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Characterization of infectious laryngotracheitis virus from outbreaks in Swedish chicken hobby flocks

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SUMMARY

Infectious laryngotracheitis (ILT) is a respiratory tract infection of gallinaceous birds, which is caused by Gallid herpes virus 1 (GaHV-1), also called infectious laryngotracheitis virus (ILTV). In its most virulent form it can cause acute respiratory symptoms such as marked dyspnoea, gasping, expectoration of bloody mucus, high mortality (up to 70%) and production losses, such as decreased growth and egg production.

In Sweden, there are regular outbreaks of ILT in hobby flocks with chickens and occasionally in other gallinaceous birds, but very few outbreaks have been diagnosed in commercial flocks. There are vaccines available and the most commonly used vaccine in Sweden is a live attenuated vaccine of chicken embryo origin (CEO). However, vaccination against ILT is not routinely practiced in commercial flocks in Sweden but the vaccine is administrated in some hobby flocks. In several countries where ILT vaccines are used in commercial flocks there have been outbreaks caused by vaccine-related viruses that have regained virulence though mutation (so called reversion) or recombined to new viruses, causing clinical outbreaks and production losses. The aim of this study was to improve the understanding of the epidemiology of ILT in Swedish chicken hobby flocks, including investigating if ILTVs circulating in Sweden are related to vaccine viruses. This was done by sequencing parts of the glycoprotein G (gG), thymidine kinase (TK) and infected cell polypeptide 4 (ICP4) genes from real time PCR ILTV positive nasal swabs from hobby chickens and comparing them to CEO vaccine strains and reference strains. These genes have previously been used to differentiate field strains from vaccine-related ILTVs. The samples originated from hobby chickens submitted for routine diagnostic necropsy with complaints of respiratory signs to the Swedish National Veterinary Institute in 2017 and 2018. The samples were also evaluated for co-infection with *Avibacterium paragallinarum* (APG), infectious bronchitis virus (IBV) and *Mycoplasma gallisepticum* (MG). Based on the three partial gene sequences (gG, TK and ICP4) the results suggested that the circulating ILTVs in Swedish hobby chickens flocks were related to CEO vaccine virus strains, including the vaccine used in Sweden for ILT vaccination. Complete genome data from a subset of the samples showed similar results, however, there were additional sequence differences in other locations of the genome. Further analyses are needed to determine the level of similarity to vaccine strains. The source of these viruses is however unknown. It was demonstrated that coinfections with other respiratory pathogens were common in the investigated Swedish hobby chicken flocks with ILT outbreaks, with the most common pathogen in the investigated ILT affected flocks being APG.

CONTENT

ABBREVATIONS

Acc.	GenBank accession number
APG	*Avibacterium paragallinarum*
AIV	Avian Influenza Virus
aPMV-1	Avian Paramyxovirus type 1 (previously known as Avian Avulavirus type 1)
CEO	Chicken Embryo Origin
ELISA	Enzyme-Linked Immunosorbent Assay
GHV-1	Gallid Herpesvirus 1
gC, gE, gG	Glycoprotein C, Glycoprotein E, Glycoprotein G
HSV-1	Herpes Simplex Virus 1
HVT	Herpesvirus of Turkey
ICP4	Infected Cell Protein 4
ICP18.5	Infected Cell Protein 18.5
ILT	Infectious Laryngotracheitis
ILTV	Infectious Laryngotracheitis Virus
IBV	Infectious Bronchitis Virus
MG	*Mycoplasma gallisepticum*
MS	*Mycoplasma synoviae*
ND	Newcastle Disease
ORFB-TK	Open reading frame B-thymidine kinase
ORF C	Open Reading Frame C
ORT	*Ornithobacterium rhinotracheale*
PCR	Polymerase Chain Reaction
RLFP	Restriction Fragment Length Polymorphism
SVA	National Veterinary Institute
TCO	Tissue Culture Origin
TK	Thymidine Kinase

INTRODUCTION

Infectious laryngotracheitis (ILT) is a respiratory tract infection of gallinaceous birds, which is caused by Gallid herpes virus 1 (GaHV-1), also called infectious laryngotracheitis virus (ILTV). Virulent ILTV can cause acute respiratory symptoms such as marked dyspnoea, gasping, expectoration of bloody mucus, high mortality (up to 70%) and production losses, such as decreased growth and decreased egg production (Guy & Garcia, 2008). Virulence differs between strains.

In Sweden, there are regular outbreaks of ILT in hobby flocks with chickens, but very few outbreaks have been diagnosed in commercial flocks (National Veterinary Institute, SVA, 2019). There are vaccines available and the most commonly used vaccine in Sweden is a live attenuated vaccine of chicken embryo origin (CEO). However, vaccination against ILT is not routinely practiced in commercial flocks in Sweden but the vaccine is administrated in some hobby flocks. In several countries where ILT vaccines are used in commercial flocks there have been outbreaks caused by vaccine-related viruses that have regained virulence from mutation (so called reversion) or recombined to new viruses, causing production losses and mortality.

The aim of this study was to improve the understanding of the epidemiology of ILT in Swedish chicken hobby flocks.

The hypotheses were the following:

1. The viruses that cause ILT outbreaks in Swedish hobby flocks are genetically variable viruses.

2. The viruses that cause ILT outbreaks in Sweden are vaccine-related.

LITERATURE REVIEW

For this literature review, information has been gathered from both textbooks and peer-reviewed scientific papers. Mostly the Swedish University of Agriculture's Library's search engine Primo and relevant journal article's reference lists have been used. Search words were: infectious laryngotracheitis, ILT, ILTV, herpesvirus, latency, virulence, immune response, co-infections, vaccine, vaccination, field virus, vaccine virus, backyard flocks, Sweden, sequencing, gG, TK, ICP4, PCR.

Infectious laryngotracheitis

Etiology

Infectious laryngotracheitis virus (ILTV) or Gallid herpesvirus 1 (GHV-1) belongs to the family *Herpesviridae* and subfamily *Alphaherpesvirinae* and the genus *Iltovirus* (Davidson *et al.*, 2009).

The genome of the herpesvirus family consists of a linear, double-stranded DNA that is located in the core (Knowles, 2011). The viral core is enclosed by a nucleocapsid, which in turn is surrounded by a globular matrix called the tegument. The outer layer is a lipid-protein envelope with numerous glycoprotein spikes.

First description

The disease name infectious laryngotracheitis (ILT) was established by the American Veterinary Medical Association, Special Committee on Poultry Diseases in 1931 (Cover, 1996). The disease laryngotracheitis had already been described in 1925 in Canada and may have occurred as early as in 1920. In Sweden, the first known case was described in 1940 in an outbreak in Lund, in southern Sweden (Magnusson, 1940).

Pathogenesis, virulence and immunity

Most avian herpesviruses have a relatively narrow host range, and so does the ILTV (Trapp & Osterrieder, 2008). The chicken is the primary natural host, but pheasants, peafowl and turkeys are also susceptible (Guy & García, 2008). Experimentally challenged ducks can be infected but develop a subclinical form of the disease.

The most common way that ILTV is introduced into a flock is by clinically healthy latently infected carrier birds shedding the virus, as ILTV can cause a latent state, i.e. a lifelong persistent infection (Knowles, 2011). The virus can also be transmitted from clinically affected chickens as well as indirectly from contaminated equipment, litter and people (Guy & García, 2008). The virus is spread with droplets that are inhaled or lands on the conjunctiva, but does not spread vertically, i.e. from parent hens through the egg to their progeny.

When the ILTV enters a host, the virus binds with its outer glycoprotein spikes to receptors on the host cell (Knowles, 2011). The virus envelope fuses with the host cell's plasma membrane and the nucleocapsid enters the host cell's cytoplasm. Then the viral core with the DNA enters the host cell's nucleus, where replication starts.

The way that ILTV replicates is similar to other viruses in the subfamily *Alphaherpesvirinae* (Guy & Garcia, 2008). Most of the approximately 70 proteins that start to transcribe once in

the host nucleus are viral structural proteins, others are proteins including enzymes that regulate the viral DNA replication. When the replication of the DNA is complete, it is packed into nucleocapsids. The envelope is then formed by budding through the nuclear membrane. Further on the virions are pooled in vacuoles in the host cell's cytoplasm and are released by cell lysis.

The ILTV invades and replicates in the epithelium of larynx, trachea and other mucus membranes such as conjunctiva, nasal mucosa, respiratory sinuses, air sacs and lungs (Guy & García, 2008).

