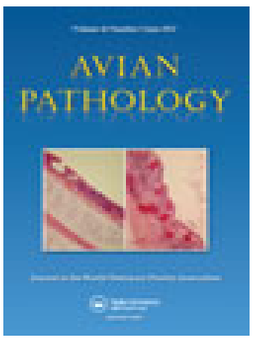

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REVIEW ARTICLE

Challenges and recent advancements in infectious laryngotracheitis virus vaccines

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Over the past 80 years, biosecurity measures and vaccines have been used to prevent the occurrence of outbreaks of infectious laryngotracheitis (ILT). Despite these control strategies, ILT continues to have an impact on intensive poultry industries. Attenuated vaccines, particularly those derived by passage in chicken embryos, have been associated with a number of side effects, including residual virulence, transmission to naïve birds, establishment of latent infections with subsequent reactivation and shedding of virus, and reversion to virulence after *in vivo* passage. Most recently, recombination between attenuated ILT vaccines in the field has been shown to be responsible for the emergence of new virulent viruses that have caused widespread disease. To address some of these issues, new-generation virally vectored recombinant vaccines have been developed and recently released in some countries. In addition, recombinant deletion mutants of ILT virus have been proposed as vaccine candidates. In this review, recent advances in the understanding of the epidemiology of traditionally attenuated ILT vaccines as well as in the development and use of new generation vaccines are examined. Next-generation vaccines, along with more appropriate immunological screening strategies, are identified as particularly promising options to enhance ILT control in the future.

Background

Infectious laryngotracheitis (ILT) is an upper respiratory tract disease of chickens caused by infectious laryngotracheitis virus (ILTV; *Gallid herpesvirus 1*), a member of the sub-family *Alphaherpesvirinae* (genus *Iltovirus*) (Davison, 2010). This virus is only transmitted horizontally, and primarily infects the conjunctiva and tracheal mucosa, causing inflammation, serous or mucous discharge, coughing and dyspnoea, as well as decreased egg production and/or weight gain. Typically, outbreaks result in high morbidity (90 to 100%) and variable mortality (5 to 70%), although the latter is usually around 10 to 20% (Guy & García, 2008; Devlin *et al*., 2011). During the lytic phase of infection, ILTV also invades peripheral nerves and establishes latent infection (Williams *et al*., 1992; Bagust & Johnson, 1995). Stress factors such as the onset of lay or transfer can reactivate viral replication and shedding (Hughes *et al*., 1989, 1991).

Shortly after ILT was first described in 1925 by May and Tittsler, immunization of chickens was achieved by inoculating birds with virulent virus via the cloaca (Brandly & Bushnell, 1934). This is considered the first effective vaccine developed for a major avian viral disease (Guy & García, 2008). Subsequently, attenuated live vaccines were developed by consecutive passage of virulent virus in cell cultures (tissue culture origin [TCO]) (Gelenczei & Marty, 1965) or in embryonated hen eggs (chicken embryo origin [CEO]) (Samberg & Aronovici, 1969). These vaccines are now commonly used in commercial poultry flocks worldwide. In recent years, recombinant vaccines have been produced using herpesvirus of turkeys (HVT) or fowlpoxvirus (FPV) expressing ILTV glycoproteins that can elicit protective immune responses in vaccinated birds (Davison *et al*., 2006; Mebatsion *et al*., 2008). These recombinant vaccines are now used commercially in some poultry-producing regions, and numerous other ILT vaccines, including additional recombinant and live attenuated vaccines, are in development in research laboratories around the world (Devlin *et al*., 2006b; Mundt *et al*., 2010; Pavlova *et al*., 2010; García *et al*., 2012).

New evidence obtained from the analyses of whole genome sequences of vaccine and field strains indicates that spontaneous, natural recombination between attenuated vaccines in the field can result in the emergence of novel virulent strains of ILTV that can then cause widespread disease (Lee *et al*., 2012). This is the first report of recombination between any attenuated live vaccines resulting in restoration of virulence in the field. This finding adds another degree of complexity to the safe use of ILT vaccines and calls for a thorough revision of the current status of live herpesvirus vaccines in use or under development.

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The purpose of this review is to examine advancements in the development, and understanding in ecology of ILT vaccines.

Live Attenuated Infectious Laryngotracheitis Vaccines

Molecular characterization. ILTV has been described as antigenically homogeneous with only a single serotype recognized. Therefore, once a flock has been vaccinated with an attenuated strain of ILTV, it is difficult to subsequently determine by serological assays whether birds have been infected by vaccine or virulent field strains. Differentiation between vaccinated and infected animals (DIVA) through serological surveillance has been proposed as a useful approach to ILT control (Bagust & Johnson, 1995) because it would allow for control strategies to be tailored based on the type of virus (vaccine or wild-type) present in the field. An important goal has therefore been to develop methods to help differentiate vaccine and field strains of ILTV based on genetic differences between virus strains.

To this end, sequence analysis of individual genes has been used by a number of research groups to genetically characterize ILTV isolates. Some of these studies have used polymerase chain reaction coupled with restriction fragment length polymorphism (PCR-RFLP) analysis (Chang *et al*., 1997; Graham *et al*., 2000; Garcia & Riblet, 2001; Han & Kim, 2001). In many of the initial studies, only a small region of the viral genome was examined and thus differences or similarities observed between strains could not be appropriately assessed. Subsequently, a combination of PCR-RFLP results from several genes has been used to characterize ILTV strains more reliably (Creelan *et al*., 2006; Ojkic *et al*., 2006; Kirkpatrick *et al*., 2006b; Oldoni & García, 2007; Neff *et al*., 2008; Oldoni *et al*., 2008). At least one of these studies concluded that most ILT outbreaks were not related to the vaccine strains in use (Kirkpatrick *et al*., 2006b); however, in many cases the restriction fragment patterns of field isolates were undistinguishable from those of vaccine strains, so the isolates were characterized as genetically similar to or closely related to vaccine strains (Creelan *et al*., 2006; Ojkic *et al*., 2006; Oldoni & García, 2007; Neff *et al*., 2008; Oldoni *et al*., 2008; Blacker *et al*., 2011) and outbreaks caused by these strains were termed "vaccinal laryngotracheitis" (Dufour-Zavala, 2008).

