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**BACKYARD POULTRY FLOCKS IN FINLAND
- AN INFECTION RISK FOR COMMERCIAL
POULTRY AND HUMANS?**

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ACADEMIC DISSERTATION

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ABSTRACT

There is increasing interest in keeping small backyard poultry flocks in rural and urban residential areas in many countries, including Finland. There is no common definition for backyard poultry flocks, but they are often defined as flocks where the birds are kept for eggs or other products consumed mainly by the owners, and for which the overall number of birds is fewer than 500, or 1000. Several studies in Western Europe and North America have identified the involvement of backyard poultry flocks in avian influenza virus outbreaks in commercial poultry. However, from the epidemiological point of view their role has been concluded to be only marginal. In addition, commonly without any signs, poultry can be carriers of enteric bacterial agents that are human pathogens. As backyard poultry flocks often live in close contact with their owners, zoonotic infections could be transmitted through fecal contact or by ingestion of contaminated poultry products, such as eggs.

In this thesis, the management and biosecurity practices among 178 backyard poultry flocks in Finland were investigated using a questionnaire. Furthermore, the main causes of mortality of backyard chickens were studied through a retrospective study of necropsy data from the Finnish Food Safety Authority Evira from 2000 to 2011. In addition, voluntary backyard poultry farms were visited during October 2012 and January 2013, and blood samples, individual cloacal samples and environmental boot sock samples were collected from 51 farms and 457 chickens. From the cloacal samples and boot sock samples, the occurrence and antimicrobial resistance patterns of *Salmonella enterica*, *Campylobacter* spp., *Listeria monocytogenes*, *Yersinia enterocolitica* and *Y. pseudotuberculosis* were studied and the occurrence of ESBL/AmpC-producing *Escherichia coli* were investigated. *Campylobacter* isolates were further typed using pulsed-field gel electrophoresis (PFGE). From the blood samples the occurrence of antibodies against infectious bursal disease virus (IBDV), avian encephalomyelitis virus (AEV), chicken infectious anemia virus (CIAV), infectious bronchitis virus (IBV), infectious laryngotracheitis virus (ILTV), avian influenza virus (AIV) and Newcastle disease virus (NDV) were studied. The occurrence of AIV, NDV and IBV were further studied from the cloacal samples of the birds and IBV strains found were genotyped by molecular methods. Additionally, IBV strains causing outbreaks in 2011 – 2013, both in Finnish commercial and backyard poultry flocks, were characterized.

The questionnaire revealed that the backyard poultry farms in Finland were mainly small (91 % \leq 50 birds) and most flocks (98 %) had access to outdoors at least for part of the year. Biosecurity practices, such as the possibilities for hand washing and changing shoes after bird contact were rare,

35 % and 13 % respectively. The birds had a possibility to be in a contact with wild birds (36 %) and visitors (84 %). The farms were mainly located distantly (94 % > 3 km) from commercial poultry farms. The subjectively reported flock health was good (96 %) and the most common health issues reported were ectoparasites (31 %), sudden death (30 %) and diarrhea (18 %). The most common postmortem diagnosis were Marek's disease (27 %) and colibacillosis (17 %).

Of the zoonotic bacterial pathogens, *C. jejuni* and *L. monocytogenes* were frequently detected on the farms, 45 % and 33 %, respectively. *Y. enterocolitica* was also frequently isolated on the farms (31 %); however, all isolates were *yadA* negative, i.e. non-pathogenic. *Campylobacter coli*, *Y. pseudotuberculosis* and *S. enterica* were each detected from only one (2 %) farm. All enteric bacteria were highly susceptible to most of the antimicrobials studied and only few AmpC- and no ESBL-producing *E. coli* were found.

AEV, CIAV and IBV antibodies were commonly found from the studied backyard poultry farms, 86 %, 86 % and 47 %, respectively. Antibodies against IBDV, ILTV, AIV and NDV were rare, 20 %, 12 %, 5 % and 0 %, respectively. The IBV detected from backyard poultry flocks were QX-type IBV strains differing from the strains found from commercial farms and also from the sole QX-strain found on a layer poultry farm in 2011, suggesting different routes of infection for commercial and backyard poultry.

The results indicate that among backyard poultry flocks pathogens circulate that can pose a risk of infection to commercial poultry production in Finland, but because of the distant locations and small flock sizes, the risk is relatively small. Notifiable avian diseases that also are of zoonotic potential (AIV and NDV) are very rare. Backyard chickens are a reservoir of *C. jejuni* strains and thus a potential source of *C. jejuni* infection for humans. Because of the lack of good hygiene after bird contact, the risk of transmission of the pathogen from birds to humans exists. Antimicrobial resistance of the zoonotic pathogens, including AmpC/ESBL-producing *E. coli*, is not common among backyard poultry flocks in Finland.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by the Roman numerals (I-IV)

- I **Pohjola** L, Rossow L, Huovilainen A, Soveri T, Hänninen ML, Fredriksson-Ahomaa M. Questionnaire study and postmortem findings in backyard chicken flocks in Finland. *Acta Veterinaria Scandinavica*, 2015; 57:3.

- II **Pohjola** L, Nykäsenoja S, Kivistö R, Soveri T, Huovilainen A, Hänninen ML, Fredriksson-Ahomaa M. Zoonotic public health hazards in backyard chickens. *Zoonoses and Public Health*, 2016; 63:420-430.

- III **Pohjola** L, Tammiranta N, Ek-Kommonen C, Soveri T, Hänninen ML, Fredriksson-Ahomaa M, Huovilainen A. A survey for selected avian viral pathogens in backyard chicken farms in Finland. *Avian Pathology*, 2016; 13:1-10.

- IV **Pohjola** LK, Ek-Kommonen SC, Tammiranta NE., Kaukonen ES, Rossow LM, Huovilainen TA. Emergence of avian infectious bronchitis in a non-vaccinating country. *Avian Pathology*, 2014; 43:244-248.

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ABBREVIATIONS

AE	Avian encephalomyelitis
AEV	Avian encephalomyelitis virus
AI	Avian influenza
AIV	Avian influenza virus
aMPV	Avian metapneumovirus
APEC	Avian pathogenic <i>Escherichia coli</i>
API	Analytical profile index
BF	<i>Bursa fabricius</i>
BPV	Buffered peptone water
CIA	Chicken infectious anemia
CIAV	Chicken infectious anemia virus
CIN	Cefsulodin-irgasan-novobiocin
CLSI	Clinical and Laboratory Standards Institute
DNA	Deoxyribonucleic acid
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
ESBL	Extended-spectrum beta-lactamase
EVIRA	Finnish Food Safety Authority
GPS	Grandparent stock
H	Flagellar antigen (<i>E. coli</i>)
HA	Hemagglutinin (AIV)
HI	Hemagglutinin inhibition
HN	Hemagglutinin-neuraminidase (AIV)
HPAI	Highly pathogenic avian influenza
IB	Infectious bronchitis
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
IBV	Infectious bronchitis virus
ILT	Infectious laryngotracheitis
ILT	Infectious laryngotracheitis virus
im	intra muscularis
LPAI	Low pathogenic avian influenza
Luke	Natural Resources Institute Finland
mCCDA	Modified charcoal cefoperazone deoxycholate
MD	Marek's disease
MDV	Marek's disease virus
MG	<i>Mycoplasma gallisepticum</i>
MLST	Multilocus sequence typing
MRSV	Modified semi-solid Rappaport-Vassiliadis

