (Poston, personal communication, 2015). Following Soncini (personal communication 2015) Salmonella deletion mutant vaccines are also available in Brazil through Merial and LAH. The deletion mutants of St can be used as vectors especially
against intestinal pathogens as has been shown experimentally with a recombinant vaccine against *Eimeria acervulina* (22) and avian influenza (24), although this approach has not yet been used commercially.

Two candidate deletion mutant vaccines have been generated for ILT. ILT-ΔgJ (31) and ILT-ΔgG (6, 28) have shown protection in challenge experiments. Both candidate vaccines can be administered *in ovo*. Glycoprotein G is of interest because it interferes with innate immune responses by binding chemokines, while gJ is important for viral egress (reviewed in 6). Immune responses to both vaccines can be differentiated from exposure to field virus, thus allowing the use of differentiating infected from vaccinated animals (DIVA) strategies. Additional deletion mutants have been reported but need further studies to demonstrate protective efficacy (5). The ILT-ΔgJ deletion mutant is not completely attenuated causing some mortality in SPF chickens after *in ovo* vaccination, which has reduced the interest in commercialization (Garcia, personal communication 2015).

Since the introduction of MD vaccines in the early 1970s MDV field strains have become more virulent over time, which led to the use of bivalent (HVT+SB-1) vaccines later followed by the HVT+CVI988 combination (40). A potential additional vaccine strain was developed by Lee et al. (25, and references therein) using the 19th passage of the very virulent MD5 strain in which the Meq gene has been deleted (rMD5ΔMeq). Following the authors this virus would provide better protection than CVI988, the current gold standard for MD protection. However this passage also causes severe thymic and bursa atrophy in MDA negative, genetically susceptible chicks. Additional cell culture passages eliminated this problem and passage 50 was reported to be safe and still protective (26). However, at this time there is no interest from the industry to commercialize rMDVΔMeq (Fadly, personal communication, 2015).

**Category 3 vaccines**

*Introduction.* This category is often referred to as vectored vaccines and in contrast to category 1 and 2, the commercial use of the vectored vaccines has exploded since the first demonstration that fowl pox virus (FPV) and HVT could be used as vectors to express genes of Newcastle disease virus (NDV) and protect against challenge (4, 33). Armour and Garcia (1) recently published an extensive review on the use of vectored vaccines, which is available through the Department of Population Health at the University of Georgia. HVT is possibly the most widely used vector, but FPV-vectored vaccines are also used as is the LaSota NDV strain. In addition, there are many publications describing the use of other vectors including transgenic plants, bacteria in addition to *St* (e.g., *Lactobacillus*) and viruses such as replication-competent fowl adenovirus (FAdV/9) (29) and replication-competent adenovirus (RCA)-free recombinant human adenovirus serotype 5 (32). Both adenoviruses have been used experimentally as vectors for avian influenza (AI) vaccines. The former may become a commercial vaccine based on current research by Avimex (Mexico City) in collaboration with Dr. Nagy (University of Guelph). However, the deletions at the left end of the FAdV/9 genome reduce the replication *in vivo* compared to wild-type FAdV/9 (8) and, as a consequence, may decrease the importance as a vector. The latter one has been evaluated by *in ovo* vaccination, titers as high as $10^9$ infectious units did reduce but not prevent virus shedding (32). Although originally seen as a highly promising approach for *in ovo* immunization (2), the human adenovirus as a viral vector was not commercialized (R. Poston, personal communication 2015).