The advent of next-generation sequencing technologies has allowed complete genome sequences of different ILT vaccine and field strains to be determined and compared (Lee *et al*., 2011a,b, 2012; Chandra *et al*., 2012; Spatz *et al*., 2012). An attenuated vaccine strain that originated in Europe (Serva ILTV, Nobilis® ILT; MSD Animal Health, Bendigo, Victoria, Australia) was the first single ILTV strain to be completely sequenced (Lee *et al*., 2011b). Subsequently, the whole genome sequences of four attenuated vaccine strains—two from Australia (SA-2 and A20; Pfizer Australia, West Ryde, New South Wales, Australia) (Lee *et al*., 2011a) and two from the USA (LT-BLEN®; [Merial Select, Gainesville, GA, USA] and Laryngovac [Fort Dodge Animal Health, Exton, PA, USA]) (Chandra *et al*., 2012)—have also been published. In addition, the complete genome sequences of four virulent field isolates from the USA (Spatz *et al*., 2012) and of two field isolates from Australia (Lee *et al*., 2012) have recently been released.

It is anticipated that the comparative analyses of the complete genome sequences of field (virulent) and vaccine ILTV strains will contribute to the elucidation of the genetic basis for vaccine attenuation and, therefore, to the generation of safer and more effective vaccines. Alignments of the whole genome sequences of two Australian CEO ILT vaccines (A20 and SA-2) revealed non-synonymous nucleotide changes in the genes *ORF B* and *UL15* (Lee *et al*., 2011a). These changes have been postulated to be associated with the comparatively greater attenuation of the A20 strain, which originated from additional passage of the SA-2 strain in cultured cells (Lee *et al*., 2011a). The further determination and analysis of complete genome sequence data will allow the development of a more accurate understanding of the phylogenetic relationships between ILTV strains, including the relationships between vaccine and field strains.

Epidemiology. In Australia, characterization of ILTV strains by PCR-RFLP of five genomic regions has led to the identification of nine different genotypes or classes of ILTV (Kirkpatrick *et al*., 2006b; Blacker *et al*., 2011). Initially, field isolates and vaccine strains were grouped into five different classes, with most isolates distinguishable from ILT vaccines (Kirkpatrick *et al*., 2006b). Shortly after the introduction of a new vaccine strain originating in Europe, four new genotypes were identified (classes 6 to 9). One of these (class 7) corresponded to the newly introduced vaccine, while all the remaining classes (classes 6, 8 and 9) corresponded to field isolates (Blacker *et al*., 2011). Phylogenetic analyses suggested that ILTV classes 8 and 9 grouped together with class 7, indicating a close genetic relationship between field isolates in these classes and the vaccine strain. Genotyping data indicated that most recent disease outbreaks were caused by one of the emergent vaccine-related ILTV classes, class 8 and/or class 9 (Blacker *et al*., 2011). New data obtained from whole genome sequence analysis of the vaccine strains (Lee *et al*., 2011a, b) and the emergent field strains (Lee *et al*., 2012) has determined that the class 8 and 9 viruses emerged as a result of independent natural recombination between attenuated vaccine strains (classes 1 and 7). It is possible that recombination may have been facilitated by the conditions under which the ILT vaccines were used, including the mass delivery of multiple vaccines to large numbers of intensively housed birds. This recent finding highlights the risk associated with the use of multiple attenuated ILT vaccines under conditions imposing high selective pressures, which may foster recombination between co-circulating viruses and selection of more virulent or transmissible progeny.

In the USA, combined PCR-RFLP analysis of four genomic regions has allowed the definition of nine ILTV genotypes (groups), two of which correspond to TCO and CEO vaccines (groups II and IV, respectively). All other groups were identified as field isolates. A number of field isolates were classified as group IV, and were thus considered to be closely related to CEO vaccines (Oldoni & García, 2007). Most ILTV field isolates studied were described as vaccine related (Oldoni & García, 2007; Oldoni *et al*., 2008). A similar situation has been seen in Western European countries, where the vast majority (98/104) of ILTV isolates examined were closely related to vaccines (Neff *et al*., 2008). A recent study from Italy

analysed PCR-RFLP patterns and nucleotide sequences of a number of ILTV genomic regions in field isolates and vaccine strains. Differences were seen between the ILTV isolates at the nucleotide level but not by PCR-RFLP, with most field isolates found to be vaccine-related strains (Moreno *et al*., 2010).

In Peru and Brazil, the analysis of the nucleotide sequence of two regions of the infected cell protein-4 gene could differentiate between field isolates and CEO vaccine strains. The ILTV isolates causing outbreaks in these countries were not related to vaccine strains and probably originated from illegally imported non-commercial birds (Chacón & Ferreira, 2009). Following the occurrence of these outbreaks, the poultry industries of Brazil and Peru elected to utilize CEO ILT vaccines (Brazil) or recombinant ILT vaccines (Peru) to control disease outbreaks (Chacón & Ferreira, 2009; Chacón *et al*., 2010). Genetic characterization of ILTV field isolates present in these countries subsequent to these different vaccination strategies being implemented has not been reported. Future studies should be directed to elucidate the influence of these different vaccines on the genetic diversity of ILTV field strains.