MS	<i>Mycoplasma synoviae</i>
NA	Neuraminidase
ND	Newcastle disease
NDV	Newcastle disease virus
NTS	Non-typhoidal <i>Salmonella</i>
O	Somatic antigen (<i>E. coli</i>)
OIE	World Organization for Animal Health
PFGE	Pulsed-field gel electrophoresis
p.i.	post infection
PCR	Polymerase chain reaction
PMV	Paramyxovirus
PS	Parent stock
pYV	Plasmid for <i>Yersinia</i> virulence
RNA	Ribonucleic acid
RT	Reverse transcriptase (enzyme)
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Spike glycoprotein (IBV)
WHO	World Health Organization
yadA	<i>Yersinia</i> adhesion A
XLD	Xylose lysine deoxycholate

1 REVIEW OF THE LITERATURE

1.1 COMMON AVIAN INFECTIOUS PATHOGENS

Avian infectious pathogens can be of viral, bacterial, protozoal and fungal origin. These infectious agents can be transmitted to the birds vertically, i.e. from hen to progeny (egg-borne diseases) or horizontally from other birds and animals, from the environment or by human activity (Bermudez and Stewart-Brown, 2008). The transmission of infectious agents is usually controlled by quarantine measures, a variety of different hygiene practices, rearing only single age birds on any one farm, pest control, vaccinations and sometimes also medications. In addition to the pathogen itself, many other factors, such as genetics, nutrition, environmental conditions and management (ventilation, temperature etc.), have an important role in the development of clinical diseases in poultry. Typically, commercial poultry is reared in large flocks with high bird density, which favors the rapid spread of contagious diseases (Hafez and Hauck, 2015). The most commonly encountered infectious and/or otherwise significant pathogens among commercial and backyard chickens are reviewed here briefly. The emergence of these diseases in Finnish commercial poultry is detailed in Table 6.

1.1.1 AVIAN INFLUENZA VIRUS (AIV)

Avian influenza virus is an enveloped RNA virus classified in the family of *Orthomyxoviridae* and genus influenza virus A (ICTV, 2015; <http://www.ictvonline.org/virustaxonomy.asp>). Wild birds in the orders of Anseriformes (screamers, ducks, swans and geese) and Charadriiformes (shorebirds) are the natural and usually asymptomatic carriers of AIV and may directly or indirectly transmit viruses to poultry (Franca and Brown, 2014). Although AIV infections in humans are rare, infections of subtypes H5, H7 and H9 have been reported (Pepin et al., 2013).

The pathogenesis of AIV is complex and the ability of the virus to produce disease in avian species is dependent on the virulence of the strain, host (age and species), concurrent infections and environmental factors, not all of which are yet completely understood (Swayne and Halvorson, 2008). The eight genome segments of AIV encode 10 or 11 proteins of which hemagglutinin (HA) and neuraminidase (NA) are the most important regarding antigenicity (Chen et al., 2001; Peiris et al., 2007). Virus strains are named according to their HA and NA subtypes. To date, sixteen HA (H1 to H16) and nine NA (N1 to N9) subtypes have been recognized in aquatic birds and these subtypes seem to be able to assort in all possible combinations (Webster et al., 1992;

Fouchier et al., 2005). The matrix gene is a highly conserved genome region of diagnostic importance (Fouchier et al., 2000).

The AIV are divided into two categories: highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). The virus is HPAI if the intravenous pathogenicity index in six-week old chickens is greater than 1.2 or if it causes at least 75 % mortality in four to eight-week old chickens infected intravenously (OIE, 2015). In addition, the pathogenicity of a strain is determined depending on the amino acid sequence at the HA cleavage site. In LPAI strains the HA cleavage site requires a trypsin protease. Trypsin proteases are available only in the mucosal epithelial cells of the respiratory and intestinal tract, which limits the tissue distribution of these viruses and the infection usually remains localized (Klenk et al., 1975). In HPAI viruses any protease is suitable for the HA cleavage, resulting in a wide range of target tissues and a greater capability for systemic infection. These strains contain several basic amino acids (arginine, lysine) at the HA cleavage site (OIE, 2014). Two subtypes (H5 and H7) are known to give rise to HPAI virus in chickens and turkeys (Peiris et al., 2007).

AIV is excreted through nasal, oral and ocular routes and in feces. It is transmitted by direct contact with an infected bird to another or by indirect contact through aerosols or fomites (Swayne and Halvorson, 2008). In gallinaceous birds, clinical signs of LPAI infections are many times mild or nonexistent. In some cases, there are signs of typical respiratory disorders: coughing, sneezing, rales, rattles and lacrimation. A drop in egg production and quality can commonly be detected in mature birds. Morbidity is high but mortality usually ranges from moderate to low (< 5 %). Gross lesions appear in the respiratory tract: catarrhal to fibrinous rhinitis, sinusitis, laryngitis, tracheitis, bronchopneumonia and airsacculitis. Hens in egg production can have egg-yolk peritonitis, ovaria regression, salpingitis and eggs can be misshapen and lack pigmentation (Swayne and Halvorson, 2008; Franka and Brown, 2014).

In HPAI infections, death typically occurs among some of the flock before the first disease signs are detected. Birds are markedly lethargic and depressed and neurological signs occur, such as tremors of neck and head, torticollis and opisthotonus as well as respiratory signs, although usually milder than in LPAI. Also decrease in water and feed consumption and severe egg drop is seen. Morbidity and mortality rates are high and can reach 100 %. Edema and hemorrhages of the skin of the face, comb, snood, wattles, upper neck and feet are typical. The conjunctiva and trachea may be congested, edematous and hemorrhagic. Hemorrhages may be seen also in serosal and mucosal surfaces of the gastrointestinal tract, especially in the proventriculus and ventriculus (Swayne and Halvorson, 2008).

AIV can cause a wide spectrum of signs and lesions and therefore a definitive diagnosis is made using direct detection methods, such as virus isolation or reverse transcriptase-polymerase chain reaction (RT-PCR) and indirectly by serological methods such as enzyme-linked immunosorbent assay (ELISA) or hemagglutinin inhibition (HI) test. In countries where virus eradication is not possible, various vaccination technologies and programs have been developed (Swayne, 2004).

1.1.2 NEWCASTLE DISEASE VIRUS (NDV)

The causative agent of Newcastle disease (ND) is a paramyxovirus-1, an RNA-virus of the *Paramyxoviridae* family (ICTV, 2015; <http://www.ictvonline.org/virustaxonomy.asp>). ND has an enormous impact on the poultry industry all over the world. It also has a zoonotic potential: NDV-causing conjunctivitis has been reported in humans (Alexander and Senne, 2008).

NDV is divided into four different pathotypes based on the severity of the disease. Velogenic ND causes a lethal infection in chickens of all ages and it can be either viscerotropic or neurotropic. The mesogenic pathotype usually results in mortality only in young chickens and the lentogenic pathotype causes little mortality and variable degrees of respiratory signs. The fourth pathotype, asymptomatic-enteric type, causes no obvious disease (Alexander, 2000; Cattoli et al., 2011). An intracerebral pathogenicity index of ≥ 0.7 in day-old chicks and/or at least three arginine or lysine residues at the C-terminus of the fusion protein cleavage site (113 – 117) are the universally recognized measures to categorize the virulence of NDV strains (OIE, 2012b).