*Important considerations in selecting a vector.* There are a number of practical considerations which may influence the general use or specific selection of a vectored vaccine. Probably the most important consideration is the effect of the deletion of vector gene(s) and the subsequent insertion of the transgene on the replication *in vitro* (cell cultures or embryonated chicken eggs) and *in vivo* (the vaccinated birds). The former is important because a decrease in replication will increase the cost of the vaccine which may make the vectored vaccine economical uninteresting. A decrease in *in vivo* replication may affect the immunity against the vector. For example, if HVT is used as the vector for VP2 of IBDV, a
rapid induction of nonspecific (natural killer cells, NO, etc.) and HVT-specific CTL immune responses is of crucial importance for the MD protection especially when early challenge is expected. On the other hand, protection against IBD needs to be present at the time that IBDV-specific MDA no longer protect against challenge. If there is a delay in the replication of the HVT vectored vaccine, MD protection by the vectored vaccine may be compromised and full MD protection by e.g., CVI988 will become even more important (15). This concern is probably of less importance in broilers than in breeders and layers. In this context it is also important to note that rHVT vaccines cannot be combined with HVT because HVT may replicate faster than the rHVT and therefore reduce the effectiveness of the rHVT vaccine. For the same reason it is not recommended that two different rHVT vaccines are used in the same birds. Thus careful evaluation of possible differences in in vivo replication is an important aspect of the development of rHVT vectored vaccines (15).

A second important factor is the susceptibility of the vector and the expressed transgene to antibodies and cell-mediated immunity (CMI). This seems to be especially important for the use of FPV as a vector. Both CMI and antibodies, including MDA, to FPV are important and can interfere with the replication of FPV (12) and impair the immune response to the transgene. Swwayne et al. (47) examined the importance of prior vaccination with FPV on the efficacy of rFPV-H5 and found a lack of consistent protection against HPAI. They concluded that this may limit the use of rFPV to chickens without previous fowl pox vaccinations. In addition, prior exposure to field fowl poxvirus could be expected to limit protection induced by this vaccine. CEVA-Biomune actually states on their webpage for the use of rFPV that “Chickens and turkeys receiving this vaccine must not have been previously exposed to fowl poxvirus either by vaccination or field exposure.” Because HVT is cell-associated, the susceptibility to neutralization by MDA is minimal and because vaccination with HVT is performed in ovo or at one day of age, anti-HVT CMI responses are not present at the time of vaccination. At this time there is little information available on interference of MDA with Lasota vectored vaccines, but if the vaccine is used by spray, drinking water or eye drop there may be little or no interference by MDA.

A third important aspect is the application of the vectored vaccine. For broilers the in ovo application of vaccines is almost a requirement in the USA (2) and also in Brazil (Ibiara Almeida Paz, personal communication 2015; Soncini, personal communication 2015). All HVT and some but not all FPV vectored vaccines have been approved for in ovo vaccination. In ovo vaccination may not be feasible for Lasota-based vaccines because this may cause embryo mortality unless specific mutations are made in the F and HN genes as suggested by Ramp et al. (35). On the other hand, this type of vaccine may be applied by aerosol or other mass vaccination approaches.

As mentioned before in the section “Important general aspects of vaccine-induced immune responses,” the complexity of the pathogen against which the transgene needs to induce protection is also important. Both HVT and FPV vectors will induce cell-mediated and humoral immune responses and most likely both types of responses will also be generated to the expressed transgene. Unfortunately, the relevant antigens involved in CMI are poorly characterized for many pathogens. This lack of basic knowledge complicates the selection of appropriate genes for insertion in vectored vaccines if CMI is crucial for protective immunity.

On the other hand, if antibody responses, especially of the IgY class, are the key to protection, vectored vaccines are “easy” to develop and expected to be successful.

The selection of the proper promoter to drive the expression of the transgene is also important. Overexpression of the transgene by using a strong promoter may actually decrease protection in the presence of MDA against the transgene product (16, 45). This is not a practical concern for the selection of vectored vaccines by the poultry industry, because it is assumed that the manufacturers have selected the correct promoter to prevent this problem.

Commerically available vectored vaccines. Table 1 summarizes currently available vectored vaccines in the Americas. Based on analysis of the websites of vaccine companies combined with personal communications to verify that the current information is correct,
three US vaccine manufacturers produce vectored vaccines (Ceva-Biomune, Merck Animal Health, and Merial), while Lohman Animal Health (Elanco) and Zoetis do not produce vectored vaccines. A LaSota-based vaccine against H5 avian influenza is produced by Avimex in Mexico (38). Interestingly, the antigen-antibody complex vaccine (Transmune, CEVA-Biomune) against IBD is preferred over the HVT-vectored vaccine against IBD. Transmune is delivered in ovo or at one day of age (Soncini, personal communication, 2015).