In general, ILTV genotyping data have provided support for the hypothesis raised by earlier studies that vaccine viruses tend to displace wild-type viruses in the field (Chang *et al*., 1997; Graham *et al*., 2000). The variations in the genotyping data obtained from different geographical areas emphasize the value of molecular characterization of ILTV isolates in the adoption of appropriate control strategies, especially in areas where traditionally attenuated vaccines, which do not allow serological differentiation from field strains, are used. It is possible that in areas where certain ILTV genotypes predominate in the field, the deliberate introduction of new (different) attenuated ILT vaccines with distinct genotypes may increase the opportunity for ILTV to recombine into fitter, more virulent or transmissible forms, as a different pool of genes is made available for recombination and selection. In such circumstances, the introduction of recombinant viral-vectored vaccines may be a safer alternative that could reduce the risk of bringing new gene populations into play.

Recombinant infectious laryngotracheitis vaccines

A number of ILTV genes have been targeted for the generation of recombinant viral-vectored vaccines or for the creation of deletion mutants. Table 1 presents a summary of the genes that have been targeted and their role during viral replication. Much of our current understanding of the functions of these genes has been derived through extrapolation from what is known of their homologues in other alphaherpesviruses, or through characterization of the resultant ILTV deletion mutants.

Virally vectored infectious laryngotracheitis vaccines. New molecular technologies available in recent years have been used to create recombinant viruses that express immunogenic antigens of ILTV. These vaccines have the advantage of providing protective immunity without the risk of the re-emergence of latent virus in carrier individuals (Bagust & Johnson, 1995; Davison *et al*., 2006; Sun *et al*., 2008).

Currently, a recombinant FPV-vectored vaccine (Vectormune® FP-LT; Ceva Animal Health, Lenexa, KS, USA) expressing genes from ILTV is commercially available in some areas of North and South America. This vaccine expresses the ILTV glycoprotein B (gB) and *UL-32* genes and is currently registered for administration via wing-web puncture in 1-week-old birds, or *in ovo* in 18-day-old embryos. Studies have shown that this vaccine confers adequate protection, measured in terms of gross tracheal pathology, against challenge with ILTV when administered by wing web injection (Davison *et al*., 2006). A more recent study using this vaccine delivered *in ovo* demonstrated partial protection against challenge, and only a small reduction in the replication of the challenge ILTV in the tracheal mucosa at 5 or 8 days after challenge (Johnson *et al*., 2010). The authors speculated that this may have been due to the inability of the vector virus to replicate in the trachea of vaccinated birds, and thus the absence of a local immune response capable of preventing viral replication. In a similar study, Guy *et al*. (2010) found that the protective immunity provided by this same recombinant vaccine inoculated *in ovo* was somewhat less than that provided by TCO or CEO vaccines administered by eye-drop or drinking water, respectively. However, the vaccine was capable of preventing mortality, reducing clinical signs and lesions, and improving weight gain. In addition, although the vaccine could not completely prevent the replication of challenge virus, it could reduce the extent of this replication and shortened its duration (Guy *et al*., 2010).

An FPV-vectored vaccine co-expressing the Newcastle disease virus fusion and haemagglutinin-neuraminidase

Table 1. *Genes that have been targeted for generation of viral-vectored or deletion mutant recombinant ILTV vaccines.*

Gene name	Function
Glycoprotein B	Viral entry, involved in envelope-membrane fusion[a]
Glycoprotein C	Viral attachment to the cell surface[b]
Glycoprotein D	Viral entry mediator[c]
Glycoprotein G	Viral chemokine binding protein[d]
Glycoprotein I	Viral cell-to-cell spread[e]
Glycoprotein J	Viral egress[f]
Thymidine kinase	DNA synthesis[g]
UL0	Regulation of viral gene expression, DNA synthesis or encapsidation[h]
UL32	Cleavage and encapsidation of the viral genome[i]
UL47	Virion maturation in the cytoplasm. Gene regulation or particle assembly in the nucleus[j]

[a]Poulsen & Keeler (1997). [b]Kingsley *et al*. (1994) and Kingsley & Keeler (1999). [c]Spear & Longnecker (2003). [d]Devlin *et al*. (2006b, 2010). [e]Devlin *et al*. (2006a). [f]Mundt *et al*. (2011). [g]Griffin & Boursnell (1990) and Keeler *et al*. (1991). [h]Veits *et al*. (2003). [i]Lamberti & Weller (1998). [j]Helferich *et al*. (2007).

genes and the ILTV *gB* gene has been developed in China. Under experimental conditions and when administered by scarification of the wing-web, this vaccine candidate has been able to induce effective immunity to ILTV challenge, equivalent to a traditionally attenuated vaccine (Sun *et al.*, 2008). Previously, an FPV-vectored ILT vaccine expressing ILTV gB only, developed by the same group of researchers, had been shown to provide immunity against a lethal challenge dose of ILTV. However, replication of challenge ILTV could not be completely prevented (Tong *et al.*, 2001). More recently, the immune responses elicited by FPV-vectored vaccine candidates expressing both ILTV gB and chicken interleukin-18 (IL-18), or gB alone, were examined and compared using inoculation by wing-web puncture. Challenge studies indicated that the expression of chicken IL-18 by the recombinant vaccine induced a more effective immune response in chickens as measured by detection of ILTV DNA by PCR at 15 days after challenge (Chen *et al.*, 2011). This suggests that IL-18 may be used as an adjuvant in recombinant FPV-vectored ILT vaccines and highlights the importance of Th1-type immune responses in protection against ILTV infection and disease. The use of IL-18 or other molecules with immunomodulatory effects may play a major role in the development of new poultry vaccines, including ILT vaccines.