Latency

As previously stated, the ILTV can cause a latent infection characterized by a subclinical state and no virus replication. Williams *et al.* (1992) suggested the trigeminal ganglion to be the predominant site for ILTV latency. This has been confirmed by Oldoni *et al.* (2009), who showed that the virus could be detected as early as two days post infection in the trigeminal ganglia. Most knowledge about the establishment of latency has been obtained from the human herpes simplex virus (HSV-1), which also belongs to the subfamily *Alphaherpesvirinae* (Nicoll *et al.*, 2011, Thilakarathne *et al.*, 2019). Viruses in this subfamily appear to infect the sensory nerve ends in the tissue where the primary infection and virus replication take place. The virus is then transported retrogradely to the neuron bodies where it enters the nucleus. In the nucleus the viral DNA binds to histones and the lytic gene expression is suppressed and latency is established. Reactivation of herpesviruses can occur and is often associated with stress, such as the onset of lay and re-housing (Guy & García, 2008). A latent reactivated infection is usually asymptomatic but the birds may shed the virus, as having ongoing active replication of the virus in the upper respiratory tract. This can lead to infection of susceptible/uninfected animals (Trapp & Osterrieder, 2008).

Immune response

The most important immune response against ILTV seems to be the local cell-mediated response in the upper respiratory tract, and not the humoral response with mucosal antibodies. This was shown by Fahey & York (1990) in a study involving drug-induced bursectomised chickens (in growing birds, the bursa of Fabricius is necessary for the maturation of B-lymphocytes). The chickens were ILT vaccinated and then challenged with ILTV. The bursectomised chickens cleared the ILT infection as effectively as the intact chickens. This shows that the mucosal antibodies do not have a critical role in immunity from vaccination or recovery from ILTV infection.

As in other herpesviruses it is the glycoproteins in ILTV that stimulate both a humoral and a cell-mediated immune response (Guy & García, 2008). The glycoprotein G (gG) gene is present in most herpesvirus genomes, ILTV included (Coppo *et al.*, 2013a). The gG gene codes for a protein that has been demonstrated in some herpesviruses in mammals to be cell-membrane bound and/or secreted. The secreted protein can function as a chemokine-binding protein (Bryant *et al.*, 2003). This means that it can bind to the host's chemokines and thus prevent the chemokines from binding to both its receptors and glucosaminoglycans. The binding may inhibit the function of the chemokines and reduce the cell-mediated immune response. It suggests that gG has a significant role in the pathogenesis of herpesvirus infection (Coppo *et al.*, 2013a).

Virulence

The virulence describes the capacity for the virus to cause disease in a host (Knowles, 2011). The term is relative and makes it possible to compare different strains with each other. The determinants of virulence are often associated with multiple genes, i.e. several genes contribute to the virulence of the virus, which is probably the case in ILTV. For example, Devlin *et al.* (2006) confirmed the gG gene to be an important virulence factor for ILTV, by demonstrating that chickens infected with gG deficient viruses had milder clinical signs and greater weight gain than chicken inoculated with a wild type virus. Similar results were obtained when inoculating an open reading frame C (ORF C) gene-deleted recombinant virus in chickens, which suggests that also the ORF C gene is a virulence factor (García *et al.*, 2016).

Morbidity and mortality

The morbidity in an infected flock can vary from 5% (mild form) to 90–100% (severe form) (Guy & García, 2008). The mortality in a flock can be 0.1–2% for the mild form, while it can vary from 5–70% for the severe form. The most common mortality rate for the severe form is 10–20%.

Clinical signs

The incubation period is between 6 and 12 days (Guy & García, 2008). Clinical signs can vary from unthriftiness, decreased egg production, watery eyes, conjunctivitis, swelling of infraorbital sinuses, persistent nasal discharge, haemorrhagic conjunctivitis to marked dyspnea, expectoration of blood-stained mucus and death in the more severe form.

The recovery time is usually between 10 and 14 days, but in some situations it may take up to 4 weeks (Guy & García, 2008).

Pathology

Gross pathology

Lesions can be detected in the conjunctiva and the respiratory tract, especially in the larynx and trachea (Guy & García, 2008). Depending on the severity of the disease, the lesions can vary. In mild cases, conjuctivitis, sinusitis and tracheitis as well as excess mucus in the trachea may be observed. In more severe forms there can be diphteric changes with necrotic laryngeal and tracheal mucus membranes and/or haemorrhage. This can cause casts consisting of blood and exudate that can occlude the larynx or trachea leading to asphyxia (Jansson *et al.*, 2006). The inflammation can also extend to the bronchial epithelium, the air sacs and lungs (Guy & García, 2008).

Microscopic pathology

The microscopic findings depend on the stage and severity of the disease (Guy & García, 2008; Trapp & Osterreider, 2008; Jansson *et al.*, 2006). In the early stages of the acute infection there are pathognomonic lesions in the conjunctiva, larynx and trachea. These consist of intranuclear inclusion bodies in epithelial cells and syncytia (multinucleated cells originating from fused ILTV infected epithelial cells). The changes can no longer be observed when the infection progresses as a result of necrosis and desquamation.

Co-infections and secondary infections

Co-infections are important factors to take into consideration as they can contribute to ILT disease severity and slow recovery (Blakey et al., 2019). As ILT can have a latent state, coinfections can potentially be a stress factor that triggers the virus to reactivate.

There are several infectious microorganisms that can cause respiratory disease in chickens and the most important are shown in table 1 (without mutual order) (National Veterinary Institute, 2019). Further, secondary bacterial infections may occur in birds with ILT.

Table 1. *Infectious respiratory pathogens in poultry*

Mycoplasma gallisepticum (MG, chronic respiratory disease)
Mycoplasma synoviae (MS)
Ornithobacterium rhinotracheale (ORT)
Avibacterium paragallinarum (APG, infectious coryza)
Avian paramyxovirus type 1 (aPMV-1, Newcastle disease)
Avian influenza virus (AIV, avian influenza)
Infectious laryngotracheitis virus (ILTV, infectious laryngotracheitis)
Infectious bronchitis virus (IBV, infectious bronchitis)
Aspergillus spp. (aspergillosis)
Syngamus trachea (gape worm)

Occurrence of respiratory diseases in chicken hobby flocks

There is limited information on the occurrence of respiratory disease in hobby poultry from Sweden and elsewhere. In a study by Blakey *et al.* (2019) from the USA, it was shown that the most common coinfections in hobby flocks with ILT were *Mycoplasma synoviae* (MS) and *Mycoplasma gallisepticum* (MG).

In another study from USA, Derksen *et al*. (2018) showed that the most seroprevalent respiratory coinfection in hobby flocks were *Ornithobacterium rhinotracheale* (ORT, 97.5%), avian paramyxovirus type 1 (aPMV-1, 77.5%), infectious bronchitis virus (IBV, 75%), MS (75.6%), MG (70.7%), and ILTV (46.3%). They also reported that chickens in hobby flocks in close vicinity of a commercial poultry facility were more likely to be seropositive for aPMV-1 and MG, while chickens in hobby flocks located far from a commercial facility were more likely to be seropositive for ORT. The authors suggested that the presence of antibodies in chicken close to a commercial facility may be related to the commercial facilities' vaccination protocols.

High seroprevalence in hobby flocks against respiratory pathogens has also been reported from Europe by Haesendonk *et al.* (2014): ORT 100%, IBV 91.1%, MS 96.4%, MG 73.2%, ILTV 64.3%; and by Wunderwalt & Hoop (2002): ORT 100%, aPMV-1 17.6%, IBV 92.5%, MS/MG 82.5%, and ILTV 64.3%. In Finland, Pohjola *et al.* (2017) demonstrated the seroprevalence for respiratory disease in hobby flocks to be lower compared to the above studies (IBV: 47.1%, ILTV: 11.8%, aPMV-1: 0%). Information from Sweden on the occurrence of respiratory disease in non-commercial poultry is very limited. In a recent report investigating Swedish flocks with respiratory complaints (Jansson *et al.*, 2019), ILTV was detected in 62.2%, APG

in 66.1% and MG in 54.2% of the flocks. Combination of pathogens was found, the most common being MG+APG (23.7%) and MG+APG+ILTV (22.0%).

Diagnostics

Infectious laryngotracheitis can be diagnosed based on the pathognomonic histopathological changes in the early phase of the disease (Guy & García, 2008; Jansson *et al*., 2006). This method has a high specificity but a low sensitivity as the lesions disappear when the disease progresses. Moreover, samples need to be obtained from dead or euthanised birds. If using this diagnostic method, several birds from an affected flock should be analysed in the early stage of the disease.

There are several additional ways to diagnose ILT according to the OIE Terrestrial Manual (2019). Polymerase Chain Reaction (PCR) and serology e.g. antigen enzyme-linked immunosorbent assay (ELISA), can be used to screen flocks and confirm the presence of ILTV. Virus isolation and immunofluorescence are also considered suitable methods. Antibodies may be detected with antibody-ELISA and virus neutralisation.