### Table 1. Vectored poultry vaccines available in the Americas\(^{1,2}\)

<table>
<thead>
<tr>
<th>Company</th>
<th>Vector</th>
<th>Targeted pathogen</th>
<th>Available in</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Brazil(^3)</td>
</tr>
<tr>
<td>Avimex</td>
<td>LaSota</td>
<td>AIV H5</td>
<td>No</td>
</tr>
<tr>
<td>Ceva-Biomune</td>
<td>FPV</td>
<td>ILTV, MG, NDV</td>
<td>Yes(^3)</td>
</tr>
<tr>
<td></td>
<td>HVT</td>
<td>IBDV, ILTV, NDV</td>
<td>Yes(^3)</td>
</tr>
<tr>
<td>Merck Animal Health</td>
<td>HVT</td>
<td>ILTV, NDV</td>
<td>Yes</td>
</tr>
<tr>
<td>Merial</td>
<td>FPV</td>
<td>NDV</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>FPV</td>
<td>AIV H5</td>
<td>No</td>
</tr>
</tbody>
</table>

\(^1\)Based on their websites and confirmed by personal communications.  
\(^2\)Abbreviations: AIV=avian influenza virus, FPV=fowlpox virus, HVT=herpes-virus of turkeys, IBDV=infectious bursal disease virus, ILTV= infectious laryngotracheitis, LaSota=vaccine strain for NDV, MG=Mycoplasma gallisepticum, NDV=Newcastle disease virus.  
\(^3\)Some but not all products are available in Brazil  
\(^4\)Licenced in the USA for emergency use only. Current USDA regulations do not allow vaccination of chickens against avian influenza.

Theoretical advantages and disadvantages of vectored vaccines over traditional vaccines  
The advantages of vectored vaccines providing protection against two or more diseases are:  
1) Reduction in the number of vaccinations thus less stress for the chickens,  
2) Reduced risk of adversary vaccine reactions as can be caused by traditional vaccines,  
3) Absence of reversal to virulence especially with the HVT vectored vaccines because HVT is nonpathogenic in chickens,  
4) Ability to vaccinate in ovo using the HVT- and some FPV-vectored vaccines and  
5) Vaccinate in the presence of MDA against the transgene product.

There are also disadvantages such as:  
1) The limited number of inserts in a vector (one to two inserts for HVT and potentially a few more for FPV), which may cause insufficient protection against complex pathogens,  
2) Interference of immunity to FPV-vectored vaccines when birds are already exposed to FPV,  
3) Short protection duration after vaccination with FPV-vectored vaccines (15),  
4) The inability to use more than one HVT-vectored vaccine or HVT + HVT-vectored vaccine in the same birds,  
5) Slower in vivo replication of the vectored vaccine compared to the original vaccine, which may be the explanation for the inability to combine the different HVT vaccines (15).

Comparative studies of traditional versus vectored vaccines  
There are few comparative studies in peer-reviewed journals evaluating traditional versus vectored vaccines.  
Vagnozzi et al. (50) vaccinated broilers with traditional or vectored ILT vaccines (see Table 2 for details) and challenged the birds at 35 and 59 days of age. Vaccine doses were adjusted to the typical doses used in the USA. Protection was evaluated based on prevention of clinical signs, reduction in virus replication of the challenge virus, and maintenance of body weight gain. Key results for 5 days post challenge at 35 days of age are summarized in Table 2. The results at 59 days of age are of less interest, because broilers are typically going to the processing plant before 59 days of age. However, the level
of protection had improved when birds vaccinated with the Innovax vaccines were challenged at 59 days, suggesting that the development of complete immunity to ILT required more than 30 days. This raises an important question concerning the concomitant immunity to MD compared to the HVT vaccine. Challenge experiments to demonstrate MD protection are complex, but measuring the HVT copy numbers at 14 days of age in the feather follicles can provide an indication how many birds reached the threshold level of HVT copies associated with protection (3).