The genome of NDV is composed of six genes that encode six structural proteins: nucleoprotein, phosphoprotein, matrix, fusion, hemagglutinin-neuraminidase, and RNA polymerase (Chambers et al., 1986). Genetically, NDV strains are divided into two classes (I and II) based on the phylogenetic analysis of the partial or complete nucleotide sequences of the *Fusion* gene (Peeters et al., 1999; Miller et al., 2010). Currently, nine genotypes of class I viruses and ten of class II have been identified (Miller et al., 2010).

NDV can infect many avian, as well as non-avian, species, but chickens are the most susceptible hosts. Many wild birds, such as pigeons and mallards, can be reservoirs of avian paramyxoviruses (Teske et al. 2013; Tolf et al., 2013). Infected birds excrete the virus as aerosols, respiratory discharges and feces. In the case of velogenic ND infection, onset of the disease is rapid and birds may suddenly die without any visible signs. Other typical signs are listlessness, edema around the eyes and head, green diarrhea, neurological signs such as muscular tremors, torticollis, paralysis and opisthotonus. Respiratory signs can be severe or absent. Mortality can reach 100 % in fully susceptible flocks.

A gross lesion in velogenic ND can be absent, but typically hemorrhagic lesions in the mucosa of proventriculus, ceca and intestines are observed (Alexander and Senne, 2008).

There are no pathognomonic signs or lesions associated with ND. The diagnosis is typically done using RT-PCR. NDV is controlled by vaccinations. Finland and Sweden have a vaccination-free status for ND and the use of ND vaccines is banned (European Union; 94/963/EY).

1.1.3 AVIAN ENCEPHALOMYELITIS VIRUS (AEV)

Avian encephalomyelitis virus (AEV) is an RNA virus that belongs to the *Picornaviridae* family (ICTV, 2015; <http://www.ictvonline.org/virustaxonomy.asp>). It is ubiquitously spread, and in addition to chickens, it can also infect pheasants, quails and turkeys (Tannock and Shafren, 1994; Calnek, 2008). AEV can be divided into two distinct, but serologically similar, pathotypes (Calnek, 2008). The enterotropic pathotype, represented by natural field strains, is pathogenic to chicks only by vertical transmission or by early horizontal transmission (Calnek et al., 1960; Springer and Schmittle, 1968). After infection by the oral route, the virus replicates primarily in the duodenum, which is followed by viremia and subsequent infection in the visceral organs (pancreas, spleen, liver) and the central nervous system (Springer and Schmittle, 1968). Consequently, the virus is excreted in the feces and infection spreads rapidly from bird to bird (Calnek et al., 1961; Butterfield et al., 1969; Shafren and Tannock, 1991). In vertical transmission, susceptible, recently AEV-infected hens excrete the virus a short period of time to their eggs (Ikeda and Matsuda 1976). Virus replication occurs during embryogenesis and the virus can be found from the brain, liver and intestines already in 20-day-old chicken embryos (Calnek et al., 1960). The second pathotype, an embryo-adapted strain (also called Van Roekel strain), is not discussed here because it is not a natural field strain (Van Roekel et al., 1938).

AE is a disease of young chickens, commonly at the age of 1 - 2 weeks, and it is characterized by dullness, ataxia progressing to paralysis and rapid tremors usually followed by prostration and death. Mortality averages 25 % (AAAP, 2013). Some chicks may survive but they usually develop cataracts later. Older (> 3 weeks) chickens are usually resistant and do not show any clinical signs. In mature birds a temporary drop in egg production and possibly also decreased hatchability is evident (Taylor et al., 1955; Calnek, 1988; Calnek et al., 2008). Gross lesions are very minute but whitish areas (masses of lymphocytes) in the muscularis of the ventriculus can be observed (Calnek et al., 2008).

The presumptive diagnosis is usually made according to the history of the parent flock (susceptible to AEV, egg drop), typical age and signs of the progeny and by histopathological findings. Antibodies to AEV are most commonly measured using commercial ELISA kits. The control of AE is achieved by vaccination of the breeder flocks before the beginning of lay.

1.1.4 CHICKEN INFECTIOUS ANEMIA VIRUS (CIAV)

Chicken infectious anemia virus (CIAV) causing a disease called blue wing disease, is a DNA virus of the family *Anelloviridae* (ICTV, 2015; <http://www.ictvonline.org/virustaxonomy.asp>). Two serotypes have been described but the significance of the second serotype is not clear (Spackman et al., 2002a, b). The infection has been reported in chickens and turkeys and it has spread to all major chicken-producing countries (Schat and van Santen, 2008; AAAP, 2013).

CIAV spreads both vertically and horizontally by the feco-oral route. Vertical transmission occurs when susceptible hens become infected through horizontal infection. Hens themselves do not usually show any clinical signs (Hoop, 1992). The virus is shed into eggs and replicated in newly hatched chicks and the clinical disease is seen in young chickens, typically aged between 2 to 4 weeks, and it is characterized by aplastic anemia and lymphoid atrophy. In horizontal exposure the disease develops in 8 to 10 days post infection (p.i.) (Miller and Schat, 2004).

The clinical outcome varies depending on the age, presence of protective antibodies and secondary infections. CIAV replicates in hemocytoblasts (bone marrow) and thymocytes (thymus), which are important for the development of innate and acquired immune responses (Sharma, 2008). The infection leads to cell apoptosis and a decrease in blood erythrocytes, thrombocytes and granulocytes. The gross lesions are associated with marked thymic, splenic and bursal atrophy, pale bone marrow and hemorrhages. Characteristic skin lesions (anemia dermatitis), which are prone to secondary bacterial infections, are common. Mortality levels of 5 – 15 % are typical (Lucio et al., 1990; Hoop, 1992; McIlroy et al., 1992; Todd, 2000). The susceptibility to anemia rapidly decreases after 3 weeks of age, largely due to the ability to produce virus-neutralizing antibodies, but the chickens remain susceptible to the immunosuppression at older ages (Goryo et al., 1985; Markowski-Grimsrud et al., 2003).

The diagnosis is based on typical clinical signs and the presence of the virus, which can be confirmed by PCR (bone marrow, spleen, thymus) or by virus isolation in susceptible cell lines (Yuasa et al., 1983). Commercial ELISA kits are available for detecting antibodies. Prevention of the disease is best achieved by ensuring that the hens develop antibodies against CIAV before

they begin to lay. This is usually achieved by vaccinating the hens before 15 weeks of age using live vaccines (Schat and van Santen, 2008).

1.1.5 AVIAN METAPNEUMOVIRUS (AMPV)

Avian metapneumovirus (aMPV) is an RNA virus belonging to the *Pneumoviridae* family and is the causative agent of turkey rhinotracheitis and avian rhinotracheitis (ICTV, 2015; <http://www.ictvonline.org/virustaxonomy.asp>). Four subtypes (A - D) of the virus have been identified based on the divergence in the surface glycoprotein gene, which is responsible for cellular attachment (Juhasz and Easton 1994; Seal, 1998; Băyon-Auboyer et al., 2000; Cook and Cavanagh, 2002). Subtypes A and B are prevalent and are the most important ones in Europe. Subtype C appears to be important in turkeys in the USA and subtype D is rare (Jones, 2010; AAAP, 2013).