Treatment

There is no treatment for ILT (Guy & García, 2008; Jansson *et al.*, 2006). If there is a confirmed secondary bacterial infection, antibiotics can potentially relieve signs of disease and shorten the course of the disease.

Outbreaks in Sweden

The first outbreak of ILT in Sweden was described in a laying hen flock in 1940 (Magnusson, 1940). From 1959, there were no diagnosed cases until an outbreak occurred in January 1997 in Dalarna (Jansson *et al.*, 2006; Engström *et al.*, 2011). Since then, there have been outbreaks every year. From 1997 to 2011 there were more than 100 outbreaks diagnosed in hobby flocks across the country and only a very few in commercial flocks. The number of outbreaks is probably much higher than the reported cases (Jansson *et al.*, 2006).

A large majority of the ILT outbreaks in Sweden occur in hobby flocks (National Veterinary Institute, SVA, 2019). The outbreaks vary in severity, which is likely associated with different levels of virulence of the virus, management, current immune status of the flock and occurrence of respiratory coinfections (Jansson *et al.*, 2006). In the most severe cases, Swedish hobby flock owners have reported a rapid disease progression in the entire flock with mortality up to 75%. In milder cases owners have reported a slower disease progression with milder symptoms. It is assumed that the virus is mainly transmitted by latently infected clinically healthy chickens, often as a result of trading. Outbreaks have also occurred after poultry shows.

Outbreaks in commercial flocks have been demonstrated in four cases, which occurred between 2007 and 2013 (National Veterinary Institute, SVA, 2019). Apart from these outbreaks, the commercial sector is considered free from ILTV.

In 1999 the National Board of Agriculture in Sweden changed the status of ILT from being classified as an epizootic disease to a notifiable disease (National Veterinary Institute, SVA, 2019), which means that the disease is not included in an eradication policy. The decision was

based on serological results that suggested the virus to be endemically established in Sweden and that chances of eradication were small.

Vaccination against ILT

Type of ILT vaccines

There are two commercially available types of approved vaccines against ILT, live-attenuated vaccines and recombinant viral vectored vaccines (review by García & Zavala, 2019). There is also potential new vaccines being developed, most of these are recombinant gene-deleted attenuated vaccines.

Live attenuated vaccines

To prepare a live attenuated vaccine several virus sources can be used. For ILTV the virus is attenuated by repeated passages through either chicken embryos (CEO, chicken embryo origin) or in tissue culture (TCO, tissue culture origin) (García, 2016). During each passage nucleotide substitutions (mutations) accumulate as the virus adapts to replicate in an embryo or a cell culture, which leads to reduced virulence (MacLachlan & Dubovi, 2011).

- *Transmission and regaining of virulence*

The CEO and the TCO vaccines are effective in reducing the clinical ILT signs in chickens, but they do not prevent infection, virus replication and transmission of the virus (Guy *et al.*, 1991, Rodríguez-Avila, 2007). This means that the vaccine virus (and wild-type viruses) can still infect the birds and be transmitted to other birds as well as establish a latent infection. Further, as transmission is not prevented by vaccination, the bird-to-bird (*in vivo*) passage in a flock can allow the CEO vaccine virus to regain its virulence, a process called reversion (Guy *et al.*, 1991). This can cause disease outbreaks if the vaccine virus is spread to unvaccinated birds due to insufficient biosecurity measures, mixing of vaccinated and unvaccinated birds or if vaccine administration is done incorrectly, leading to a situation where not all birds in the flock have developed immunity.

Several outbreaks of ILT have been genetically associated to a vaccine virus (Blakey *et al.*, 2019; Coppo *et al.*, 2013b; García & Zavala, 2019; Menendez *et al.*, 2014; Moreno *et al.*, 2010; Neff *et al.*, 2008; Ojkic *et al.*, 2006).

In USA, Blakey *et al.* (2019) sequenced the infected cell protein 4 (ICP4) gene in 15 samples from hobby flocks that were ILTV positive by real time-PCR. Nine of these were 100% identical to a CEO vaccine strain, and five were 98.8% identical. Neff *et al.* (2008) analysed virus isolates with PCR and restriction fragment length polymorphism (RFLP), and sequencing of the thymidine kinase (TK) gene. Isolates were extracted from 104 samples from outbreaks of ILT in Western Europe during the years 1973–2006, including early isolates from Sweden. The analyses revealed that the majority of western European isolates (98 of 104 samples in total) were related to vaccine strains. Of the 104 isolates, 14 isolates came from hobby flocks in Sweden, of which 13 had genetic similarity to the vaccine strains, despite that they had been collected prior to the start of vaccination in Sweden (Jansson, D., personal communication 2019). One Swedish isolate differed in RFLP pattern and was considered to be one of the wild-type strains circulating in Europe, although limited in regional spread and number of infected cases. In another study, Ojkic *et al.* (2006) investigated ILT outbreaks in Ontario, Canada. Here

as well it was demonstrated that some of the included isolates were genetically related to vaccine strains. García & Zavala (2019) recently reported that there are currently four main viral genotypes that are considered to cause ILT outbreaks in the USA, of which three are closely related to live attenuated vaccines.

- *Recombination of vaccine strains*

There is also a possibility that vaccine viruses can recombine with each other and with wild-type ILTVs and create new virulent field strain. This has been reported from Australia (Lee, 2012) where new classes of viruses have started to circulate when a live attenuated vaccine from Europe was introduced. Further, Agnew-Crumpton *et al*. (2016) demonstrated that one of the new virus classes had replaced the previous dominant classes of viruses in some regions in Australia and seems to be more virulent than previous circulating virus classes.

Recombinant viral vector vaccines

As there is a risk for the attenuated live vaccine viruses to regain virulence, there has been a new generation of vaccines developed for chickens: recombinant viral vector vaccines (reviews by García & Zavala, 2019; Coppo *et al.*, 2013b). For ILT there are two types of recombinant viral vector vaccines that are commercially available.

The principle behind recombinant viral vector vaccines is to insert one or several genes (in this case from the ILT genome) coding for antigens that stimulate an immune response, into a genome of another DNA virus, i.e. the vector (MacLachlan & Dubovi, 2011).

Recombinant viral vector vaccines against ILT have been shown in several studies to reduce the severity of clinical signs of ILT and improve the birds' performance (reviews by Coppo *et al.*, 2013b; García & Zavala, 2019). They are however not as effective as the live attenuated CEO vaccines in terms of reducing shedding of challenge virus (Johnson *et al.*, 2010).

Recombinant gene-deleted live attenuated ILTV vaccines

There is a potential new type of vaccine against ILT, recombinant gene-deleted live attenuated ILTV vaccines (García, 2016). In this type of vaccine non-essential genes that contribute to virulence are deleted from the genome of the vaccine virus candidate (MacLachlan & Dubovi, 2011). An advantage is the possibility to differentiate serologically between vaccinated and unvaccinated birds (differentiating infected from vaccinated animals, DIVA, strategy), if the protein that the deleted gene is coding for can be used as a capture antigen in ELISA.

The most extensively investigated candidate for a recombinant gene-deleted live attenuated vaccine for ILT is the ΔgG ILTV vaccine strain, where the virulence factor gene gG has been deleted (Korsa *et al.*, 2018).

Vaccination strategies and control of the disease

Disease control is based on biosecurity, management and vaccination (García & Zavala, 2019). In countries where vaccination is used against ILT, vaccination is commonly done to prevent and control disease in breeders, layers, broilers and hobby flocks (review by Menendez *et al.*, 2014; National Veterinary Institute, SVA, 2019). Although new vaccine types have become available, the live attenuated CEO vaccines are still being used to get the highest disease protection in USA, Europe and Asia (review by García & Zavala, 2019). It is a common

practice in commercial flocks to administer a combination of vaccines during the life span of a flock depending on the needs of the producer and what is permitted in the country. As an example, in USA, commercial layer flocks can receive a subcutaneous vaccination at one day of age with a recombinant viral vector vaccine and then be revaccinated with a CEO or TCO vaccine at between 8–12 weeks of age to protect birds from ILT.

In Sweden, an outbreak of ILT on a multi-age laying hen farm in 2007 was eliminated with the help of vaccination and various biosecurity measures, such as manure storage in closed houses, change in delivery schedules to the farm and cleaning and disinfection of transport vehicles (Engström *et al.*, 2011).

Administration routes

There is a variety of administration routes for ILTV vaccines including eye drop, coarse spray, in drinking water, subcutaneous injection, wing web application and *in ovo* injection. Drinking water and coarse spray are most commonly used in commercial flocks as mass vaccination is easy to perform at low cost (reviews by Coppo *et al.*, 2013b and Menendez, 2014).