A recombinant cell-associated Marek's disease vaccine using HVT as a vector for the genes encoding for ILTV glycoproteins I (gI) and D (Innovax®–ILT; Intervet International B.V., Whitehouse Station, NJ, USA) has recently been released in the USA (Mebatsion *et al.*, 2008). This vaccine has been registered for subcutaneous administration to healthy 1-day-old chicks, and for *in ovo* inoculation of 18-day-old chicken embryos, with onset of immunity from 4 weeks post inoculation and lasting up to 60 weeks (Intervet International B.V., 2010a, b). Recent studies have demonstrated that this recombinant HVT vaccine does replicate in the chickens' tissues following *in ovo* and subcutaneous inoculations. However, comparatively lower levels of expression of the ILTV *gI* gene were detected in the lung than in the spleen (Gimeno *et al.*, 2011). Authors have speculated that this may explain the comparatively lower levels of protection observed in birds inoculated with this vaccine compared with those vaccinated with a conventional attenuated ILT vaccine (Guy *et al.*, 2010). In their study, Guy *et al.* (2010) determined that this HVT-vectored vaccine delivered *in ovo* provided significant protective immunity in vaccinated chickens, but was not as effective as that provided by TCO or CEO vaccines delivered by traditional routes of inoculation.

A different HVT-vectored ILT vaccine has most recently been released in the USA (Vectormune® HVT-LT; Ceva Biomune, Lenexa, KS, USA). This product is registered for subcutaneous administration in 1-day-old chicks and *in ovo* inoculation in 18-day-old embryos. There have been no reports characterizing this new recombinant vaccine at the time of submission of this review.

When both FPV-vectored and HVT-vectored vaccines were used in combination, increased protection was observed compared with that provided by each recombinant vaccine alone, and comparable with that granted by a TCO vaccine (Guy *et al.*, 2010). Furthermore, when either FPV-vectored or HVT-vectored vaccine or a combination of both vaccines was administered prior to the inoculation of a CEO vaccine at 14 days of age, viral detection following CEO vaccination was markedly reduced, indicating that prior *in ovo* vaccination with these recombinants induced immunity that reduced CEO viral replication (Guy *et al.*, 2010). This is a significant finding because shedding of ILT vaccines following inoculation is a recognized safety concern. This combined approach also provided levels of protective immunity, measured in terms of body weight and clinical signs that were not significantly different to those provided by the CEO vaccine alone.

Further studies are warranted to investigate the ability of these novel recombinant vaccines to prevent the establishment of latency by challenge ILTV, especially in light of recent experimental studies that have shown these recombinant viral-vectored vaccines are unable to completely prevent replication of challenge ILTV in trachea (Guy *et al.*, 2010; Johnson *et al.*, 2010; Vagnozzi *et al.*, 2012). Field evidence has also shown that these recombinant viral-vectored vaccines fail to fully protect birds against disease in regions where there is a high level of challenge (Johnson *et al.*, 2010). This may be a limitation of virally-vectored ILT vaccines that do not replicate in respiratory tissues.

Deletion mutant infectious laryngotracheitis vaccines. The development and establishment of chicken hepatoma cell lines suitable for the propagation of ILTV (Kawaguchi *et al.*, 1987; Scholz *et al.*, 1993) have facilitated the generation of ILTV gene-deletion mutants by homologous recombination. Deletion mutants of ILTV lacking the genes *UL0* or *UL47*, or genes encoding thymidine kinase (TK), glycoproteins C (gC), G (gG) or J (gJ) have been examined in pathogenicity and functional studies. Deletion of these genes resulted in reduced virulence *in vivo*, and the deletion mutants have been proposed as potential vaccine candidates (Schnitzlein *et al.*, 1995; Veits *et al.*, 2003; Fuchs *et al.*, 2005; Devlin *et al.*, 2006b; Helferich *et al.*, 2007; Mundt *et al.*, 2010; Pavlova *et al.*, 2010).

The first ILTV gene-deletion mutant to be proposed as a vaccine candidate was a TK-deficient mutant strain. Intratracheal inoculation revealed that this mutant was highly attenuated, at a level comparable with that of a traditional attenuated ILT vaccine. It was also capable of inducing protection against a lethal challenge dose of virulent ILTV (Schnitzlein *et al.*, 1995). Later, Han *et al.* (2002) reported the creation of a different TK-deletion mutant, expressing green fluorescent protein as a marker. This TK-deleted mutant grew normally *in vitro*, had reduced virulence *in vivo* after intratracheal administration and induced protection against challenge in specific pathogen free chickens. Similarly, deletion of the unique ILTV gene *UL0* resulted in attenuation in birds inoculated by eye-drop but maintaining the capacity to induce protection against challenge, as demonstrated by the absence of viral shedding in most vaccinated birds after challenge. The insertion of the avian influenza virus haemagglutinin gene (H7) into the ILTV genome (to replace UL0) resulted in concurrent induction of protection against both homologous avian influenza and ILT viruses (Veits *et al.*, 2003).

An ILTV recombinant lacking gJ has also been reported to be attenuated *in vivo*, as demonstrated by comparatively lower clinical scores and mortality in

experimentally inoculated specific pathogen free birds when administered intratracheally. It has also been reported to induce protection against challenge, as demonstrated by the complete abolition of ILTV replication and shedding after challenge, and by the lack of clinical signs in vaccinated birds after challenge (Fuchs *et al*., 2005). Fuchs *et al*. (2005) have reported that this gJ deletion mutant grew only to low viral titres in chicken embryo kidney cells. This is probably related to the role of gJ in the egress of ILTV from infected cells, through mechanisms not yet fully understood (Mundt *et al*., 2011), and may present challenges for large-scale commercial vaccine production. Most recently, it has been reported that broiler birds vaccinated by eye-drop or *in ovo* with a different gJ deletion mutant developed by Mundt *et al*. (2010) were protected against challenge as evidenced by a reduction of clinical signs and viral loads in the trachea (García *et al*., 2012). Also a gC ILTV deletion mutant has been proposed as a vaccine candidate and shown to be attenuated following combined intratracheal and eye-drop inoculation of chickens, and to be capable of preventing shedding of the challenge virus (Pavlova *et al*., 2010).

