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ORIGINAL ARTICLE

Emergence of avian infectious bronchitis in a non-vaccinating country

Leena K. Pohjola[1*], S. Christine Ek-Kommonen[2], Niina E. Tammiranta[2], Eija S. Kaukonen[3], Laila M. Rossow[4], and T. Anita Huovilainen[2]

[1]*Department of Production Animal Medicine, Faculty of Veterinary Medicine, University of Helsinki, Saarentaus, Finland,* [2]*Veterinary Virology, Finnish Food Safety Authority Evira, Helsinki, Finland,* [3]*HKScan Finland Oy, Eura, Finland, and* [4]*Production Animal and Wildlife Health, Finnish Food Safety Authority Evira, Helsinki, Finland*

Infectious bronchitis virus (IBV) is a coronavirus of the chicken. It is a highly contagious pathogen and in addition to causing respiratory and kidney diseases can affect the reproductive organs, resulting in loss of production and poor egg quality. Despite the global distribution of IBV, Finland has been free of clinical cases for almost three decades. Since April 2011, outbreaks involving genotypes QX, D274-like and 4/91-like have occurred in southern Finland. The clinical samples studied were submitted to the Finnish Food Safety Authority Evira from different regions of Finland during 2011 to 2013 and originated from a voluntary health monitoring programme, a national survey for avian influenza and diagnostic specimens from both commercial poultry production and hobby flocks. The sources of the infections are not known, but strains D274 and 4/91 are widely used in vaccines elsewhere.

Introduction

Infectious bronchitis virus (IBV), first described in the 1930s in the USA (Schalk & Hawn, 1931), is a coronavirus of chickens (*Gallus gallus*). IBV is a highly contagious virus and in addition to causing respiratory and kidney diseases can affect the reproductive organs, resulting in loss of production and poor egg quality (Cavanagh & Gelb, 2008). IBV belongs to the family *Coronaviridae* in the order *Nidovirales* and to the genus of the gamma-coronavirus (Gonzalez *et al*., 2003). It has a single-stranded, positive-sense RNA genome. The 3′ end of the genome encodes four structural proteins—spike (S), envelope, membrane and nucleocapsid—and small non-structural proteins (Cavanagh, 2007). The spike is formed by post-translational cleavage of two parts, S1 and S2. The carboxy-terminal S2 subunit is a conserved structure found in coronaviruses of different species that anchors S1 to the viral envelope and is also responsible for membrane fusion (Cavanagh, 2007). The amino-terminal subunit S1 is important in viral attachment and is a major virus-neutralizing antibody site (Cavanagh *et al*., 1986; Casais *et al*., 2003). S1 contains hypervariable regions that are able to change very easily through mutation or recombination (Kusters *et al*., 1990; Cavanagh *et al*., 1992). The molecular identification of IBV is based mainly on the analysis of the S1 protein gene (De Wit, 2000).

IBV represents a severe economic burden for the poultry industry worldwide (Cavanagh & Gelb, 2008). Infectious bronchitis (IB) can be managed through sound biosecurity principles and vaccination. However, control by vaccination is difficult because of antigenic variation in the virus. Outbreaks of the disease can occur even in vaccinated flocks following small changes to the amino acids in the S1 protein of IBV, which can result in a decrease in cross-protection (Cavanagh *et al*., 1992). However, in some cases there is a high level of cross-protection against other strains with a low homology, for which the term protectotype was introduced (Dhinakar Raj & Jones, 1996; Cook *et al*., 1999). To date, many different genotypes of IBV have been identified. A survey revealed that the predominant ones in European countries were 793B (4/91), Massachusetts, Italy02 and QX (Worthington *et al*., 2008).