As TCO vaccines must be administered through eye drop to each bird, this is not as commonly used as the CEO vaccines (review by Menendez *et al.*, 2014). If the TCO is administered as a mass vaccination, for example in drinking water, the level of immunity may be insufficient in a subset of the birds, which may increase the risk for reversion to more virulent vaccine strains (M. García, pers. comm. in García & Zavala, 2019).

ILT vaccination in Sweden

Vaccination against ILT of commercial poultry is not routinely practiced in Sweden (National Veterinary Institute, SVA, 2019). As mentioned earlier, there has been one case of vaccination in a commercial flock, as a measure to eliminate an ILT outbreak (Engström *et al.*, 2011; National Veterinary Institute, SVA, 2019). Vaccination of hobby flocks started in 2006 due to an increased number of outbreaks and in response to the wishes of individual poultry owners. Two different CEO vaccines have been used (personal communication D. Jansson, National Veterinary Institute, SVA, Uppsala, Sweden, 2019):

- AviPro ILT; Lohmann Animal Health; Hudson strain (Menendez *et al.*, 2014)

- Nobilis ILT; Intervet/MSD Animal Health; Serva strain (Menendez *et al.*, 2014)

AviPro ILT was used from 2006 to around 2014, when it was replaced with Nobilis ILT. The use of Nobilis ILT is licensed to the National Veterinary Institute (SVA) and a prescription from a veterinarian is necessary for a hobby flock owner to get access to the vaccine. The vaccination of hobby flocks in Sweden is voluntary. The vaccine is administered by the bird owners as an eye drop vaccine.

Virus strains

Vaccine virus and wild-type virus

The CEO vaccines were developed from field viruses in the 1950s and the 1960s (Menendez *et al*., 2014). Today the following vaccine strains are still in use: Cover, Hudson, Samberg, A2,

A20 and Serva strain. The vaccine strains originate from USA (Cover and Hudson), Israel (Samberg), Australia (A2 and A20) and Europe (Serva).

As all ILTVs belong to the same serotype, it is not possible to separate infection with field viruses from vaccine viruses with diagnostic assays based on antigenic differences (Menendez *et al.*, 2014; Coppo *et al.*, 2013b). Instead, molecular analysis is necessary, such as RFLP and DNA sequencing. These methods use the multilocus genotyping schemes targeting various combinations of genes. The genes targeted for sequencing vary depending on the geographic region (Menendez *et al.*, 2014). In Europe the gG, TK, ICP4, glycoprotein E (gE), open reading frame B-thymidine kinase (ORFB-TK) and infected cell protein 18.5 (ICP18.5) genes have been used for characterisation of field isolates and vaccine strains (Neff *et al.*, 2008; Moreno *et al.*, 2010).

Complete genome sequencing

Complete genome sequencing of ILT vaccine viruses and field isolates can be done to provide data on genetic variation (Menendez *et al.*, 2014). This can make it possible to compare and associate for example the level of attenuation with nucleotide changes (Coppo *et al.*, 2013b). Complete genome sequencing can also be used to systematically describe virus genotypes that are present in an area or country.

MATERIALS AND METHODS

Study population

Owners of hobby flocks have a possibility to submit dead or euthanized chickens for routine diagnostic necropsies to SVA. The samples used in this study came from such chickens. The enclosed limited medical history provided by the flock owners indicated that most flocks had a moderate to severe outbreak of respiratory disease with mortality at the time of submission. However, detailed clinical information was not available, including development of clinical signs and mortality rates in later stages of the outbreaks. In a majority of the cases included in this study, ILT was confirmed by histopathology and in some cases ILTV was detected by real time-PCR. The chickens were submitted during 2017 and 2018. Cases were those from which samples where available for further analysis and are named as 17-ALDXXXXX or 18-ALDYYYYY, where 17 and 18 represents the year, ALD is the laboratory code followed by a unique sample number.

Samples (swabs from the nasal cavity or trachea from the necropsied chickens) originated from 29 flocks with confirmed ILT outbreaks. Of these, 13 flocks were sampled in 2017 and 16 in 2018. The chickens were submitted from different parts of Sweden (Table 2). There were 27 chickens submitted in 2017 and 39 in 2018, with a total of 21 swabs available from 2017, and 36 swabs from 2018.

Table 2. *Number of flocks per region (county) in 2017 and 2018 that were included in this study. The numbers in brackets represents number of submitted chickens per flock.*

County	**2017**	**2018**
Skåne	3 (1,3,1)	1 (3)
Halland	1 (2)	1 (3)
Östergötland	2 (4,2)	1 (2)
Stockholm	2 (3,2)	4 (4,2,2,2)
Västra Götaland	3 (2,1,2)	3 (1,3,2)
Västmanland	1 (3)	1 (3)
Gästrikland/Hälsingland	1 (1)	1 (3)
Dalarna	0	1 (3)
Närke	0	1 (1)
Södermanland	0	1 (3)
Småland	0	1 (2)

There was no information if the flocks were vaccinated against ILT or not. The laboratory work was carried out on the Department of Microbiology, SVA.

Method

Extraction of nucleic acid

Nucleic acids were extracted using Viral NA Magnetic Beads kits in an ArrowTM 2 extraction robot (DiaSorin, Saluggia, Italy). For the extraction, 250 µL of transport media was added to separate 1.2 mL Eppendorf tubes together with 10 µL ≥800 U/mL proteinase K (Sigma-Aldrich, Saint Louis, MO, USA), and run in the extraction robot according to the manufacturer's recommendations. Positive and negative controls were included in each extraction. The vaccine used in Sweden, Nobilis ILT, was used as the positive control. The other vaccine that has been used in Sweden (AviPro ILT) was not available for analysis.

Real time PCR

The presence of ILTV DNA in the extracted samples (including positive and negative controls) was determined by real-time PCR ILTV assay, as described previously by Callison *et al.* 2007. Primers and probes were designed based on a conserved region of the glycoprotein C (gC) gene. The assay was customised using an AgPath-ID One-Step RT-PCR kit (Applied Biosystems, ThermoFisher Scientific) that is routinely in use.

To screen for co-infections, all samples were analysed for APG, IBV and MG nucleic acid by real time-PCR. Previously published primer and probe sequences targeting the GTP-binding protein (yihA) genes of APG (Corney *et al.*, 2008), the 5′-untranslated region of IBV (Callison *et al.*, 2006) and the CTP synthetase (pyrG) and the cytadhesin (mgc2) gene of MG (Sprygin *et al.*, 2010) were used for specific detection of the respective pathogen. Positive and negative controls were used.

Sequencing

Choice of genes and primer design

The TK, gG and ICP4 genes were selected for amplification and sequencing as these genes can help to differentiate virus strains, as previously described (Neff *et al.*, 2008; Moreno *et al.*, 2010; Menendez *et al.*, 2014). The gG gene codes for a glycoprotein on the surface of the virion (Devlin *et al.*, 2006), while the TK gene codes for an enzyme that catalyses one of the reactions involved in the DNA synthesis (Coppo 2013b). The ICP4 is a major regulatory protein of gene expression during the early infection phase (Johanson *et al.*, 1995). These genes have conserved regions, which means that they have not changed during natural selection and have a similar DNA sequence across strains, which is an advantage when detecting any differences in the sequences (Johnson *et al.*, 1995; Griffin & Boursnell, 1990; Devlin *et al.*, 2006). There are many sequences to use for comparison in the GenBank database. GenBank is an online database that is built and distributed by the United States National Institute of Health and has gathered all publicly available DNA sequences (Benson *et al.*, 2013).

Primers targeting the gG and TK genes were designed by S. Zohari, Department of Microbiology, SVA, Sweden (2010) and primers used for ICP-4 were according to Madsen *et al.*, 2013 (Table 3).

Table 3. *Primers used with sequence*

Gene	Name of primer	Forward (F)/ Reverse (R)	Sequence
gG	ILTgG-F58	F	5'-gtaactgactacgcatc-3'
gG	ILTgG-R1165	R	5'-ttagcaacagacacgca-3'
TK	ILT-TKIP-F4614	F	5'-cttagcggaacctatgcaag-3'
TK	ILT-TKIP-R5273	R	5'-tagcgtctggtcgattgaag-3'
ICP4	ILTV-ICP4-1F-Madsen2013	F	5'-actgatagcttttcgtacagcacg-3'
ICP4	ILTV-ICP4-1R	R	5'-catcgggacattctccaggtagca-3'
ICP4	ILTV-ICP4-2F-Madsen2013	F	5'-cagaggaccagcaaagac-3'
ICP4	ILTV-ICP4-2R	R	5'-ctaactgttccactggcatc-3'

Conventional PCR

Amplification by conventional PCR targeting the gG and TK genes (all samples) and the ICP4 gene (13 samples selected based on geographic origin and year) was done according to the method routinely used at the Department of Microbiology, SVA, Sweden. PCR products were separated by gel electrophoresis in Lonza FlashGel™ DNA kits according to the manufacturer's instructions. A DNA molecular weight marker as well as a positive control was loaded on each gel (Figure 1).