Deletion of the *UL47* gene, which encodes a virion protein abundant in the tegument, has also been associated with *in vivo* attenuation and the mutant induced protection against challenge. However, it has been suggested that this deletion mutant would not be suitable for a serological DIVA approach as the protein encoded by gene *UL47* is not recognized by the humoral immune response following experimental infection (Helferich *et al*., 2007).

A number of studies have been carried out to assess the suitability of an ILTV mutant lacking gG (Devlin *et al*., 2006b) as a vaccine candidate (Devlin *et al*., 2007). These studies have demonstrated the attenuation and immunogenicity of this mutant and its suitability for mass administration through eye-drop or drinking-water (Devlin *et al*., 2007, 2008; Coppo *et al*., 2011). More recently, experimental studies have shown that ILTV gG functions as a viral chemokine binding protein *in vivo* and *in vitro*. The lack of gG appears to result in a shift in the immune response from a humoral (non-protective) to a cell-mediated (protective) response (Devlin *et al*., 2010). In experimental settings this deletion mutant has been shown to be capable of reducing transmission of challenge ILTV in a population of birds vaccinated by eye-drop (Devlin *et al*., 2011). When delivered by eye-drop, this deletion mutant has displayed levels of safety and efficacy comparable with those of other commercially available attenuated ILT vaccines (Coppo *et al*., 2011). A recent study has also characterized the viral replication, safety and efficacy of this vaccine candidate following *in ovo* delivery at 18 days of embryonation. Vaccination did not affect weight gain, while a dose-dependent response was observed in terms of protection after challenge, as higher levels of protection, measured in terms of weight gain and tracheal pathology, were observed in birds that were inoculated with a higher viral dose (Legione *et al*., 2012a).

It is currently unknown whether any of these recombinant vaccines or vaccine candidates establish latency, or prevent latency being established by challenge strains. This is an important consideration as it relates to the capacity of these vaccines to displace currently prevalent vaccine-related strains present in the field.

Differentiation between vaccinated and infected animal control strategies. The advent of recombinant and gene-deleted ILT vaccines has brought with them the potential to differentiate serologically between infected and vaccinated birds and thus the potential to utilize DIVA control strategies. This is an important consideration in eradication programmes (Bagust & Johnson, 1995). Serological surveillance tools are generally preferred in DIVA control strategies as they are usually more broadly available to commercial laboratories than molecular tools (i.e. PCR-RFLP), which have typically been established for research purposes in reference laboratories. The lack or presence of a specific humoral response against specific proteins/antigens (marker proteins) should be readily detectable using serological screening methods (Veits *et al*., 2003; Fuchs *et al*., 2005; Devlin *et al*., 2007; Mundt *et al*., 2010; Pavlova *et al*., 2010; Shil *et al*., 2012). Similarly, the presence or absence of specific genetic markers would also be readily detectable using molecular methods (i.e. PCR) and these molecular methods could complement serological methods during disease outbreak investigations and the response to disease outbreaks.

A number of studies has reported the use of serological screening tools to detect antibodies against specific ILTV glycoproteins, providing evidence of the potential for development of DIVA tests to accompany the use of recombinant vaccines (Chang *et al*., 2002; Fuchs *et al*., 2005; Johnson *et al*., 2010; Pavlova *et al*., 2010; Shil *et al*., 2012). Indirect immune-fluorescence assays were developed to detect antibody targeting gB, gC, gI and gJ in sera collected from birds inoculated *in ovo* with one of the commercially available FPV-vectored or HVT-vectored ILT vaccines. The FPV-vectored ILT vaccine, which expresses ILTV gB, elicited an antibody response against ILTV gB in a proportion of the vaccinated birds. In addition, these birds had significantly higher antibody titres against gB after challenge than those inoculated with a CEO vaccine. In contrast, antibodies against ILTV gI were not detected in those that had been inoculated with the HVT-vectored vaccine that expresses ILTV gI, and there were no significant differences in the antibody titres against ILTV gI after challenge between those vaccinated with the HVT-vectored ILT vaccine and those vaccinated with a CEO vaccine (Johnson *et al*., 2010). These results suggest that the FPV-vectored ILT vaccine elicited a relatively stronger antibody response against the vectored ILTV glycoprotein than the HVT-vectored ILT vaccine. However, neither vaccine elicited an antibody response that was detectable by a commercial ILTV enzyme-linked immunosorbent assay (ELISA) kit. It is unclear whether these results are the consequence of low immunogenicity of the vaccines *per se* or of the route of inoculation used for this study, which may have failed to deliver the vaccine successfully to the embryos.

The presence of specific antibodies against gC, but not against gJ, was demonstrated using indirect immune-fluorescence tests in sera collected from birds inoculated with a gJ-deficient ILTV mutant developed by Fuchs *et al*. (2005). Similarly, serological screening tests detecting antibody against gJ and gC by indirect immune-fluorescence have been proposed for differentiation of wild-type infected and vaccinated birds with a gC deletion ILTV mutant (Pavlova *et al*., 2010). Experimental *in vivo* studies with a gG deletion mutant have

shown that infection, following inoculation via different routes, results in low antibody levels against ILTV, possibly because of the abolition of the immuno-modulatory role of gG (Devlin *et al.*, 2006b, 2007, 2008, 2010; Coppo *et al.*, 2011). Despite the low antibody response observed in experimentally vaccinated specific pathogen free birds, a recent report has described the use of a recombinant gG ELISA that could be used as a diagnostic companion to this vaccine candidate in DIVA control strategies (Shil *et al.*, 2012). The possibility of using PCR-based methods in combination with serological tools in a DIVA approach have also been proposed for this vaccine candidate, as the reduced humoral immune response induced by this gG-deleted vaccine may limit the application of this serological screening tool.