Despite the global distribution of IBV, Finland was free of clinical IB cases for almost three decades. The last known natural outbreaks were in the 1970s but the strain or strains causing the outbreaks are not known. Since then, blood samples taken from layer and broiler parent flocks for a voluntary national health monitoring programme have been negative for IB antibodies. Vaccination with live IB vaccine has been prohibited nationally and vaccination with an inactivated IB vaccine has been practiced only in broiler grandparent flocks. In Finland, broiler grandparents are imported and raised by a single company that supplies three separate Finnish companies.

In 2007 there was one small, clinically asymptomatic outbreak of IB that was suspected to have originated from a vaccine, although vaccination against IB was not allowed at that time. A further epidemiological survey showed that the outbreak was restricted to one large farm, consisting of several separate units. After a few years, in April 2011, an outbreak of IB was reported in a layer flock. Since then, a series of outbreaks have been reported in broiler parent flocks, in layer flocks and also in backyard chicken flocks. The objective of the study was to investigate the distribution

*To whom correspondence should be addressed. Tel: +358 44 3543498. Fax: +358 29 5304363. E-mail: leena.pohjola@helsinki.fi
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of avian IBV in a non-vaccinating country (Finland) and also to carry out molecular characterization of IBV isolates in Finland.

Materials and Methods

Sampling. The samples used in this study were submitted to the Finnish Food Safety Authority Evira from different regions of Finland during 2011 to 2013. The samples derived from a voluntary health monitoring programme, a national survey for avian influenza and diagnostic specimens, and included commercial poultry production and hobby flocks. All samples were from chickens that were not IB vaccinated.

Serology. The serum samples were tested for IBV antibodies using the IDEXX IBV Ab test (IDEXX Corporation, Westbrook, Maine, USA), which is an indirect enzyme-linked immunosorbent assay. The kit was used according to the manufacturer's instructions. Newcastle disease and avian influenza were tested for using the haemagglutination inhibition test according to the Council Directive 92/66/EEC (Community Measures for the Control of Newcastle Disease, 1992) and the EU Diagnostic Manual for Avian Influenza (Commission Decision 2006/437/EC).

The organ or swab samples collected from clinically ill birds were pooled (one to five samples per pool) in antibiotic medium to make 10% suspensions, then homogenized and clarified by centrifugation. The supernatants were used for further egg inoculation studies and for examination by an IBV reverse transcriptase-polymerase chain reaction (RT-PCR) (see below). The eggs were candled daily, and eggs with dead or dying embryos plus all remaining eggs after 6 days inoculation were chilled at 4°C and the allantoic fluids harvested. The harvested allantoic fluids were tested for haemagglutinating activity using chicken red blood cells and the embryos were monitored for abnormalities. The samples examined in each case are detailed in the Results section.

Polymerase chain reaction to detect IBV. For PCR studies, the RNA was extracted from organ/swab suspensions or allantoic fluids using the QIAamp Viral Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The IBV genome was detected using the OneStep RT-PCR kit (QIAGEN), with primers as published by Keeler *et al.* (1998). The following thermal profile was used: a single cycle of reverse transcription for 30 min at 50°C, 15 min at 94°C for RT inactivation and DNA polymerase activation followed by 35 amplification cycles of 45 sec at 94°C, 45 sec at 48°C and 2 min at 72°C. After agarose gel electrophoresis the positive bands were cut from the gel and DNAs were extracted with the Qiaquick Gel Extraction Kit (QIAGEN).

The sequencing was performed with the primers used in the PCR, BigDye Terminator Cycle sequencing kit v3.1 and ABI3100 Avant automatic sequencer (Applied Biosystems, Foster City, CA, USA). The sequences were edited and the nucleotide identities calculated with the EMBOSS package (Rice *et al.*, 2000). The sequences were aligned with the ClustalW program and the neighbour-joining phylogenetic tree (Figure 1) was created with the MEGA 5.05 program (Tamura *et al.*, 2011). The data were bootstrapped 1000 times and only values higher than 85% were shown.