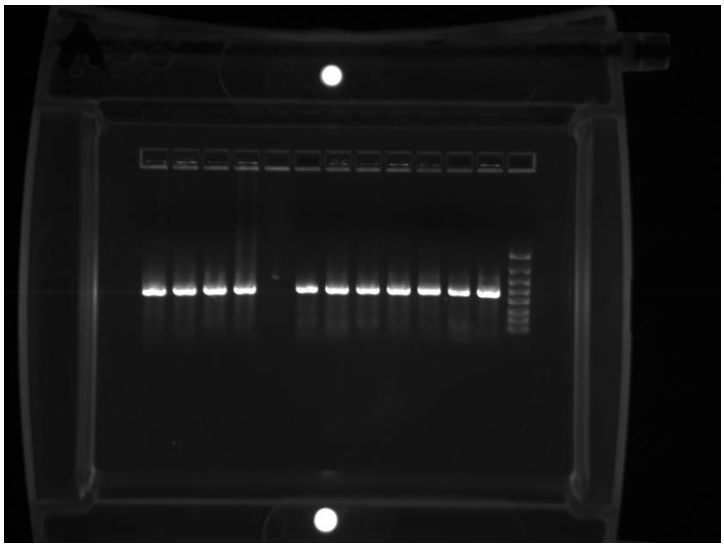

Figure 1. *Example of product of gel electrophoresis. Well number 5 is negative and well numbers 1-4 and 6-12 are positive. Well number 13 contains the DNA marker. Numbering from the left to the right side of the picture.*

Sanger sequencing with capillary electrophoresis

Sequencing of the PCR products for gG, TK, ICP4 and positive control (CEO vaccine Nobilis ILT) was performed. The PCR products were cleaned with FastAP™ Thermosensitive Alkaline Phosfatase and Exonuclease I (Thermo Fisher Scientific Inc.), followed by cycle sequencing with a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Art.). Further, sequencing clean up was done with a Montage SEQ_{96} sequencing Reaction Cleanup

kit (EMD Millipore Corp) before the capillary electrophoresis in a 3500 Genetic Analyser from Applied Biosystems, Hitatchi. Each analysed sample resulted in a chromatogram and a DNA sequence. All procedures were done according to the manufacturer's recommendations.

Processing of DNA sequencing products

Editing of chromatograms and assembly of nucleotide sequences was performed in the free software "BioEdit Sequence Alignment Editor" (Informer Technologies Inc, 2020). The sequences were aligned with reference sequences from the GenBank database in the online tool BLAST® (U.S. National Library of Medicine, 2019) to receive phylogenetic information. Commonly used vaccine strains were used as reference strains as well as field strains selected based on similarity with study samples and relation with vaccine strains.

Alignment of multiple sequences and phylogenetic analysis of the obtained gG, TK and ICP4 gene sequences were done in the free software "Mega – Molecular Evolutionary Genetics Analysis" version 6 (Tamura *et al*., 2013) and version X (Kumar *et al.*, 2018) Phylogenetic reconstruction was done using maximum likelihood (Tamura-Nei) analysis with bootstrap values expressed as percentage of 1000 replications.

Next generation sequencing

The complete genome was sequenced for four samples. Selection was based on virus concentration and geographic location. The samples represented four different regions and had been obtained in 2017 and 2018. There was no known epidemiological link between the flocks of origin. The sequencing was done with a next generation sequencing (NGS) system: library preparation with a Nextera XT DNA Library Prep kit (Illumina) followed by sequencing with a MiSeq Reagent Kit v3 600 cycles (Illumina). Quality control was done before sequencing with an Aglient High Sensitivity DNA kit on a Bioanalyzer 2100 (Agilent Technologies). Alignement as well as phylogenetic information, analysis and reconstruction were done as described earlier in "Processing of DNA sequencing products".

RESULTS

Real time PCR

ILTV

ILTV was detected by real time-PCR in 52 out of 57 samples (21 samples from 2017 and 31 samples from 2018), which corresponds to 51 out of 54 birds (21 birds in 2017 and 30 birds in 2018). The five negative samples in 2018 came from two flocks, where there was a real time-PCR positive sample from at least one other bird in the same flock. Out of the 52 positive samples there were 4 samples with a ct value over 30, which is considered less reliable as the virus concentration is low.

Co-infections

Out of the 29 sampled flocks, 8 flocks (28%) had one or more chickens that were confirmed MG positive by real time-PCR (4 flocks in 2017 and 4 flocks in 2018); 25 flocks (86%) were confirmed APG positive in one or more chickens (10 flocks in 2017 and 15 flocks in 2018) and 8 flocks (28%) had chickens positive for IBV (4 flocks in 2017 and 4 flocks in 2018).

Of the 54 sampled chickens, 15 (28%) were confirmed MG positive by real time-PCR (7 chickens in 2017 and 8 chickens in 2018); 47 (87%) were confirmed APG positive (15 in 2017 and 32 in 2018) and 11 (20%) were confirmed IBV positive (5 in 2017 and 6 in 2018) (Table 4).

Table 4. *Percentage of flocks and chickens confirmed positive by real time-PCR for MG, APG or IBV*

Pathogen	Number (%) positive flocks	Number (%) positive chicken
MG	28	28
APG	86	87
IBV	28	20

c-PCR

The PCR products amplified for the gG, TK, ICP4-1 and ICP4-2 genes were detected as a DNA band on gelelectrophoresis of approximately 1200 bp, 650 bp, 700 bp and 600 bp respectively. The gG and TK DNA band were weak for six and one sample, respectively, and no visible band for three and one sample, respectively.

Sequencing of the gG, TK and ICP4 genes

gG gene

Fifty-two samples and the positive control (Nobilis ILT vaccine) were sequenced for a part of the gG gene. Seven samples were not included in the final analysis due to lack of sequence quality (one of these samples was 18-ALD003986, see TK gene below). The gG sequence that was aligned extended from nucleotide position 131023 to 132025 (1003 bp) in the ILTV

genome strain 4787/80 (GenBank accession number (acc.) KP677885.1). Four complete ORFs were found from nucleotide position 53 to 930, 526 to 930, 715 to 930 and 745 to 930.

Computer analysis showed a 100% nucleotide identity both between samples and the Nobilis ILT vaccine (sequenced in this study) in the aligned sequence, except for four samples (Table 4). For these samples, single nucleotides could not be completely confirmed: four in 17-ALD000302 (position 592, 747, 754 and 909), two in 17-ALD007245 (position 687 and 909), two in 18-ALD003193 (position 840 and 971) and one in 17-ALD008418 (position 861). Further, sample 17-ALD007245 differed in one nucleotide in position 861 (C to T), this led to no change in amino acid. Sample 18-ALD003193 differed in two nucleotides in position 972 (T to C) and position 978 (C to G). This led to change in amino acid to an unknown amino acid (position 972) and a change from cysteine to tryptophan (position 978). For position 840 in 18-ALD003193, if C to T there would be no change in amino acid, but if C to G, the amino acid would change from cysteine to tryptophan.

The sequences were also compared with commonly used vaccine strains from other countries (Table 5). 41 samples showed a 100% nucleotide identity. The four samples that differed showed a 99.6–99.9% nucleotide identity with the vaccine strains.

Table 5. *Results of partial sequencing of the gG gene. Numbers show single nucleotide positions with polymorphisms. Polymorphic positions are highlighted in bold. An asterisk (*) represents the same nucleotide as in the row titled Shared sequence of 41 samples. Reference vaccine strains are Nobilis ILT (sequenced in this study), LT Blen (acc. JQ083493.2), LaryngoVac (acc. JQ083494.2) and live attenuated Serva strain (acc HQ630064.1)*

Nucleotide position / Sample/strain	592	687	747	754	840	861	909	971	972	978
Shared sequence of 41 samples	T	C	G	C	C	C	G	C	T	C
17-ALD000302	**A/T**	*	**G/T**	**A/C**	*	*****	**G/C**	*	*	*
17-ALD007245	*	**C/T**	*	*	*	**T**	**G/C**	*	*	*
18-ALD003193	*	*	*	*	**G/T**	*	*	**C/T**	**C**	**G**
17-ALD008417	*	*	*	*	*	**C/T**	*	*	*	*
Vaccines:										
Nobilis ILT	*	*	*	*	*	*	*	*	*	*
Laryngo-Vac	*	*	*	*	*	*	*	*	*	*
LT Blen	*	*	*	*	*	*	*	*	*	*
Serva	*	*	*	*	*	*	*	*	*	*

TK gene

Fifty-two samples and the positive control Nobilis ILT vaccine were sequenced for a part of the TK gene. The TK sequence that was aligned extended from nucleotide position 33607 to 34234 (628 bp) in the ILTV genome strain 4787/80 (acc. KP677885.1). No complete ORFs were found in the TK sequence.