Systematic vaccination with a DIVA vaccine for a long enough period of time would be expected to decrease transmission of wild-type viruses among vaccinated birds. Once a low prevalence of wild-type infection, detectable by the DIVA test, has been achieved, the last sources of infection may then be suitable for eradication (van Oirschot *et al.*, 1996). This strategy has been used successfully to control porcine pseudorabies virus in the Netherlands, where intensive vaccination programmes using a gI deletion mutant significantly reduced the seroprevalence against wild-type virus infections over a period of 2 years (Stegeman *et al.*, 1994a, b). Clearly for ILT this strategy would only be possible through the concerted efforts of all poultry producers in a particular geographical area, where biosecurity measures such as extended downtime of poultry houses and restrictions to bird or manure movements were followed by all (Dufour-Zavala, 2008). This approach assumes that the DIVA vaccines would decrease transmission of wild-type viruses among vaccinated flocks. In this regard, horizontal transmission dynamics of challenge ILTV following vaccination have only been reported for one of the ILTV deletion mutant vaccine candidates, under experimental conditions (Devlin *et al.*, 2011). This strategy also assumes that vaccines can prevent the establishment of latency by challenge viruses in DIVA vaccinated birds. The ability of the recombinant vaccines to prevent latency being established by challenge strains has not yet been reported for any ILT vaccine. Observations that some recombinant vaccines do not prevent the replication of challenge virus in the trachea (Johnson *et al.*, 2010; Vagnozzi *et al.*, 2012) have led to the hypothesis that challenge strains would be able to further colonize the peripheral nervous system during initial stages of infection and thus establish latent infections. Further experiment work to investigate this hypothesis is warranted.

Serological surveillance using DIVA tests in areas where virally-vectored recombinant vaccines have been utilized over the past few years may help to better understand the epidemiology of ILTV under these new conditions and help to elucidate whether the new recombinant vaccines can indeed displace wild-type or vaccine-related ILTV strains present in the field, or prevent their transmission.

Vaccine Safety, Protection and Administration Routes

Safety and protection. Modified live virus vaccines can exhibit varying levels of residual virulence depending on the vaccine strain and the age of the birds. Clinical signs of disease, tracheal lesions, reduced weight gain and mortality have been used in a number of studies to assess vaccine virulence as a measure of safety (Guy *et al.*, 1990; Kirkpatrick *et al.*, 2006a; Devlin *et al.*, 2008; Oldoni *et al.*, 2009; Coppo *et al.*, 2011). This residual virulence can increase after bird-to-bird passage, as demonstrated experimentally for CEO vaccines (Guy *et al.*, 1991).

Challenge-protection models have long been used to evaluate the efficacy of ILT vaccines. However, differences in the route of inoculation (intratracheal, eye-drop, *in ovo*), viral strain and the dose used, as well as age and breed of the birds can all influence the parameters that are commonly measured to assess protection following challenge. This hinders direct comparison between vaccines assessed in different studies (Fulton *et al.*, 2000; Tong *et al.*, 2001; Davison *et al.*, 2006, 2007, 2008; Rodriguez & Garcia, 2008; Rodríguez-Avila *et al.*, 2008; Sun *et al.*, 2008; Johnson *et al.*, 2010; Vagnozzi *et al.*, 2010a, b; Coppo *et al.*, 2011). Official regulatory agencies from Europe and the USA require vaccinated birds to be protected from severe clinical signs, gross pathology and death following challenge with a lethal dose of ILTV (Anonymous, 2003; European Directorate for the Quality of Medicines & Healthcare, 2007). Nevertheless, these vaccine efficacy assessment methods have failed to provide the poultry industry with vaccines that are able to completely control the threat of ILT outbreaks. In addition, reversion to virulence after multiple passages *in vivo* has not been examined for many of the ILT vaccines currently in use.

In order to tackle some of these limitations, vaccine replication and transmissibility have been recently re-examined in an attempt to better understand the origins of field outbreaks of ILTV (Rodríguez-Avila *et al.*, 2007; Coppo *et al.*, 2012a, b). Differences in replication between the vaccine strains Serva and SA-2, as well as differences in their ability to transmit to in-contact naïve birds, have been shown to occur under experimental conditions (Coppo *et al.*, 2012a). Differences in transmission between CEO and TCO vaccines have also been demonstrated, with the former replicating and spreading more rapidly than the latter (Rodríguez-Avila *et al.*, 2007). It is possible that ILTV strains that transmit more readily within or between flocks may be the origin of vaccine-related ILT outbreaks. Notably, contact exposure to live attenuated vaccine strains does not necessarily protect against challenge with virulent field ILTV strains (Rodríguez-Avila *et al.*, 2008). These findings highlight the importance of identifying vaccines that could prevent transmission of challenge strains within vaccinated flocks (Devlin *et al.*, 2011) as a new approach for assessing vaccine efficacy and improving the utility of ILT vaccines.

A recent experimental study has identified further difficulty in achieving adequate protection against ILT. Deficiencies in the protection granted by attenuated TCO ILT vaccine were observed when administered in combination with vaccines against infectious bronchitis virus and Newcastle disease virus, which is current industry practice (Vagnozzi *et al.*, 2010a). Validation of these experimental observations in a field setting would be useful to fully assess their practical significance.