In an earlier study by the same authors (19) commercial broiler embryos were vaccinated at 18 days of embryonation (DOE) with rHVT-LT (Innovax ILT, Intervet-Scherwing-Plough) or FPV-LT (Vectormune FP-LT, CEVA-Biomune) and non-vaccinated hatchmates were vaccinated at 21 days of age (DOA) with the CEO vaccine Trachivax (Intervet-Scherwing-Plough) or mock vaccinated. All birds were challenged at 37 days of age. At 5 days post challenge the CEO vaccinated birds had a clinical score of 0, while the scores for rHVT-LT and rFPV-LT were 4.0 and 2.0, respectively, while mock vaccinated birds had a score of 6.0. The differences between the CEO, rFPV-LT and rHVT-LT were all significant while the difference between rHVT-LT and the mock-vaccinated birds was not significant. The viral load of the challenge virus was not significantly different between the birds that received rHVT-LT, rFPV-LT or were mock vaccinated, while the virus load in CEO-vaccinated chickens was significantly lower. It is not clear why the results are reversed for the rHVT-LT and rFPV-LT in these two studies. Perhaps the interpretation of clinical signs is an inexact science for ILTV challenge studies or protection provided by the two recombinant vaccines may be variable. Based on these two publications it will be difficult to make a choice between the two recombinant vaccines.

Recently, two groups showed that vaccination with LaSota NDV expressing gD (21, 51) or gB (51) of ILTV protected against challenge. Interestingly LaSota-gB did not protect in the study by Kanabagatte Basavarajappa et al. (21) but did in the study by Zhao et al. (51). It will be interesting to determine how these potential vaccines compare with the HVT- and FPV-vector vaccines.

Gimeno et al. (13) examined the replication of HVT and recombinant HVT expressing gD and gI of ILTV (rHVT-LT) in lung, spleen, and feather pulp. Vaccines were titrated before use. It is important to note that plaques were detected at 3 and 5 days post inoculation for HVT and rHVT-LT, respectively, indicating that the in vitro replication rate of the recombinant virus was delayed. SPF and commercial broiler embryos and chicks were vaccinated at 18 and 19 DOE or at 1 DOA. The authors concluded that there were no significant differences at 7 and 14 days post vaccination between the two vaccines and that the route of vaccination also did not influence the outcome. Unfortunately the two types of vaccines were obtained from different, non-identified manufacturers, which prevents drawing definitive conclusions because the in vitro passage levels may have been

Table 2. Key results of a comparative vaccination trial for ILT vaccines published by Vagnozzi et al (50)