Computer analysis showed a 100% nucleotide identity between samples in the aligned sequence, except for two samples and the Nobilis ILT vaccine (sequenced in this study), that differed by one nucleotide each. Sample 18-ALD002339 in position 601 (C to T), this led to a change in amino acid threonine to isoleucine. Sample 18-ALD003986 in position 347 (T to C), this led to no change in amino acid. The same nucleotide change as in 18-ALD003986 was found in the Nobilis ILT vaccine.

When compared to GenBank retrieved vaccine strains from other countries (LT Blen, acc. JQ083493.2; LaryngoVac, acc. JQ083494.2; live attenuated Serva strain, acc HQ630064.1), the same nucleotide change as in 18-ALD003986 and Nobilis ILT vaccine was seen. The nucleotide identity for the vaccine strains was 100% for 18-ALD003986, 99.6% for 18-ALD002339 and 99.8% for the remaining 50 samples.

ICP4 gene

Thirteen samples and the positive control Nobilis ILT vaccine were sequenced for a part of the ICP4 gene. The ICP4 sequence that was aligned extended from nucleotide position 146998 to 148032 (1035 bp) in ILTV genome strain 4787/80 (acc. KP677885.1). Three complete ORFs were found, from nucleotide position 305 to 613, 668 to 838 and 802 to 1035.

Computer analysis showed a 100% nucleotide identity between samples in the aligned sequence, except for one sample (18-ALD002509) that had one additional nucleotide (a C) inserted at position 11.

When compared to GenBank retrieved vaccine strains from other countries (LT Blen, acc. JQ083493.2 and live attenuated Serva strain, acc HQ630064.1) there was a 100% nucleotide identity for 12 of the samples. For 18-ALD002509 there was a 99.9% nucleotide identity.

Sequence analyses

Figure 2 shows the genetic relationship for the combined gG and TK genes; and Figure 3 for the combined gG, TK and ICP4 genes.

The dendrogram grouped the nucleotide sequences, where the study samples are in one group and the vaccine strains are in another group. Bootstrap value is 62%.

Reference strains included in the dendrograms (Fig. 2–4) were complete genomes from GenBank available for all three gene parts and the complete genome analysis. They were selected based on relation to vaccine virus (wild type viruses: Rus/Ck/Penza/2013/270, acc. MF405080.1 and 4787/80 Italy, acc. KP677885.1; vaccine related virus: 40798/10/Ko Korea, acc. MH937566.1) and similarity with study samples. The USDA reference (acc. JN542534.1) was selected as an outgroup in the complete genome analysis (Fig. 4) and were also included in Figure 2 and 3 for uniformity.

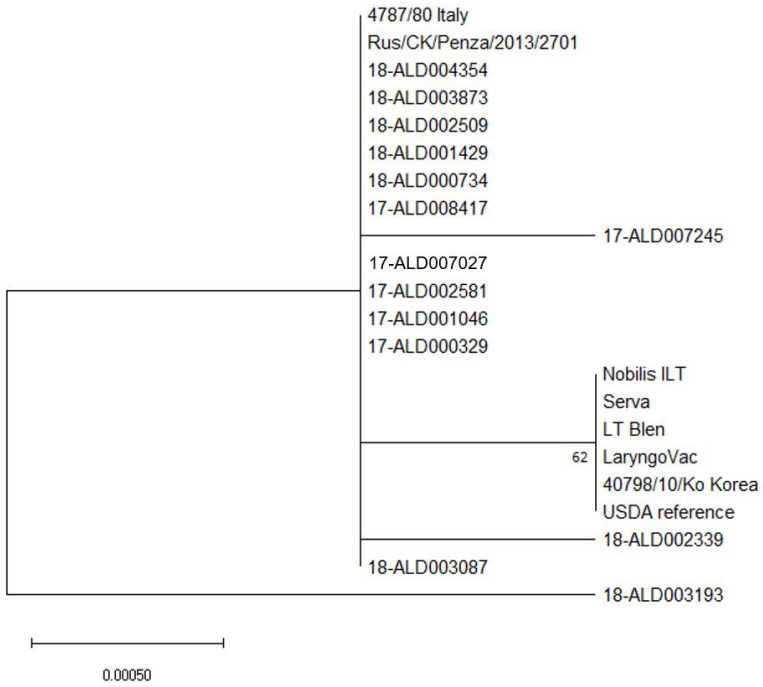

Fig. 2. *Genetic relationship of the combined gG and TK sequences for 14 samples, reference strains and vaccine strains. The partial gene sequences were aligned with the Nobilis ILT vaccine strain sequences (sequenced in this study) and GenBank retrieved reference strains (40798/10/Ko Korea, related to vaccine strain, acc. MH937566.1; Rus/Ck/Penza/2013/270, acc. MF405080.1; 4787/80 Italy, acc. KP677885.1 and the USDA reference, acc. JN542534.1) and vaccine strains (LT Blen, acc. JQ083493.2 and live attenuated Serva strain, acc. HQ630064.1 and LaryngoVac, acc. JQ083494.2).*

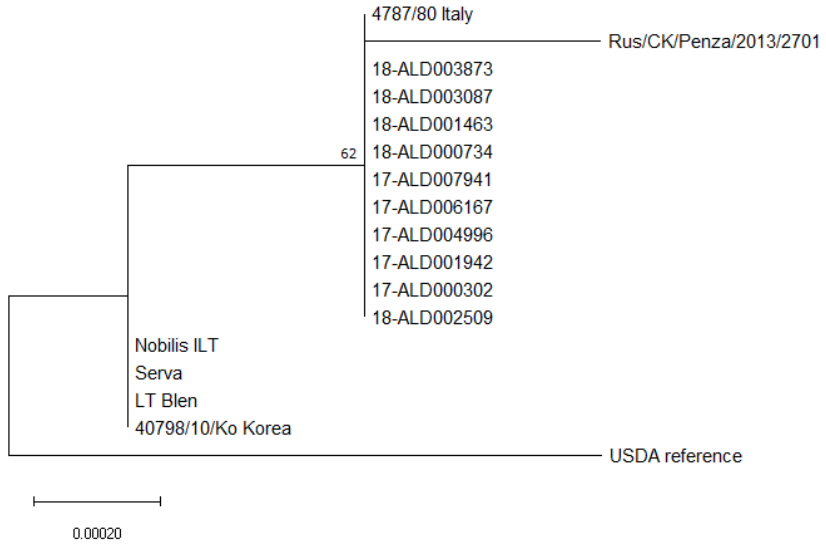

Fig. 3. Genetic relationship *of the combined gG, TK and ICP4 sequence for 13 samples, reference strains and vaccine strains. The partial gene sequences were aligned with the Nobilis ILT vaccine strain sequences (sequenced in this study) and GenBank retrieved reference strains (40798/10/Ko Korea, related to vaccine strain, acc. MH937566.1; Rus/Ck/Penza/2013/270, acc. MF405080.1; 4787/80 Italy, acc. KP677885.1 and the USDA reference, acc. JN542534.1) and vaccine strains (LT Blen, acc. JQ083493.2 and live attenuated Serva strain, acc. HQ630064.1).*

Complete genome sequencing

Each of the four complete genome sequences made from field viruses in the samples were aligned with their respective gG and TK sequences. As only 13 samples were sequenced for the ICP4 gene, the ICP4 sequence aligned was not from the same sample as the complete genome except for one sample. The aligned sequences showed a 100% nucleotide identity.

Figure 4 shows the genetic relationship for the full genome for the four samples. The dendrogram grouped the nucleotide sequences into three clades (A, B and C) where the four study sequences and two reference sequences are in clade A, the vaccine strains and one reference strain (related to vaccine strains) are in clade B and one reference strain is in clade C. The nucleotide identity was 96.3% (USDA reference) and between 99.8-99.9% for the remaining reference strains.