Turkeys and chickens are natural hosts of aMPV. It is horizontally transmitted and vertical transmission has not been reported even though the virus can be detected from the reproductive tract of infected hens (Jones et al., 1988; Kehra and Jones, 1999). Typical clinical signs in young birds include acute respiratory infection, such as tracheal rales, sneezing, swollen sinuses, and nasal and ocular discharge. In older birds coughing and head shaking are commonly seen. Management factors, such as poor ventilation and overstocking, can exacerbate the signs (Gough and Jones, 2008). In laying hens, egg-drop, peritonitis and poor shell quality can be evident (Jones et al., 1988). Mortality ranges from negligible to as high as 50 %. Particularly among broilers and broiler breeders, aMPV infection, together with secondary *E. coli* infection, can cause swollen head syndrome, which is characterized by swelling of the periorbital and infraorbital sinuses, torticollis, disorientation and opisthotonus and gross lesions such as airsacculitis, pericarditis, pneumonia and perihepatitis. (Gough and Jones, 2008). In laying hens various reproductive tract lesions, such as egg peritonitis, misshapen eggs and regression of ovaries and oviduct are reported. Also prolapsed oviduct due to violent coughing has been reported (Jones et al., 1988; Gough and Jones, 2008).

aMPV detection is usually done with RT-PCR. Most commonly the virus is detected from ocular and nasal secretions, sinus/turbinate scrapings and trachea and lung. Also several commercial ELISA kits have been developed to detect antibodies (Gough and Jones, 2008). Infections are controlled by the use of live attenuated and killed vaccines. Infections can be successfully eradicated in areas of low flock density (Jones, 2010).

1.1.6 INFECTIOUS BRONCHITIS VIRUS (IBV)

Infectious bronchitis virus (IBV) is an RNA virus belonging to the *Coronaviridae* family (ICTV, 2015; <http://www.ictvonline.org/virustaxonomy.asp>). IBV was first isolated in Massachusetts in the 1930s and since then hundreds of different IBV variants have been discovered. Today it is one of the most important causes of economic loss within the poultry industry (Cavanagh, 2007).

The IBV genome encodes four major structural proteins, the spike glycoprotein (S), the membrane glycoprotein, the nucleocapsid protein and the envelope or small membrane protein (Cavanagh, 2007). The spike is formed by post-translational cleavage of two subunits, S1 and S2. The subunit S2 is a conserved structure found in coronaviruses of different species and it anchors subunit S1 to the viral envelope and thus is responsible for membrane fusion. The subunit S1 is needed in the viral attachment and is a major virus-neutralizing antibody site as well as playing an important role in host cell specificity (tissue tropism) (Cavanagh et al., 1986, Casais et al., 2003). It is now known that even very minute changes in the amino acid sequence of the S protein can result in the development of new antigenic variants (Cavanagh et al., 1992).

Worthington et al. (2008) conducted a survey of IBV genotypes in commercial poultry flocks of selected Western European countries. The four predominant IBV types during that time were 793B, Massachusetts, Italy02 and QX. In USA, the most commonly isolated IBV types have been Arkansas, Delaware, Conn and Mass (Jackwood et al., 2005; Jackwood, 2012). Currently, genotyping the gene that encodes the S1 subunit is the most commonly used system for grouping different IBV strains (de Wit et al., 2011; Valastro et al., 2016).

Despite the tissue tropism of the strain, IBV initially infects the upper respiratory tract's ciliated and mucus-secreting cells. Infection damages the epithelial cells resulting in deciliation and predisposes the host to secondary bacterial infections such as *E. coli* and avian mycoplasma. In addition to respiratory tissues, IBV also replicates in many other epithelial cells, such as those of the alimentary tract, kidney, testes and oviduct (Boltz et al., 2004; Cavanagh et al., 2007). IBV is shed via respiratory tract excretions and feces and only horizontal transmission is known to occur. Typical gross lesions are serous to caseous exudate in the trachea, nasal passages and sinuses. Swollen and pale kidneys are typical of nephropathogenic infections (Cavanagh and Gelb, 2008).

IBV causes respiratory disease in chickens of all ages, but especially among young ones. Typical signs are nasal and ocular discharge, sneezing, rales and lethargy, and in some cases also mortality, although usually it is associated

with secondary bacterial infections. In mature hens, loss of production and poor egg quality are usual (Cavanagh 2007, Cavanagh and Gelb, 2008). With certain strains, especially the QX variant of IBV, severe nephritis and false layer syndrome are reported (Cavanagh and Gelb, 2008). Some nephropathogenic strains do not produce clinical respiratory infection lesions (Glahn et al., 1989).

Vaccinations against IBV have been practiced for a long time. Both live attenuated and inactivated vaccines are in use. However, the protection offered by the vaccination is generally short-lived (9 weeks) (Cavanagh et al., 2007). Frequently vaccinations with two antigenically different live vaccines are used (such as Mass and 4/91) for broader cross-protection against different IBV variants (Cook et al., 1999; Terregino et al., 2008). The use of live-attenuated vaccine strains, which are not circulating in the area, are not recommended because of the ability of IBV to mutate rapidly and recombine with other IBV (Jackwood et al., 2012).

1.1.7 INFECTIOUS BURSAL DISEASE VIRUS (IBDV)

The causative agent of infectious bursal disease (IBD), also known as Gumboro disease, is an RNA virus that belongs to the *Birnaviridae* family (ICTV, 2015; <http://www.ictvonline.org/virustaxonomy.asp>). IBDV can cause clinical disease only in young chickens. The disease has a worldwide distribution and is present in all major poultry producing areas (Etteradossi and Saif, 2008).

There are two IBDV serotypes recognized. Serotype 1 causes clinical disease in young chickens and serotype 2 is non-pathogenic (McFerran et al., 1980). A wide range of IBDV serotype 1 pathotypes of highly variable pathogenicity has been reported to exist. The pathotypes are classified into sub-clinical, classic virulent and very virulent groups (van den Berg et al., 2004). Very virulent IBDV was first identified in Belgium and has now spread to nearly all poultry-producing countries in the world (Chettle et al., 1989).

IBDV replicates in the gut-associated (duodenum, jejunum, cecum) macrophages and lymphoid cells and enters the portal circulation via the liver, leading to primary viremia, after which it reaches the *Bursa fabricius* (BF) and secondary viremia occurs. The target cells are the bursal B lymphocytes. The stage of B cell differentiation in the BF is important for viral replication because stem cells and peripheral B cells do not support replication of the virus. The infection causes massive destruction of B lymphocytes in the BF, resulting in lymphopenia and immunosuppression (Sharma et al., 2000; Etteradossi and Saif, 2008). The most severe clinical signs are seen in chicks 3 - 6 weeks old, which is the age when BF approaches its maximal stage of development. Birds under 2 weeks of age are usually less susceptible because of maternal antibodies. However, in young (< 2 weeks) birds without maternal

antibodies, IBDV infection causes bursal lymphoid depletion, resulting in immunosuppression and possible secondary infections. Birds over 6 – 10 weeks develop antibodies against IBDV but do not usually express any clinical signs (Etteradossi and Saif, 2008; Mahgoub, 2012).