Routes of administration. The route of vaccination is extremely important. Drinking water and coarse spray are routinely used in the broiler poultry industry (Robertson & Egerton, 1981; Devlin *et al*., 2008; Guy & García, 2008), but in laying hens vaccination by eye-drop has been shown to provide more uniform immunity than vaccination by drinking-water (Fulton *et al*., 2000). It has been suggested that drinking-water vaccination may lead to the establishment of an "underground" or unnoticed infection cycle between vaccinated and naïve birds in the same shed or flock (Fulton *et al*., 2000; Coppo *et al*., 2012a, b). A recent study that characterized and compared the replication and transmissibility of a CEO ILT vaccine strain following eye-drop or drinking-water vaccination under experimental conditions found significant differences in the extent of viral replication associated with administration via each of the different routes, with drinking-water vaccination resulting in a longer viral persistence in the trachea compared with eye-drop vaccination (Coppo *et al*., 2012b). Viral replication in contact-exposed birds in this and other studies has provided evidence of frequent bird-to-bird transmission of vaccine viruses between vaccinated and naïve birds. Vaccines that are readily transmitted between birds may have a heightened potential for reversion to virulence due to the potential for multiple *in vivo* passages after initial vaccination and selection of variants with greater transmissibility within the vaccine.

To avoid the limitations of traditional routes of ILTV vaccination, the safety and vaccine efficacy of ILT vaccines delivered by other methods have been investigated. In particular, *in ovo* administration, which could provide greater vaccination coverage and more uniform immunity within vaccinated flocks, has been assessed in a number of recent studies. *In ovo* vaccination has been used extensively in the broiler poultry industry to control Marek's disease (Bermudez, 2008). More recently, viral-vectored vaccines against Newcastle disease virus and infectious bursal disease virus have expanded the use of this technology to enable delivery of multiple vaccine antigens simultaneously to more than 50,000 eggs per hour (Williams & Zedek, 2010). A study on the efficacy of either FPV-vectored or HVT-vectored ILT vaccines following *in ovo* administration found that these vaccines did induce partial immunity against challenge in terms of body weight gain and clinical signs, but did not reduce challenge virus loads in the trachea (Johnson *et al*., 2010). More recently, Vagnozzi *et al*. (2012) compared the protection afforded by commercially available FPV-vectored and HVT-vectored ILT vaccines administered *in ovo* or subcutaneously. The HVT-vectored vaccine appeared to be more effective than the FPV-vectored vaccine in reducing clinical signs of ILT after challenge, while the FPV-vectored vaccine appeared to afford better protection against challenge when administered subcutaneously than *in ovo*. Nevertheless, regardless of the route of administration, both vaccines mitigated clinical signs although they both failed to reduce challenge viral loads in trachea. The authors suggested that these vaccines may be unable to control disease or the circulation of virulent ILTV in situations where there is an overwhelming field challenge (Vagnozzi *et al*., 2012). Clearly, field-based challenge-protection studies and epidemiological analyses are needed to examine the performance of these vaccines in the field more comprehensively and thus better contextualize the results from these experimental studies.

To date there have been no studies to assess the safety or efficacy of traditional attenuated ILT vaccines following *in ovo* inoculation. However, there have been very recent studies examining viral replication, safety and protection of ILTV deletion mutants lacking gG or gJ delivered by this route (García *et al*., 2012; Legione *et al*., 2012b). Compared with eye-drop inoculation, the gJ deletion mutant delivered *in ovo* at 18 days of embryonation afforded comparable levels of protection, as measured in terms of the proportion of protected birds and reducing clinical signs and challenge viral replication (García *et al*., 2012). The gG deletion mutant delivered *in ovo* at the same age of embryonation was also protective against challenge as assessed in terms of body weight gain, tracheal gross and microscopic pathology (Legione *et al*., 2012b). However, both vaccine candidates delivered *in ovo* were unable to completely abolish the replication of challenge ILTV in vaccinated birds. This is an important issue related to the potential establishment of bird-to-bird transmission between birds within a vaccinated flock. As similar observations have been made in birds inoculated *in ovo* with viral-vectored ILT vaccines, further characterization of this inoculation route is necessary to better understand its limitations when used to deliver ILT vaccines.

Assessment of flock immunity and protection. The routine assessment of ILT immunity in vaccinated flocks is problematic because of the poor predictive value of serological data in determining protection from disease. Cell-mediated immunity, but not serum or local antibody responses, has been found to be correlated with protection against ILTV (Fahey *et al*., 1983, 1984; Fahey & York, 1990; Honda *et al*., 1994a, b). Therefore, quantification of antibody concentration in sera from vaccinated flocks may not necessarily correlate with protection. Earlier field studies found lower mean virus neutralizing antibody titres in birds vaccinated via drinking water than in eye-drop vaccinated birds, but both groups of birds were sufficiently protected against challenge (Hayles *et al*., 1976). Subsequently, Sander *et al*. (1997)) used a commercial kit and suggested that geometric mean ELISA antibody titres above 400 were indicative of protection against virulent challenge. However, some commercially available ELISA kits (including the one used by Sander *et al*.) are more suited to qualitative detection of antibody against ILTV than to quantification of antibody titres in sera (Bauer *et al*., 1999; Fulton *et al*., 2000). Unfortunately, cell-mediated immunity detection methods, which could be better correlated with protection, are technically more difficult and costly to implement and use than serological screening methods to detect antibody against ILTV; therefore, despite their limited value, ELISAs are the only commercially available methods to assess flock protection and are broadly used.

Serological testing to assess the proportion of birds with serum antibodies to ILTV in a flock represents another method to estimate protection against challenge. A recent experimental study to investigate transmissibility of wild-type challenge ILTV within a group of birds vaccinated via eye-drop with a gG deletion mutant (Devlin *et al*., 2011) found the reproduction ratios (the average number of secondary infectious cases from a

typical infectious case) to be <1, meaning that each infected individual would produce less than one new infected individual (Heffernan *et al.*, 2005), and therefore vaccination would prevent the challenge ILTV strain from spreading within that vaccinated flock. However, there are no field or experimental data indicating the minimum proportion of birds within a flock that need to have been exposed (directly by vaccination or indirectly by contact) to an ILT vaccine for the flock to be considered protected from challenge (i.e. reproduction ratio <1). In their study, Devlin *et al.* (2011) vaccinated all birds via eye-drop, an ideal route that is impractical under field conditions for broiler flocks. New field studies utilizing commonly used inoculation routes are necessary to assess and optimize current industry practices in order to achieve optimum protection in vaccinated flocks. In addition, the development of more reliable screening methods that better correlate with protection and that are easy to use would be valuable when assessing the immunological status of vaccinated flocks. Antigen-specific lymphoproliferation assays have been found useful for screening for human herpesvirus infections in blood of healthy individuals (Leroux *et al.*, 1985). Similar approaches for routine screening for cell-mediated immune responses elicited by ILT vaccines in chickens may be useful and need to be further investigated.