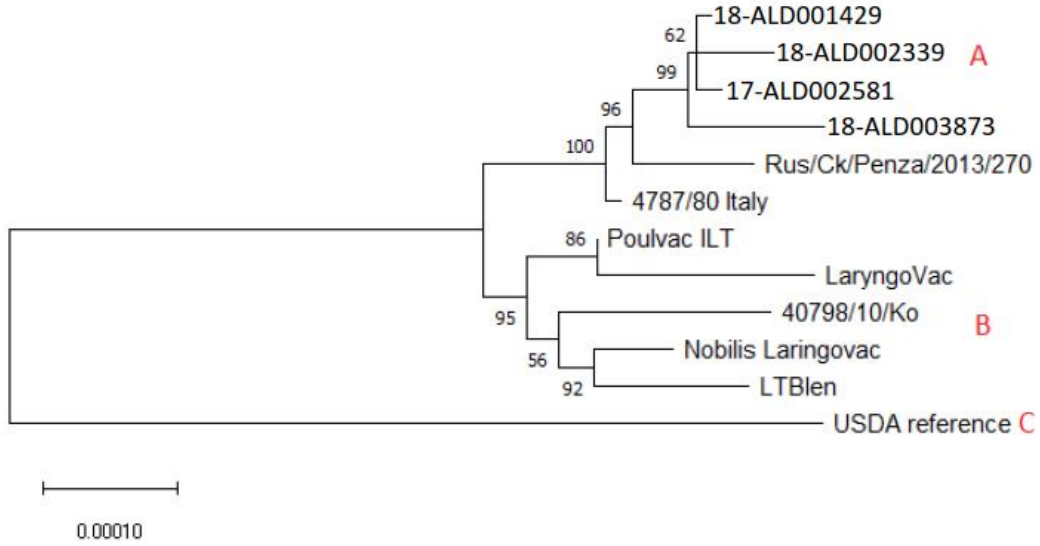

Fig. 4. *Genetic relationships of the complete ILTV genome from 4 samples, reference vaccine strains and reference strains. Full genomes used for the analyses were:17-ALD002581,18-ALD001429, 18-ALD002339 and 18-ALD003873 from the present study and GenBank retrieved vaccine strains and reference strains (LT Blen, acc. JQ083493.2; LaryngoVac, acc. JQ083494.2; Nobilis Laringovac, acc. KP677881.1; Poulvac, acc. KP677882.1; strain 4787/80 Italy, acc. KP677885.1; strain Rus/CK/Penza/2013/270, acc MF405080.1, the Korean vaccine related strain 40798/10/Ko, acc. MH937566.1 and the USDA reference, acc. JN542534.1). Clades are designated as A, B and C.*

DISCUSSION

This study investigated both the genetic variation and relation to vaccine strains in samples from ILTV outbreaks in 2017 to 2018 in chicken hobby flocks in Sweden. Respiratory co-infections were also assessed. The aim was to improve the understanding of the ILT epidemiology in Sweden. The samples were analysed by PCR to detect ILTV and partial sequences of three genes previously used to assess similarity to vaccine-related viruses obtained. Complete genome sequences were determined from four samples.

Field virus versus vaccine virus

From the analyses of the partial sequences of the gG, TK and ICP4 genes it appears that the ILT virus strains circulating in Sweden during 2017–2018 were vaccine-like strains, as the sequences from samples obtained during clinical ILT outbreaks showed a sequence similarity of at least 99.6% to vaccine virus strain. This assumption confirms the results from a study by Neff *et al.* (2008) that suggested that most viruses circulating in Swedish hobby flocks during the first years of the return of ILT in this population of chickens and prior to the use of ILT vaccines in Sweden were related to live attenuated vaccine strains.

When comparing the partial TK gene sequence from Neff *et al.* (2008; GenBank acc. EU360946) that belonged to the same clone (1, CH04) as the Swedish isolates in the same study, with the part that overlaps the partial TK gene sequenced in this study, it was identical in 50 out of 52 samples. The partial TK gene in vaccine strains from the same study were identical with our sample 18-ALD003986 and the vaccine used in Sweden, ILT Nobilis, also sequenced in the present study. This indicates that it might be the same virus that circulated in Sweden in 2017–2018, as during the years 1998–2006 when the samples used in Neff *et al.* (2008) were obtained. However, this assumption is based on only one sequence from the TK gene. It would be interesting to investigate whether our samples have the same cleavage pattern as the samples in Neff *et al.* (2008) if conducting an RLFP analysis.

In previous publications, reviewed by Menendez *et al.* (2014), the method of choice to differentiate strains has until recently been multi-locus RLFP, which is now steadily replaced with DNA sequencing of combinations of genes or whole genome sequencing. It has been shown that if two separate regions of the ICP4 gene are sequenced in field viruses and compared with vaccine viruses, it is possible to differentiate between ILTV field and vaccine strains (Chacón & Ferreira, 2009). The present study sequenced parts of three genes that have been used earlier to differentiate field and vaccine viruses (gG, TK and ICP4 genes). When combining the gG, TK and ICP4 gene the dendrograms (Fig. 3 and 4) separated the study samples and vaccine strains into different groups. The bootstrap value was however quite low (62%) for separating the vaccine strains in a different group. When analysing the genes separately, vaccine strains fell into the same group as study samples for the gG and ICP4 sequences. For the TK sequence the samples and vaccine strains are separated into two groups, except for sample 18-ALD003986 that is grouped together with the vaccine strains. This indicates that it is the differences in the TK gene that separated the study samples and vaccine strains into different groups when combining all three genes together. As the difference is only one nucleotide, this strengthens the assumption that the majority of ILT viruses analysed in this study were vaccine-like strains.

When aligning and comparing the complete genome of four samples from this study with complete genomes of vaccine strains and reference strains from GenBank, the genetic relationship showed that samples from this study were grouped in a different clade than the vaccine strains (Fig. 4, clade A and B). This suggests that there are additional sequence differences in other parts of the genome compared to vaccine strains. It may indicate that the partial gG, TK and ICP4 genes investigated in this study were not optimal for differentiating between field and vaccine viruses for currently circulating ILTVs in Sweden. However, the reference strain "USDA reference", which showed a nucleotide identity of 96.4% with the study samples and were grouped in clade C (Fig. 4) can be considered an outgroup. This might indicate that the complete genome of study samples and vaccine strains are very similar. In future studies it would be interesting to evaluate a higher number of complete genomes and analyse where possible differences are found when comparing field strains with vaccines.

Coppo *et al.* (2013b) commented on the importance to systematically describe the virus genotypes that circulate in an area or country. This makes it possible to evaluate any vaccination strategy and to tailor a possible control strategy. As mentioned earlier, vaccination of hobby flocks in Sweden was started on a voluntary basis in 2006 (Engström *et al.*, 2011; National Veterinary Institute, 2019b). This, together with our results suggest that ILT viruses from outbreaks which were presumably related to vaccine virus were circulating already before vaccination started in Sweden. In this case it means that vaccination itself has not necessarily contributed to the spread of the vaccine-related viruses. How the vaccine-related virus was originally introduced in Sweden is not known, but the results from this and Neff *et al.* (2008), may suggest introduction from one or a few virus pools. However, more detailed data is needed to support this hypothesis.

Due to the ability to create latency it should assumingly be possible to get a positive real time PCR result in both healthy vaccinated chickens and healthy unvaccinated previously infected chickens, if they are currently shedding the virus. It would be interesting to analyse presence of virus in healthy vaccinated and unvaccinated chicken and to compare the genetic relationship with the samples in this study and vaccine virus. As one common way of introducing the ILTV into a hobby flock is by a latent carrier, the ILTV from the vaccinated healthy birds should assumingly be genetically alike the samples in this study.

Gene by gene

For the gG gene there were four samples with nucleotides not completely confirmed in one to four positions. The chromatograms for these samples showed some background noise, meaning that there were other DNA fragments present, which interfered with the interpretation of the sequence. Of these, it was only sample 18-ALD003193 that was interpreted as having a nucleotide that completely differed (C to G/T). As this sample also had two additional nucleotide changes, it suggests that it belongs to a separate virus strain or that there was too much background noise for the sample to produce reliable results. In sample 17-ALD007245 there was one nucleotide that differed. However, there was another sample from this flock that did not have this change. This suggests that there was too much background noise for sample 17-ALD007245 to produce reliable results.

The sample 18-ALD002339 that differed by one nucleotide in the TK gene is suggested to belong to a different strain, as can be seen in the dendrogram (Fig. 2). The sample 18-ALD-

003986 had the same nucleotide change as the vaccine strain. This sample was not present in the presented figures as only the sequence for the TK gene could be obtained. As this sample had an identical sequence as the vaccine strains it suggests that this was a potential escaped vaccine virus that had regained virulence.

For the ICP4 gene it was only sample 18-ALD002509 that differed with an insert of one nucleotide. This sample was in the same branch as the other samples in the dendrogram.

Genetic variation of the viruses

This study shows that the majority of birds included in the study were infected with ILTVs belonging to the same group, with small genetic variation (Figures 2, 3 and 4). This suggests that there are only a few ILTV strains that circulate in Sweden. Engström *et al.* (2011) described that most outbreaks of ILT in Sweden were diagnosed following introduction of new chickens into existing flocks or following poultry shows. As ILT can become a latent infection and be reactivated when the bird is stressed (Guy & García, 2008), it is likely that the majority of ILT outbreaks are caused by trading within the non-commercial population and that this leads to circulation of closely related viruses. Latent infections are favoured in hobby flocks compared to commercial flocks, as hobby flocks often are long lived and of a multi-age structure, which allows the circulation of virus from latent carriers to other birds in the flock and between generations. Further, commercial flocks are often managed according to an "all in-all out" principle, which can stop circulation of pathogens present in the flock.