Typical clinical signs are watery diarrhea, anorexia, depression and ruffled feathers. Infected birds suffer from dehydration and finally die (Cosgrove, 1962; Etteradossi and Saif, 2008). In susceptible flocks the morbidity is high and mortality can range from nil to very high (90 - 100 %) depending on the pathotype and type of bird (Chettle et al., 1989; Etteradossi and Saif, 2008). Typical gross findings are hemorrhages in the thigh and pectoral muscles and lesions in the BF. Seventy-two hours p.i. the BF begins to increase in size and becomes edematous and hyperemic. At day 4 p.i., the weight of the BF is usually doubled after which it starts to atrophy. At day 5 p.i., BF is again at its normal weight and at day 8 p.i., it is one-third of its original weight. BF lesions in the early stages of the disease are critical in the differential identification of acute IBD because BF atrophy can be caused by many different pathogens, including NDV, CIAV and IBV (Etteradossi and Saif, 2008; Maghoub, 2012).

Diagnosis is based on the typical signs and BF gross lesions as well as histopathological examination of BF. The laboratory diagnosis is usually based on the detection of specific antibodies against the virus, or on detection of the virus in tissues, using immunological or molecular methods (OIE, 2016). Breeder chickens are commonly vaccinated with live IBDV vaccine and boosted later with an inactivated vaccine. This gives the progeny maternal antibodies via the egg yolk that last at least until 4 weeks of age (Maghoub, 2012).

1.1.8 INFECTIOUS LARYNGOTRACHEITIS VIRUS (ILTV)

Infectious laryngotracheitis virus (ILTV) is a DNA virus that belongs to the subfamily *Alphaherpesvirinae* (ICTV, 2015; <http://www.ictvonline.org/virustaxonomy.asp>). It causes upper respiratory tract infection of chickens, pheasants and peafowl, but the chicken is the primary natural host (Crawshaw and Boycott, 1982).

Although ILTV strains are antigenically homogenous, they vary in their virulence and the infection can be separated into a milder enzootic form and a severe epizootic form (Kirkpatrick et al., 2006; Guy and Garcia, 2008). The characteristics of the mild form are nasal discharge, conjunctivitis, swelling of infraorbital sinuses, decreased egg-production and general unthriftiness. In the severe form, marked dyspnea, gasping and coughing of blood-stained mucus is characteristic. The severe form is associated with high mortality (10 – 70 %) (Guy and Garcia, 2008). All ages are affected, but chickens older than 3 week are most susceptible to ILTV (Fahey et al., 1983).

ILTV is transmitted horizontally through the upper respiratory and ocular routes and replicates only in the respiratory tissues, such as in the epithelium of the larynx and trachea, (Beaudette, 1937; Bagust et al., 1986). The infection results in severe epithelial damage and hemorrhage because of the cytolytic effects of the virus. Typical gross lesions are hemorrhagic conjunctivitis and tracheitis with excess mucus or blood. Also diphtheritic lesions can develop in the larynx and trachea (Linares et al., 1994; Guy and Garcia, 2008). An important part of the persistence of the virus is the ability to establish latent infections by spreading to the trigeminal ganglia (Bagust et al., 1986). In its severe epizootic form ILTV can be quite reliably diagnosed on the basis of high mortality associated with the expectoration of blood (Guy and Garcia, 2008). In microscopic histopathological examination, intranuclear inclusion bodies in the epithelial cells of respiratory tissues are pathognomonic for ILTV (Guy et al., 1992).

Since vaccination can also result in latent carrier birds, vaccinations are recommended only in areas where the disease is endemic (Guy and Garcia, 2008). Vaccination is done with live-attenuated vaccines, which have been attenuated by sequential passages in cell culture or sequential passages in chicken embryos. The use of live-attenuated chicken embryo origin vaccines has been associated with adverse effects such as spreading the vaccine to non-vaccinated chickens, insufficient attenuation, production of latent carriers and even gaining in virulence and resulting in outbreaks of vaccinal laryngotracheitis (Guy et al., 1991; Guy and Garcia, 2008).

1.1.9 MAREK'S DISEASE VIRUS (MDV)

Marek's disease virus (MDV) is a DNA virus belonging to the subfamily *Alphaherpesvirinae* (ICTV, 2015; <http://www.ictvonline.org/virustaxonomy.asp>). It is highly contagious and induces lymphoproliferative disease in chickens, quails, turkeys and pheasants (Schat and Nair, 2008).

MDV is divided into serotypes 1-3 (von Bülow and Biggs, 1975a). Serotype 1 is a pathogenic strain, serotype 2 is a naturally avirulent strain and serotype 3 is an avirulent herpesvirus of turkeys (Tulman et al., 2000). Serotype 1 is classified into four pathotypes based on the ability of the virus strain to induce lymphoproliferative lesions in immunized chickens: mild (or classical) MDV, virulent MDV, very virulent MDV and very virulent plus MDV (Witter, 1997; Witter et al., 2005). Virulence of MDV strains has increased over the years and currently mild MDV pathotype strains have not been recognized among recent isolates (Witter et al., 2005).

The feather follicle epithelium is considered to be the major or sole source of natural virus transmission. Vertical transmission does not occur. The

sequential pattern of the pathogenesis of MD is very complex and not yet completely understood (Schat and Nair, 2008). It is divided into four phases: early cytolytic infection; latent infection; late cytolytic infection and immunosuppression; and transformation (Calnek, 1986). The infection occurs by the respiratory route via inhalation of feather dust. The virus initially replicates in the lungs and is then transferred to the lymphoid organs (spleen, thymus, BF) by macrophages (Barrow et al., 2003). The target cells in the lymphoid organs are B cells that undergo cytolytic infection and destruction (Baigent and Davison, 1999; Barrow et al., 2003). Also T cells are activated and cytolytic cell death occurs (Calnek et al., 1984a, b). The death of lymphocytes results in immunosuppression of the host and atrophy of the lymphoid organs (Payne et al., 1976). After 6 – 7 days, T and B cells are latently infected (Calnek et al., 1984a; Lee et al., 1999). If chickens are genetically resistant, the infection may remain latent (Witter et al., 1971). The development of a second phase of cytolytic infection depends on the strain (virulence) and host (genetic resistance) (Addinger and Calnek, 1973). T cells (and to lesser extent also B cells) undergo a complex transformation process and they infiltrate nerves and visceral organs, resulting in the development of lymphomas (Schat et al., 1991).