Results

Case 1. In March 2011 blood and organ samples were received by Evira from birds of a commercial layer flock in southeast Finland that had experienced a sudden drop in egg production, poor egg quality and mild respiratory signs in the birds. The signs had spread rapidly to three separate compartments (flock size 1800 to 4000) in the farm and the egg production drop was severe (67 to 74%), although mortality was low (0.03 to 0.69% a month). In the post-mortem examinations, enlarged spleens, pale, swollen kidneys and atrophic ovaries were recorded. Histological examination of the kidneys revealed a moderate multifocal lymphocytic interstitial nephritis. The blood samples were tested serologically against IBV, Newcastle disease and avian influenza, and were positive only for IB. Organ suspension (kidneys and oviducts) was tested for IBV using embryo inoculation and RT-PCR. The embryos showed changes typical of IBV infection, including curling and decreased growth and weight. The RT-PCR was positive for IBV and further phylogenetic analysis showed that the virus (Li3817/2011) belonged to the genotype QX, sharing a minimum of 97% nucleotide identity with strains circulating widely in Europe (Yu *et al.*, 2001). The entire flock was killed and the premises were cleaned and disinfected thoroughly.

Since there was no evidence of prior clinical IB infections in Finland and vaccination against IB was prohibited, epidemiological studies concerning the origin of the infection were initiated. The farm had contacts with small backyard poultry flocks in different parts of Finland. The biosecurity practices on this farm were poor and there were regular visitors to the bird premises. All known contact farms, as well as surrounding farms, were traced and the birds were tested for IBV and found to be serologically negative. The source of the infection remains unknown.

Case 2. In May 2011 cloacal specimens from an emaciated hen from a backyard poultry flock (11 chickens) in western Finland, some 300 km from the location of Case 1, were found to be positive for IBV by RT-PCR (second egg passage material). No evident gross pathological alterations were observed during post-mortem examination, except for an inflamed trachea. Histological examination of the trachea, lungs and kidneys revealed a multifocal lymphocytic inflammation. No haemagglutinating agents were found in the allantoic fluids of the eggs inoculated with the sample, thus excluding avian influenza and Newcastle disease. The virus (Li5811/2011) was partially sequenced and the genotype of the virus was established as QX, sharing only 91.5% nucleotide identity with the Li3817/2011 strain isolated from the layer flock in March 2011 (Case 1). Interestingly, the isolate from Case 2 had a unique amino acid sequence "LDKG" at amino acids 117 to 122 of the S1 protein (numbering is according to the strain CK/SWE/0658946/10, JQ088078; Abro *et al.*, 2012), including a deletion of two amino acids. Most QX strains possess the amino acid sequence "SSGSGS" in this position.

After this second QX isolation, serological testing of 45 backyard flocks from different parts of Finland was performed and antibodies to IBV were detected in 73% of them.

Cases 3 and 4. Later in the period under investigation, signs such as sneezing, coughing, diarrhoea, decrease in egg production of approximately 10% and loss of appetite were observed in several broiler parent flocks (flock size 2500 to 10,000 chickens) in southwestern Finland. Almost simultaneously a drop in egg production (7 to 10%) and respiratory signs were noted in parent layer flocks (flock size 1400 to 4100 chickens) in the same area. IB antibodies were detected in these flocks and IBVs were isolated from cloacal swab material during the first embryo passage. The serological samples were mostly negative when the clinical signs first appeared in the flock and became positive for IB antibodies as the disease proceeded, which supported the PCR results. The broiler parent flocks were closely monitored and spread of the virus among parent flocks of the company was recorded. The infection also spread to several broiler flocks within the company and re-infections were recorded in several farms. The viruses that spread in both the commercial companies shared approximately 98%