<table>
<thead>
<tr>
<th>Vaccine</th>
<th>Manufacturer</th>
<th>Route of vaccination</th>
<th>Challenge at day 35</th>
<th>Protection at 5 dpc based on</th>
<th>Weight gain between day 35 and 42</th>
<th></th>
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<tr>
<td>PBS</td>
<td>NA</td>
<td>NA</td>
<td>No</td>
<td>0.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>-0.27&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>PBS</td>
<td>NA</td>
<td>NA</td>
<td>Yes</td>
<td>5.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.22&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>HVT io</td>
<td>Merial</td>
<td>18 DOE</td>
<td>Yes</td>
<td>6.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.63&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>HVT sc</td>
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<td>1 DOA</td>
<td>Yes</td>
<td>6.4&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>CEO Trachivax</td>
<td>Intervet-SP</td>
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<td>0.0&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.25&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HVT-LT io Innovax ILT</td>
<td>Intervet-SP</td>
<td>18 DOE</td>
<td>Yes</td>
<td>1.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HVT-LT sc Innovax ILT</td>
<td>Intervet-SP</td>
<td>1 DOA</td>
<td>Yes</td>
<td>0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.41&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.32&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PPV-LT io Vectormune</td>
<td>Ceva</td>
<td>18 DOE</td>
<td>Yes</td>
<td>4.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.56&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.22&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>PPV-LT sc Vectormune</td>
<td>Ceva</td>
<td>1 DOA</td>
<td>Yes</td>
<td>3.9&lt;sup&gt;c&lt;/sup&gt;</td>
<td>3.70&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.30&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>NA—Not applicable. DOE—days of embryonation, DOA—days of age.
<sup>b</sup>Clinical scores are approximate values estimated from Fig 1a in (50). Values with a different superscript are significantly different.
<sup>c</sup>Viral load measured by real-time PCR. Values with a different superscript are significantly different.
<sup>d</sup>Approximate average weight gains in kilograms during the 7 day period estimated from Fig 2 in Vagnozzi (50). Values with a different superscript are significantly different.
quite different which may have influenced the \textit{in vivo} replication rate. In addition, it would have been of interest to look at time points between 3 and 7 days post vaccination to more precisely analyze the early replication rates of both vaccines.

\textbf{Future possible developments: good and bad (strictly my opinion)}
There are several publications suggesting that incorporation of specific cytokine genes can enhance protective immunity (see 1 for references). Thus far, the results have not been encouraging, which is not surprising in my opinion. The cytokine network is a fine-tuned network with numerous feedback loops (20). By inserting a specific cytokine in a vaccine this network may become deregulated resulting in all kind of unexpected effects. As mentioned earlier, a better approach would be to include ligands for the TLR, which are essential to initiate innate and acquired immune responses.

Thus far, there are only a few vectors that are appropriate for use in poultry and each vector has a limited number of insertion locations. It may be possible to mix rFPV and rHVT vaccines together for \textit{in ovo} vaccination to provide protection against several diseases, but careful evaluation of this approach will be needed especially if products from different manufacturers are combined. There are some suggestions to use CVI988 and SB-1 MDV vaccine strains as vectors in addition to HVT. In my opinion, this is not a good idea especially if HVT-vecorred vaccines induce a suboptimal protection against MD. We will then need the best possible CVI988 and SB-1 vaccines (probably with the lowest cell culture passages available) to protect against MD. Moreover, based on the fact that HVT cannot be combined with rHVT vaccines I expect that the same will be true for CVI988 and SB-1.

Several publications have suggested that the combination of rHVT-NDV \textit{in ovo} or at 1 DOA with live NDV vaccines would increase the protection against challenge with NDV (36, 37). This is an interesting suggestion, but it also raises some questions. The first question is why is this combination needed if the recombinant vaccine works so well? The second questions relates to the potential negative effects of live traditional vaccines, which is one of the main reasons to use vectored vaccines.

\textbf{My conclusions}
Vectored vaccines are here to stay and VLP vaccines and deletion mutant viral vaccines are on the horizon. The choice to use a vectored vaccine or a traditional vaccine will depend on a number of scenarios. In general vectored vaccines against “simple” pathogens (e.g., IBDV) will be successful, but keep in mind that MD protection cannot be compromised! The situation may be different for more complex pathogens. The two papers comparing ILT vaccines indicate that recombinant vaccines for ILT may not provide strong enough protection and perhaps deletion mutant vaccines will become the future for protection against this disease. Finally, any recombinant vaccine must be cheap like traditional vaccines to provide a positive balance between price and improved protection. Any new developments like improved adjuvants, use of ligands for TLR must be based on this principle.

Compared with my 2001 paper (39) vectored vaccines have become widely used since 2001, but some of the promises such as the insertion of cytokine genes in vectored vaccines and the use of DNA or plant-based vaccines have remained promises for the future. It is likely that there will be a place for both traditional and recombinant vaccines in the foreseeable future.

\textbf{Disclaimer:} Mentioning of specific products or vaccine manufacturers does not imply an endorsement of the products or the manufacturer.

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