MD consists of several distinct pathological syndromes: lymphoproliferative, lymphodegenerative, central nervous system related and vascular related syndromes. Lymphoproliferative syndromes are most frequently seen and can be divided in four lesion groups: lymphomas, paralysis, skin leucosis and blindness, and signs vary according to the syndrome (Schat and Nair, 2008). In general, signs are related to the dysfunction of peripheral nerves such as incoordination, stilted gait, progressive paresis and paralysis. In lymphomas signs are many times non-specific (chronic wasting, diarrhea, depression) and death results from dehydration and starvation. In the ocular form there is unilateral or bilateral blindness and in skin leucosis typical swollen feather follicles (tumors) are observed (Schat and Nair, 2008). The onset of lymphomas and paralysis occurs 4 - 12 weeks p.i. and most commonly the clinical disease is seen in birds between 12 and 30 weeks of age (Payne and Biggs, 1967; Niikura et al., 2004). In susceptible flocks mortality can be up to 30 - 60 %. In addition, MDV of high virulence can cause early mortality syndrome that occurs already 8 – 16 days p.i. (Witter et al., 1980). Also transient paralysis, a paralytic syndrome involving the brain, has been described in field flocks. Most birds with transient paralysis recover completely within 24 - 48 hours (Cho et al., 1970; Kenzy et al., 1973).

Typical gross lesions are enlarged peripheral nerves (especially in *plexus coeliacus*, *p. brachialis* and *p. iliaci*) that often are edematous and discolored gray or yellow (Goodchild, 1969). Lymphomatous lesions can be found from many visceral organs and no organ is without occasional involvement. Usually

visceral lymphomas are diffuse enlargements but alternatively they can appear also as focal or nodular lesions (Schat and Nair, 2008).

MD diagnosis is most commonly based on characteristic pathological gross and microscopic lesions although no pathognomonic gross lesions exist and other tumor related agents such as avian leucosis virus and rethiculoendotheliasis virus can cause similar lesions (Schat and Nair, 2008). MDV control is achieved by vaccination that is administered commonly to commercial poultry chicks subcutaneously or intramuscularly before or at hatch.

1.1.10 AVIAN PATHOGENIC *ESCHERICHIA COLI* (APEC)

E. coli bacteria belongs to the family *Enterobacteriaceae*. Today *E. coli* is the most common infectious cause of bacterial disease in poultry (Barnes et al., 2008). Colibacillosis refers to any infection caused by avian pathogenic *E. coli* (APEC) and the syndromes and lesions differ vastly depending on the species, gender, age, immunity status and other diseases of the host (Kariuki et al., 2002; Barnes et al., 2008). Previously, there was a common understanding that colibacillosis is always a secondary disease, but today APEC has become accepted also as a primary pathogen, especially in young chickens (Barnes et al., 2008). It is also suggested that APEC might represent a zoonotic risk by transmitting and causing disease also in humans (Rodriguez-Siek et al., 2005; Moulin-Schouleur et al., 2007).

Serotyping of *E. coli* is most commonly based on two antigens: somatic and flagellar. To date, there are at least 180 somatic (O) and 60 flagellar (H) antigens (Stenutz et al., 2006). Thousands of different serotypes can be divided into two main groups: intestinal commensals and serotypes that can cause extra-intestinal disease. Most of the APEC strains belong to serotypes associated with extra-intestinal infections. Certain *E. coli* serotypes such as O1, O2 and O78 are more frequently associated with colibacillosis (Dziva and Stevens, 2008; Johnson et al., 2008). Compared with pathogenic *E. coli* strains causing infections in mammals, APEC strains do not commonly produce enterotoxins (Blanco et al., 1997). Pathogenicity of APEC is determined by the ability of the bacteria to cause mortality using an embryo lethality assay (Gibbs et al., 2003).

The APEC strains may be further classified based on the virulence genes they possess. The virulence genes are located in the chromosome as well as on plasmids (Ginns et al., 2000; Dozois et al., 2003). In APEC, no single common virulence factor has been identified in all strains. Factors commonly associated with pathogenicity in APEC include: F1 and Pap/Prs fimbriae for colonization, the *iss* gene associated with serum resistance, the *ibeA* gene associated with

invasion, and the *sitA* gene associated with iron acquisition (Dziva and Stevens, 2008).

All avian species and ages are susceptible to APEC, but the disease is usually most severe in young birds (Barnes et al., 2008). APEC can be transmitted both vertically and horizontally. Vertical transmission can cause high chick mortality and in newly hatched chicks the most common outcomes of APEC infections are omphalitis and yolk sack infection as well as colisepticemia (Giovanardi et al., 2005; Petersen et al., 2006). In young birds, especially in broiler chickens, localized cellulitis, lameness (bacterial arthritis) and retarded growth can frequently be observed. Also respiratory-origin colisepticemia (airsacculitis, polyserositis) is a common finding and it is frequently associated with other infectious agents such as IBV, NDV and *Mycoplasma* spp. (Kariuki et al., 2002; Barnes et al., 2008; Landman et al., 2012). Salpingitis-peritonitis syndrome is seen in layers as well as in breeders. It is an ascending infection through the cloaca, although other colonization pathways have also been reported (Vandekerchove et al., 2004). Diarrheal diseases associated with APEC are rare in poultry (Barnes et al., 2008).

Diagnosis is based on isolation of *E. coli* from typical lesions. Bone marrow cultures in septicemic birds are recommended because they are easy to collect and usually free of contaminants (Barnes et al., 2008). Colibacillosis is commonly controlled with antimicrobial agents but major current concerns are residues of antimicrobial agents in food as well as the development of bacterial antimicrobial resistance (Sojka and Carnaghan, 1961; Johnson et al., 2004; Singer and Hofacre, 2006). Improving management actions such as breeder egg hygiene and environmental conditions of the birds is usually beneficial but unfortunately often not effective enough to prevent colibacillosis (Barnes et al., 2008).

1.1.11 **MYCOPLASMA SPP.**

Mycoplasmas are very small bacteria lacking a cell wall, and they belong to the Mycoplasmataceae family. Avian mycoplasmosis most commonly includes two *Mycoplasma* spp. bacteria: *Mycoplasma synoviae* and *Mycoplasma gallisepticum*, the latter being the most important mycoplasma species among commercial poultry and the cause of major economic losses (Mohammed et al., 1987). *M. gallisepticum* occurs worldwide, but its prevalence has decreased markedly due to the implementation of compulsory eradication programs because clinical outbreaks impair international trade (EU, 2009/158/EC). Other mycoplasmas that are important to poultry are *M. meleagridis* (turkeys) and *M. iowae* (turkey embryos) (OIE, 2008). *M. gallisepticum* and *M. synoviae* are transmitted both vertically and horizontally and commonly cause diseases associated with respiratory and locomotory signs in chickens. The strains vary in infectivity and virulence and some infections may appear

clinically silent but still result in decreased production (Noormohammadi, 2007; Kleven, 2008b).

Control of avian mycoplasmosis can be divided in three separate actions. First, the chicks should be sourced only from mycoplasma-free parent flocks. Secondly, the flocks should be maintained free from mycoplasma infection by enforcing strict biosecurity and the infections should be monitored using an effective disease monitoring system. If the first action fails or is not possible, vaccination can be a useful long-term solution. Medication as the third action cannot eliminate the infection from an infected flock and is never a satisfactory long-term solution (Kleven, 2008a). Mycoplasma infections can be detected routinely using both serology and PCR. Serology is commonly used for large-scale monitoring (Feberwee et al., 2005).

1.1.12 ENDOPARASITES

1.1.12.1 *Eimeria* spp.