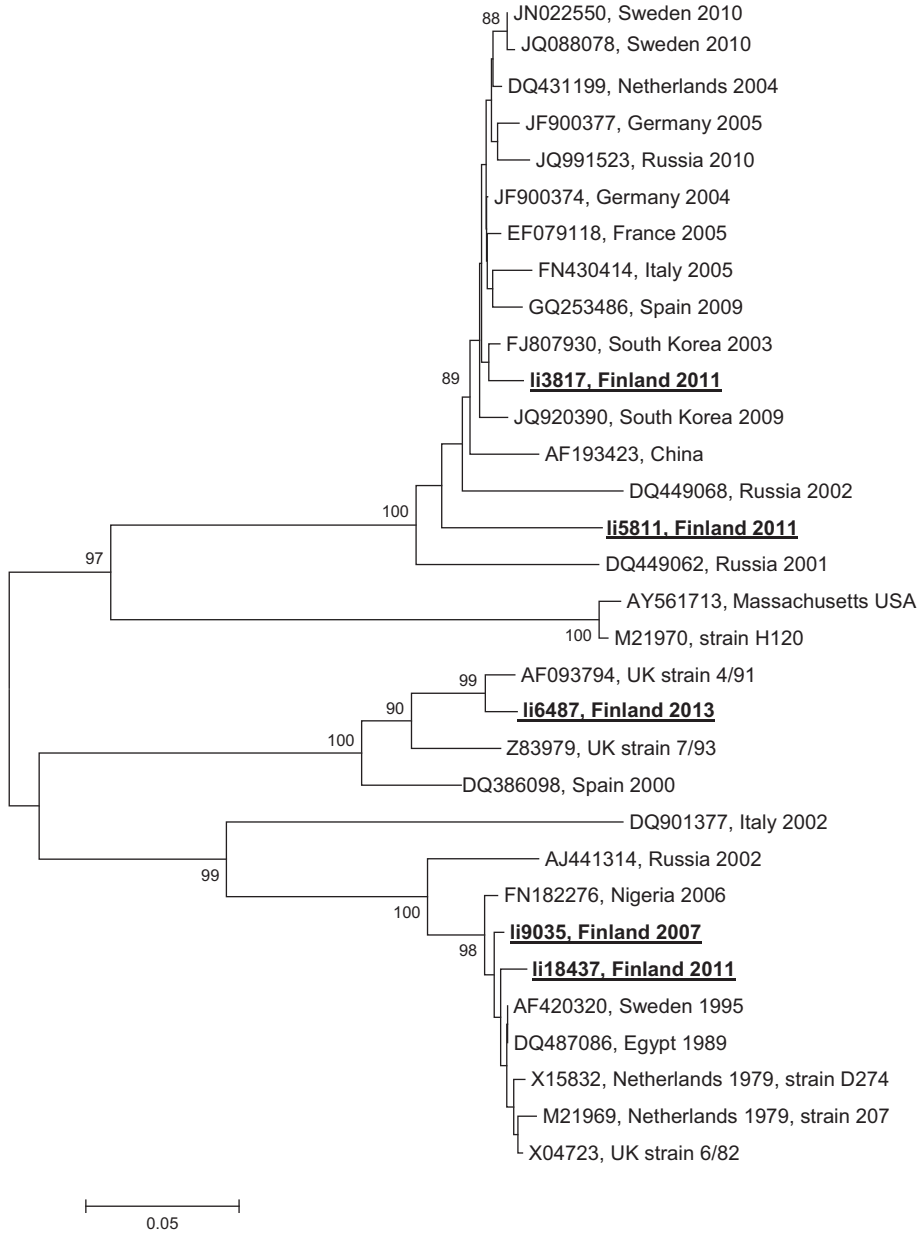

Figure 1. *Phylogenetic relationships among Finnish IBV strains and selected IBV sequences available in GenBank. The analysis is based on 425 nucleotides of the S1 gene. The GenBank accession numbers and the country and year of isolation are given for each branch. Only bootstrap values higher than 85% are shown.*

nucleotide identity and are represented by strain Li18437/2011 in Figure 1. The viruses were also closely related to strain D274, a serotype of IBV first isolated in the Netherlands, which is used in vaccines (Jordi *et al*., 1989).