Conclusions

Alphaherpesviruses such as ILTV are complex pathogens that have co-evolved with their hosts over millions of years. Recent evidence that attenuated ILT vaccines have recombined to generate fitter more virulent (or transmissible) field strains, capable of causing significant losses in the intensive poultry industry (Lee *et al.*, 2012), has added another layer of complexity to the problem of achieving control of ILT by vaccination. It is currently unknown whether recombination is a sporadic event or a common evolutionary strategy employed by ILTV and other alphaherpesviruses to facilitate their spread and persistence in host populations. The increasing availability of next-generation sequencing technologies at lower costs will assist in elucidating this as more whole genome sequences of historical and contemporary ILTV isolates are anticipated to be available to the research community. Studies should be pursued to examine the mechanisms involved in recombination so as to better understand its genesis and, if possible, prevent its occurrence. Further epidemiological studies revisiting the vaccination practices and protocols in place during the emergence of these recombinant strains may assist in this endeavour.

Considerable effort and resources have been dedicated to the control of ILT through the use of vaccines over the past 80 years. Unfortunately, no attempts have been made so far to quantify the economic impact of ILT on the poultry industry. Improved control strategies using new more effective vaccines would be likely to reduce the economic impact that results from decreased production or mortality when ILT outbreaks occur. Insight into this impact and ultimately insight into the capacity of different control strategies to prevent or decrease these economic losses would be an objective basis for selection of the most appropriate control measures. It is clear that the currently available attenuated vaccines have served the poultry industry well, yet they have been insufficient to prevent periodic ILT outbreaks. This perhaps reflects the inadequacy of current (traditional) methods of assessing vaccine safety and efficacy, which are largely based on the evaluation of the residual virulence of the vaccines and the capacity of vaccinated birds to withstand the effects of challenge using parameters such as clinical signs, weight gain and tracheal pathology. New (different) parameters, such as vaccine transmissibility and the capacity of the vaccine to prevent transmission of challenge virus, may be more appropriate measures in the light of recent studies on ILT. The capacity of vaccines to establish latent infections and prevent or limit the establishment of latency by challenge strains of ILTV also needs to be examined. Experimental studies are limited in their capacity to mimic field conditions so future research into vaccine assessment and use will need to be more focused on field conditions and practices.

The use and development of new recombinant ILT vaccines, as well as the exploration of new, more effective administration strategies, have aimed to overcome many of the obstacles preventing sustained control of ILT using vaccination. However, available experimental data have shown that currently available commercial recombinant vaccines do not afford the levels of protection against challenge that are provided by traditional attenuated vaccines (Guy *et al.*, 2010; Johnson *et al.*, 2010; Vagnozzi *et al.*, 2012). Similarly, *in ovo* vaccination, which could be a means of achieving higher and more uniform levels of immunity in vaccinated flocks, has not yet been proven to afford levels of protection comparable with those granted by traditional routes of inoculation (Guy *et al.*, 2010; Johnson *et al.*, 2010; Vagnozzi *et al.*, 2012). Larger scale population studies, where a DIVA approach is possible, are needed to fully assess the efficacy of newly available recombinant vaccines in displacing ILTV strains prevalent in the field, at least in areas with comparatively lower levels of viral challenge where these vaccines appear to be more effective (Johnson *et al.*, 2010).

Our limited understanding of the pathobiology of ILTV is an additional obstacle to the development of appropriate control strategies. New developments in avian immunology are likely to allow more in-depth investigations of viral–host interactions that are currently poorly understood, and, more specifically, of innate immune responses that have been shown to play a key role in the containment of other herpesvirus infections (Paludan *et al.*, 2011). Understanding and measuring innate immune responses to ILTV may ultimately provide a method to better assess flock protection, beyond what can be achieved by measuring serum antibody against ILTV. A better understanding of the mechanisms used by ILTV to establish latent infection, to remain latent and to reactivate is crucial for the control of this disease, as it is likely to be directly correlated with the capacity of vaccines to prevent the spread of ILTV from long-lived birds, such as layers and breeders, which can act as reservoirs of infection. The analysis of whole genome sequencing data of a larger number of field and vaccine ILTV strains may also contribute to a better understanding of the molecular bases of virulence and attenuation, which will be useful for the development of improved vaccines. In the long term, a better understanding of the viral–host interac-

tions may also lead to the development of therapeutic or control strategies targeting host proteins, rather than the virus, thus avoiding rapid evolution of counteracting strategies by the virus (i.e. recombination).

In conclusion, an ideal vaccine against ILT would need to allow for convenient and cost-effective delivery in order to achieve uniform protection across vaccinated flocks. This vaccine would ideally not establish latent infections but would prevent the establishment of latency by other ILTV strains. A short period of replication after vaccination, limited transmission of vaccine virus to in-contact birds and complete prevention of viral replication, shedding and transmission following challenge would also be key desirable features of the vaccine. Finally, an ideal vaccine would be suitable for DIVA control strategies and eradication programmes. Clearly, we have not yet achieved this ideal, but recent advancements in ILT vaccine development have made it more feasible. A combined approach where different vaccine types are used synergistically will probably bring us closer to enhanced control of this disease in the years to come.

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