Coccidiosis is a parasitic disease caused by protozoa of the genus *Eimeria*. *Eimeria* spp. are found in the domestic fowl, turkeys, geese, ducks and pigeons. Generally coccidia are highly host specific (Vrba and Pakandl, 2015). In chicken, nine different *Eimeria* spp. are described and they vary in pathogenicity (Haug et al., 2008). Coinfections with two or more species of coccidia are common (McDougald and Fitz-Coy, 2008).

The complex *Eimeria* life cycle causes intestinal tissue damage, which results in interrupting the digestive processes and nutrition absorption and in some more severe cases also dehydration and anemia. It also allows colonization by secondary pathogens such as *Clostridium perfringens*, the infective agent of necrotic enteritis (Helmbolt and Bryant, 1971; Alnassan et al., 2014). The disease is self-limiting and under normal circumstances most birds shed small numbers of oocysts in their feces without clinical signs. The occurrence of clinical disease depends greatly on the immune status of the host and also on the number of oocysts ingested. Immunity usually develops rapidly, but cross-immunity between different *Eimeria* species is reported to be poor (Johnson, 1923; Chapman, 2003). Typical clinical signs are diarrhea, retarded growth, drop in feed and water consumption and increased mortality (McDougald and Fitz-Coy, 2008).

Each *Eimeria* species has a predilection zone in the gastrointestinal tract and the diagnosis is based on the assessment of macroscopic lesions (location and gross appearance), histopathological analysis and morphological identification of oocysts in native scrapings (Johnson and Reid, 1970). The postmortem lesions are best diagnosed from freshly killed birds (< 1 hour)

(McDougald and Fitz-Coy, 2008). The traditional control of coccidiosis relies on chemoprophylaxis, i.e. the use of anticoccidial drugs in the feed and often rotation or shuttle programs are favored. However, the worldwide use of anticoccidial drugs has caused development of resistance in commercial broiler farms (Jeffers, 1974; McDougald et al., 1986). Coccidiosis vaccines are now commonly used for layers and breeders (McDougald and Fitz-Coy, 2008).

1.1.12.2 *Ascaridia galli*

Ascaridia galli is a widespread nematode of poultry and other birds (Ackert, 1931). It frequently occurs in both intensive and non-intensive poultry production sites (Permin et al., 1999; Jansson et al., 2010). The life cycle of *A. galli* is direct, with no intermediate host. The predilection site of the parasite is the small intestine and the eggs are passed with the feces. Other birds become infected by ingesting the eggs. Severe *A. galli* infestations can result in loss of appetite, weight loss, ruffled feathers, diarrhea, anemia and even mortality, but the pronounced signs are usually evident only among young chickens (Reid and Carmon, 1958; Ikeme, 1971).

1.1.12.3 *Heterakis gallinarum*

Heterakis gallinarum is commonly found in the lumen of ceca of chickens, turkeys and also other birds. The life cycle is direct and adult worms produce eggs in the ceca that are passed in the feces. The worms cause inflammation and thickening of the cecal mucosa with petechial hemorrhages, but usually clinical signs are not seen (Yazwinski and Tucker, 2008). The importance of the parasite rests in it being a carrier of the protozoon *Histomonas meleagridis* (blackhead disease) (Springer et al., 1969).

1.1.12.4 *Capillaria* spp.

Many different *Capillaria* species (threadworms) can affect birds, but among commercial poultry the most common ones are *Capillaria annulata* and *C. contorta*. These two species are found in the mucosa of the crop and esophagus. The worms are small and hair like and sometimes difficult to detect in the intestinal content (Yazwinski and Tucker, 2008). The lifecycle of *C. contorta* is direct. The lifecycle of *C. annulata* is indirect and earthworms are needed as intermediate hosts for the eggs to become infective (Wehr, 1936). The birds are infected when ingesting earthworms. In severe infestations, thickening of the esophagus and crop wall with catarrhal inflammation can be observed and the hosts can become emaciated and anemic (Permin and Hansen, 1998; Yazwinski and Tucker, 2008).

1.1.13 ECTOPARASITES

Poultry ectoparasites can cause irritation and disease to the birds and product losses to the poultry industry. In addition, they can also spread more severe diseases such as *Salmonella* and NDV (Valiente Moro et al., 2005; Valiente Moro et al., 2007). The most commonly found poultry ectoparasites in Finland are summarized here.

1.1.13.1 *Cnemidocoptes mutans*

Cnemidocoptes mutans, also known as scaly leg mite, lives primarily within unfeathered skin, under the leg scales of chickens and turkeys but also among other avian species as the mites are not host specific. The infection spreads usually from the toes upwards and the lesions can occasionally be seen also on the neck, comb and wattles. The mites cause inflammation and keratinization of the legs. Malformation of the feet due to the hyperkeratinization and, in severe cases, also lameness can be observed. The mites pass through their life cycle on the host within 10 - 14 days (Permin and Hansen, 1998; Hinkle and Hickle, 2008).

1.1.13.2 *Dermanyssus gallinarum*

Dermanyssus gallinarum, also known as poultry red mite, is an economically important ectoparasite of laying hens in Europe (Höglund et al., 1995). It is a blood-feeding parasite that can cause behavioral changes, irritation resulting in reduced weight gain and egg production, death due to anemia and poor egg quality because of blood stained eggs (Chauve, 1998; Kilpinen et al., 2005). The red mites spend most of the time hidden in colonies in the cracks of walls and come out to feed only during the dark. The life cycle is very rapid, 7 - 9 days, though the nymphs and adults can both survive several weeks without blood meals (Hinkle and Hickle, 2008). *D. gallinarum* is also known to be carrier of other poultry pathogens such as chicken pox virus, NDV and *Salmonella* spp. (Chauve, 1998; Valiente Moro et al., 2007).

1.1.13.3 *Menacanthus stramineus*

Menacanthus stramineus (chicken body louse) is a chewing louse that feeds on the scale of skin and feathers. The entire life cycle occurs on the chicken in approximately three weeks. The parasite is dependent on the host and dies in five to six days if separated. Predilection sites are the vent area and the underside of the wings. Female lice lay their eggs on feathers and they hatch as nymphs in four to seven days (Hinkle and Hickle, 2008). Lice infestation causes discomfort and irritation to the chicken and severe infestations may result in scabby skin and decreased egg production (Tower and Floyd, 1961).

1.2 COMMON ZONOTIC POULTRY PATHOGENS

Zoonoses are infectious diseases that can directly or indirectly transmit from vertebrate animals to humans, and vice-versa. It is estimated that over 70 % of emerging pathogens are zoonotic (Woolhouse, 2005; Jones, et al., 2008). In addition to the well-known viral zoonotic diseases, AI and ND, poultry can carry, commonly without any signs, bacterial agents that are human pathogens, of which *Salmonella* and *Campylobacter* are the most frequently occurring (EFSA, 2015; Hafez and Hauck, 2015). Other less commonly encountered poultry pathogens with zoonotic potential are *Chlamydia psittaci*, *Erysipelothrix rhusiopathiae* and *Mycobacterium avium* (Hafez and Hauck, 2015). According to the European Food Safety Authority (EFSA), in addition to *Campylobacter* spp. and *Salmonella* spp., *Listeria monocytogenes* and enteropathogenic *Yersinia* spp. are common foodborne zoonoses in Europe, and are briefly reviewed here (EFSA, 2015).