Case 5. In May 2013 a broiler flock (flock size 26,000) in southwestern Finland exhibited signs of mild respiratory infection, increased mortality (0.3% for a few days) and decreased growth during the last week before slaughter. IBV was detected by RT-PCR directly from an intestinal suspension and the genotype established as 4/91-like virus (Li6487/13).

The map in Figure 2 shows the distribution of the different IBV genotypes detected in Finland. Most of the infected farms are located in southwestern Finland, which has the highest poultry density in the country. The GeneBank accession numbers for the Finnish IBV strains shown in Figure 1 are as follows: KJ535506–KJ535510.

Discussion

Our study revealed that QX, D274-like and 4/91-like viruses have been circulating simultaneously in southern Finland over a short time period. The first virus isolated, belonging to the QX genotype, emerged in a layer flock in the southeast of Finland. IB strains belonging to the QX genotype have been associated with nephritis, proventriculitis and the false-layer syndrome (Wang *et al*., 1996; Yu *et al*., 2001; Beato *et al*., 2005). In Case 2, the virus belonging to the QX genotype was found in an emaciated hen in a backyard flock. The farm is located approximately 300 km from the Case 1 farm. Based on the relatively low nucleotide identity (91.5%) between the two QX viruses (Li3817/2011 and Li5811/2011), it is apparent that they had different origins. The unique ^{117}LDKG^{120} sequence in the S1-protein of Li5811/2011 might represent a recombination event between QX and unknown IB strains. Based on the published sequences in GenBank, only strains related to the Massachusetts strain

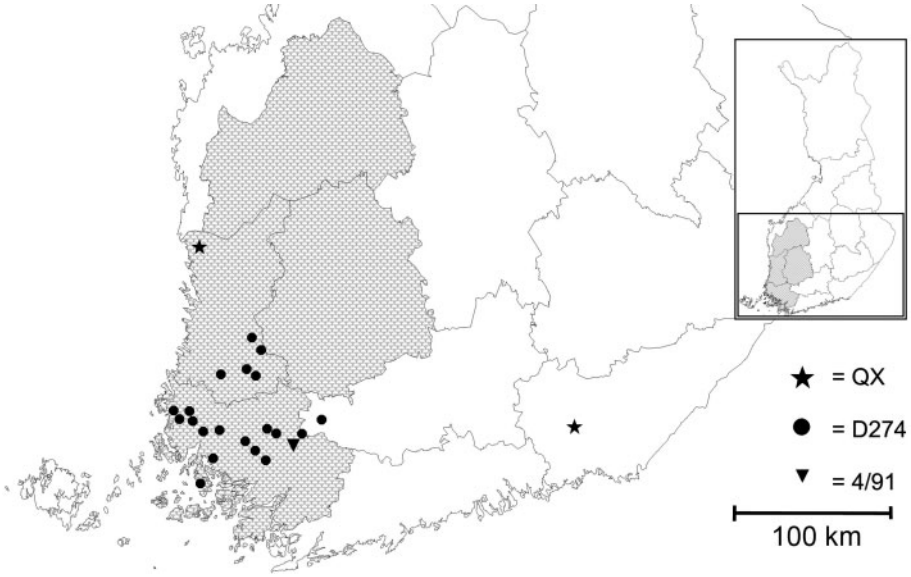

Figure 2. *Distribution of the different IBV genotypes in Finland. Most of the infected farms are located in southwestern Finland. The areas that have the highest poultry density in the country are shaded grey.*

appear to have a two amino acid deletion at this site. There has been no evidence of this virus spreading in Finland and the origins of the two sporadic infections remain unknown. Considering the highly contagious nature of IBV, it is surprising that the virus did not spread to other farms.