There is a risk for ILTV as well as for co-infections, to transmit from hobby flocks to commercial flocks. As the commercial flocks in Sweden are not vaccinated against ILTV an infection would cause major disruption in production and animal suffering.

Coinfections

Respiratory co-infections appear to be common in hobby chicken flocks in some other countries (Blakey *et al*., 2019, Derksen *et al*., 2018, Haesendonk *et al*., 2014, Wunderwalt & Hoop, 2012, Pohjola *et al*., 2017). This was also demonstrated in our study although the occurrences of investigated co-infections were lower than in some previous studies. However, diagnostic methods and selection of study flocks varied between the different studies.

APG was detected by PCR in 86% of the flocks in this study. There is very limited information regarding the occurrence of APG in hobby flocks in Sweden (Jansson *et al.*, 2019), but together this and previous results suggest that it is a widespread pathogen among chicken hobby flocks with respiratory disease. The ability of APG to establish chronic clinical or subclinical infections and the high horizontal transmissibility between birds might be a possible explanation for our finding. In this study, MG and IBV showed similar occurrence among the sampled flocks (28%). MG has not been diagnosed in commercial flocks in recent years in Sweden but is known to occur in hobby flocks (National Veterinary Institute, 2019). The occurrence of IBV in hobby chicken flocks in Sweden is not known but IB is occasionally diagnosed (National Veterinary Institute, 2019). Commercial flocks, i.e. breeder chickens and laying hens, are vaccinated against IBV in Sweden.

ILT outbreaks and respiratory co-infections might be associated with insufficient biosecurity. Derksen *et al.* (2018) evaluated the biosecurity in hobby flocks and found that in a majority of

flocks the owners did not prevent contact with wild birds and did not use dedicated shoes when working around their birds. Obtaining new birds to a flock is also a factor to consider. Many hobby flock owners in Sweden buy hatching eggs and birds and from other non-commercial farms and they sometimes bring home birds from swap-meets and visit poultry shows. Subclinically infected carrier birds may thus be introduced into the existing flock. As latent ILTV can be reactivated due to stress, as a co-infection, co-infections are an important factor to consider when evaluating factors that affect spread and outbreaks of ILTV.

Concluding remarks

The aim of this study was to improve the understanding of the ILT epidemiology in Sweden. A major limitation was the possibility to obtain representative samples from the hobby chicken population. The only available samples originated from chickens submitted to SVA from private owners, and it can be assumed that these are not fully representative of the entire non-commercial hobby chicken population. Moreover, the vaccination status of the flocks from which samples were collected was unknown. To get a broader picture of the epidemiology of ILTV in Sweden it would be interesting to investigate ILT viruses from clinically healthy vaccinated and unvaccinated chickens as previously discussed, as well as the presence of respiratory co-infections in healthy hobby flocks. However, to obtain representative samples from the non-commercial poultry population in Sweden would most likely be associated with major difficulties as flocks are not registered and the population structure is unknown. Despite these limitations, this study had provided new information on ILT viruses associated with clinical outbreaks in non-commercial chickens.

To conclude, when evaluating parts of the TK, gG and ICP4 genes this study suggested that the circulating ILTVs in Sweden are vaccine-like strains. Complete genome data from a subset of the samples showed similar results, however, there were additional sequence differences in other locations of the genome. Hence, further analyses are needed to determine whether the Swedish field strains originate from vaccine strains. Moreover, Swedish field strains showed limited genetic variation, which may speculatively suggest a common origin. This means that the first hypothesis (see page 1) stated for this study should be rejected. The second hypothesis was however more difficult to support as partial gene sequencing and complete genome sequencing did not show identical results (Fig. 2–4). The selection of individual genes for sequencing may need to be revised and additional complete genomes could provide more useful data. Further, coinfections were common in the flocks included in this study, with the most common pathogen being APG. The general occurrence of ILTV and concurrent pathogens in the healthy hobby chicken population is not known.

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POPULÄRVETENSKAPLIG SAMMANFATTNING

Infektiös laryngotrakeit (ILT) är en luftvägssjukdom som drabbar tamhöns, men även fasaner, påfåglar och kalkoner i viss utsträckning. Sjukdomen kan orsaka stort lidande hos fåglarna med akuta kliniska tecken från luftvägarna, som kan variera från rinnande ögon och näbb till mer allvarliga tecken som andfåddhet och upphostningar av blodigt slem. I värsta fall kan slemmet täppa till luftstrupen och fågeln dör. Förutom lidandet för djuren orsakar sjukdomen också ett stort produktionsbortfall, såsom minskad äggläggning och försenad tillväxt.

ILT kan skapa en så kallad latent infektion, vilket betyder att smittämnet ligger vilande i kroppen efter det att fågeln tillfrisknat. Det finns risk att viruset aktiveras igen om fågeln blir stressad, t ex vid miljöombyte, äggläggningsstart eller vid utbrott av annan sjukdom. En höna visar inte utanpå om den bär på en latent infektion. Det innebär att en höna som bär på ett vilande ILT-virus och introduceras till en tidigare frisk flock, kan smitta den friska flocken med ILT-viruset om viruset aktiveras av stressen.

Det går att vaccinera tamhöns mot ILT. Det finns levande försvagat vaccin, vilket innebär att vaccinet härmar en naturlig infektion men utan att orsaka sjukdom. Studier har visat att om vaccinationen inte utförs korrekt kan det försvagade vaccinviruset återfå sin sjukdomsframkallande förmåga. Det har lett till att nya virusstammar, alltså nya typer av viruset som är släkt med vaccinviruset, har börjat cirkulera bland höns. De nya virusstammarna kan ibland orsaka allvarlig sjukdom.

I stora delar av världen är de nya stammarna som uppkommer från vaccinvirus ett stort problem. I Sverige är ILT en ovanlig sjukdom bland de kommersiella besättningarna. Utbrott har diagnosticerats sedan slutet av 1990-talet i flockar med hobbyhöns. Den ursprungliga källan till de ILT-virus som orsakar utbrotten bland hobbyhöns i Sverige är okänt. Det utförs sällan vaccination mot ILT i kommersiella flockar, men sedan 2006 används vaccin mot ILT i vissa hobbyhönsflockar.

Syftet med den här studien var att undersöka om de ILT-virus som smittat hobbyhöns i Sverige de senaste åren (2017–2018) är släkt med de virus som används som vaccin, samt att undersöka om höns med ILT också har andra luftvägssjukdomar samtidigt som ILT. För att ta reda på det analyserades ILT-virusets arvsmassa i prover från hobbyhöns som haft kliniska tecken från luftvägarna och som skickats till Statens Veterinärmedicinska Anstalt för analys. ILT var konstaterad i de flesta fallen. Arvsmassan i dessa prover jämfördes med arvsmassan från vaccinvirus för att påvisa likheter och skillnader. I samma prover undersöktes även förekomsten av tre andra smittämnen som kan orsaka luftvägssjukdomar hos tamhöns.

Vår studie visade att arvsmassan hos ILT-virus från hobbyhönsproverna var sinsemellan mycket lika. Detta talar för att de ILT-virus som cirkulerar i Sverige har ett gemensamt ursprung. Dessutom var arvsmassan hos ILT-virus från hobbyhönsen och vaccinvirus lika varandra, vilket kan tala för att ILT-virus hos svenska hobbyhöns kan ha sitt ursprung från vaccinvirus. Vår slutsats är dock att ytterligare analyser behöver göras. Vad som också framkommit är att viruset hos hobbyhönsen ser ut att vara väldigt likt äldre virus som cirkulerade innan vaccinationen av hobbyhöns påbörjades i Sverige.

Resultaten visade också att proverna förutom ILT-virus ofta innehöll arvsmassa från andra smittämnen som kan orsaka luftvägssjukdomar. Det vanligaste smittämnet var en bakterie (*Avibacterium paragallinarum*) som orsakar sjukdomen infektiös coryza. Totalt 83 % av de undersökta flockarna bar på denna bakterie. Infektiös coryza kan, liksom ILT, spridas med kroniskt smittade friska smittbärare. En femtedel av de undersökta hobbyhönsflockarna i vår undersökning hade även arvsmassa från smittämnena infektiöst bronkitvirusvirus och bakterien *Mycoplasma gallisepticum*.

Resultaten från den här studien bidrar till ökad förståelse för sjukdomen ILTs ursprung och orsak i Sverige, vilket är viktigt för att kunna vidta åtgärder för att minska sjukdomens framtida framfart i Sverige.

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