There have been increasing numbers of IB cases in Europe where the virus implicated has been QX (Bochkov *et al*., 2006; Gough *et al*., 2008; Worthington *et al*., 2008; Valastro *et al*., 2010; Abro *et al*., 2012; Sigrist *et al*., 2012). Backyard flocks can be more susceptible to infectious diseases because of their close contact with wild birds (De Wit *et al*., 2011; Wang *et al*., 2013) and on some occasions novel infectious diseases are known to emerge first in backyard flocks (Capua *et al*., 2002). Although IBV-like coronaviruses have been recovered from wild birds, there is as yet no evidence for long-distance transmission of these viruses (Muradrasoli *et al*., 2009). Another explanation for the emergence of this genotype could be an illegal import of hobby chickens. In a small-sized hobby flock, clinical signs associated with IB can go unnoticed by the owner and the threshold to call the veterinarian may be high. Further serological testing of backyard poultry flocks revealed IB antibodies in 73% of the flocks tested. This suggests that IBV is common among such populations.

In Cases 3 and 4, D274-like virus appeared at the same time in layers and broiler parents in western Finland. Despite the sound biosecurity measures on commercial farms, one explanation for this could be the close proximity of the farms. Based on the mild clinical signs observed and the close genetic relationship (Figure 1), the D274-like and the 4/91-like (Case 5) IBVs might be vaccine-derived strains. The fact that in most samples IBV was isolated in the first egg passage also supports this hypothesis, as vaccine-derived viruses are more easily isolated since they are adapted to grow in embryonated eggs (McKinley *et al*., 2011).

It is interesting to note how a virus very closely related to a vaccine strain can lead to clinical manifestation in birds that were not previously vaccinated or in contact with IBV. The D274-like IBV genotype was also detected earlier in a small asymptomatic outbreak in 2007, the origin of which remains unknown. It was the most common IB serotype in several western European countries in the early and mid 1980s and represented the reason for initiating vaccination (Cook, 1984; Davelaar *et al*., 1984).

In Case 5, the 4/91-like genotype caused similar clinical signs in the broiler flock to the previously recorded D274-like infections. Genotype 4/91 emerged in the 1990s in Great Britain, and in addition to respiratory infections was associated with bilateral myopathy and increased mortality of adult birds (Gough *et al*., 1992). This genotype is used widely in attenuated vaccines.

The spread of the IBVs from neighbouring countries is a possibility. A study made in Russia revealed that the most frequently detected genotypes in the area are Massachusetts, 4/91 (793/B) and D274, although QX-like strains seem to be spreading rapidly (Ovchinnikova *et al*., 2011). In Sweden the QX-like strains have replaced the Massachusetts-like strains that were the predominant strains in the 1990s (Abro *et al*., 2012).

The disadvantage of live vaccines is that they spread easily in the field (Meulemans *et al*., 2001; Farsang *et al*., 2002). In Sweden there was a strong suspicion that vaccination with Massachusetts-like live attenuated vaccine led to the spread of the vaccine virus (Farsang *et al*., 2002). It is possible that the importation of vaccinated 1-day-old chicks from other European countries may have contributed to the spread of the D274 and/or 4/91 genotypes, because no vaccinations have been undertaken in Finland. The differentiation of the vaccine strain from a wild strain is difficult and needs further investigation.

This study shows that after three decades of absence of IB, viruses of the QX, D274-like and 4/91-like genotypes are present in poultry flocks in Finland. The sources of the viruses are not known, but infections with D274-like and 4/91-like viruses could be related to vaccine strain escapes. IB infection seems to have returned to both broiler and layer companies and is also present in backyard poultry. To date the Finnish poultry industry has refrained from vaccinating with live-attenuated vaccines. Currently, vaccination with an inactivated vaccine is practiced in layer parent flocks as well as in broiler grandparent flocks. The decision to use live vaccines has not yet been taken by the Finnish poultry industry.

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