1.2.1 SALMONELLA SPP.

Two species, *Salmonella enterica* and *S. bongori*, belong to the *Enterobacteriaceae* family (Gast, 2008). The genus consists more than 2500 distinct serovars that can be identified on the basis of their antigenic structure and are classified using the Kauffmann-White scheme (Ewing, 1986). All poultry-associated *Salmonella*, as well as most mammalian *Salmonella*, belong to the species *Salmonella enterica* (Table 1). Serovars Pullorum and Gallinarum can cause severe disease in poultry (Gast, 2008).

Table 1. Salmonella infections associated with poultry.

Species	<i>Salmonella enterica</i>		
Subspecies	<i>enterica</i>		<i>arizonae</i>
Serovar	Gallinarum-Pullorum	Non-typhoid <i>Salmonella</i>	
	Avian host-specific: Pullorum disease (<i>S. Pullorum</i>) Fowl typhoid (<i>S. Gallinarum</i>)	Foodborne disease of humans	Acute septicemic disease in young turkey poult

1.2.1.1 Non-typhoidal *Salmonella* (NTS)

Non-typhoidal *Salmonella* (NTS) is the most common bacterial pathogen, causing gastrointestinal infection worldwide and a global burden of 94 million cases, with 155000 deaths each year (Majowicz et al., 2010). The most common zoonotic infections are caused by serovars Typhimurium and Enteritidis (Ferris et al., 2003; Galanis et al., 2006; Siitonen et al., 2008). NTS frequently colonize poultry and other production animals and are important agents of foodborne human salmonellosis (Newell et al., 2010). Human salmonellosis outbreaks are commonly linked to the consumption of poultry products and to a lesser extent contact with live poultry (Gaffga et al., 2012; Loharikar et al., 2012; EFSA, 2015; Trung et al., 2016). Antimicrobial resistance in NTS is considered to be a serious global public health problem. However, resistance rates vary among serovars and geographic areas (Parry and Threlfall, 2008). *S. Enteritidis* is more susceptible to antimicrobial agents than *S. Typhimurium* (Su et al., 2004; Helms et al., 2005).

The most common signs of human salmonellosis are those of uncomplicated gastroenteritis: nausea, vomiting and diarrhea and it only seldom requires antimicrobial treatment. Systemic infections are rare; bacteremia occurs in 5 % of the infected patients and is commonly associated with immunosuppression, young or old age and certain *Salmonella* serovars (Olsen et al., 2001; Fisker et al., 2003; Gordon, 2008).

1.2.2 *CAMPYLOBACTER* SPP.

Campylobacter spp. are Gram-negative, obligate microaerophilic bacteria that are common colonizers of the gastrointestinal tract of a wide variety of animals. Within the genus *Campylobacter* there are three thermophilic species (*C. jejuni*, *C. coli* and *C. lari*) that are the main causative agents of human foodborne campylobacteriosis (Rautelin and Hänninen, 2003; Skarp et al., 2016). *Campylobacter* spp., especially *C. jejuni*, are among the most prevalent zoonotic pathogens associated with diarrhea in humans (EFSA 2015; Man, 2011). In Finland, *Campylobacter* has been the most common cause of infectious gastroenteritis since 1998 (Zoonoosikeskus, 2016). Most campylobacteriosis cases are sporadic but a seasonal prevalence peak during the summer months has been observed in several countries (Altekruse et al., 1999; Rautelin and Hänninen, 2000; Nylén et al., 2002).

C. jejuni commonly colonizes the intestines of avian hosts (Yogasundram et al., 1989; van de Giessen et al., 1998; Sahin et al., 2003; Sulonen et al., 2007). After horizontal transmission, *C. jejuni* colonizes the ceca, large intestine and cloaca in the mucus filled crypts without adhering to the crypt surface, but it may occasionally also be recovered from the spleen and liver (Herman et al., 2003; Cox et al., 2005). *Campylobacter* infections in poultry usually show no clinical signs and no gross or microscopic lesions are induced

(Beery et al., 1988). However, in some experimental reports diarrhea, weight loss and mortality have been observed in newly hatched chicks (Sanyal et al., 1984; Welkos, 1984).

Several studies have identified handling/eating of raw or improperly cooked poultry meat as a risk factor for human campylobacteriosis (Schönberg-Norio et al., 2004; Mughini Gras et al., 2012; Levesque et al., 2013; Strachan et al., 2013; Gözl et al., 2014). Other common sources are unpasteurized milk and natural water (Schönberg-Norio et al., 2004; Davis et al., 2016). Also travelling abroad is considered to be a major risk factor in acquiring campylobacteriosis, especially for individuals living in northern European countries (Skarp et al., 2016). In Finland, sources other than chicken meat seem to have a role at least during the seasonal summer peak (Kovanen et al., 2016). *Campylobacter* shows an increasing resistance to antimicrobials and the use of antimicrobials in poultry has been associated with the development of resistance (McDermott et al., 2002; EFSA, 2016).

Human campylobacteriosis is usually a self-limiting diarrhea, but can occasionally lead to serious p.i. sequelae such as reactive arthritis and polyradiculitis (Guillain-Barre syndrome, Miller Fisher syndrome) (Mishu and Blaser, 1993; Altekruse et al., 1999; Man, 2011; Keithlin et al., 2014).

1.2.3 **LISTERIA MONOCYTOGENES**

Listeria monocytogenes, the causative agent of listeriosis, is commonly found in soil, plants, and surface water and it can colonize a wide range of animal hosts, including arthropods as well as cold and warm-blooded vertebrates (Cossart and Lebreton, 2014). The majority of human listeriosis cases are foodborne, and they are typically linked to ready-to-eat foods because the organism grows at refrigeration temperatures and is tolerant of low pH (Sleator et al., 2003; Liu, 2006; Scallan et al., 2011; Malley et al., 2015). *L. monocytogenes* is commonly isolated from raw poultry meat products (Berrang et al., 2005; Loura et al., 2005; Malley et al., 2015). However, it is only infrequently isolated from live poultry (Milillo et al., 2012; Sasaki et al., 2014). Contamination is thought to occur more often during slaughtering and further processing (Rørvik et al., 2003; Loura et al., 2005). Both in animals and humans the most common *Listeria* infections are caused by three serotypes: 1/2a, 1/2b, and 4b, serotype 4b being the most important in humans (Gilot et al., 1996; Aarnisalo et al., 2003; Lukinmaa et al., 2003). With the exception of tetracycline resistance, most *L. monocytogenes* isolates from different sources are commonly susceptible to the antimicrobials active against Gram-positive bacteria (Charpentier and Courvaline, 1999; Hansen et al., 2005). However, emergence of multiresistant strains has occurred (Poyart-Salmeron et al., 1990; Hadorn et al., 1993; Papa et al., 1996).

Human listeriosis usually causes non-specific flu-like symptoms and gastroenteritis. However, as an opportunistic pathogen, it can most severely affect those who are immune compromised, pregnant females, neonates, and the elderly. Especially when the infection is not controlled by the immune defense system, it can develop into septicemia, meningitis, encephalitis, abortion and in some cases, death (Vázquez-Boland et al., 2001). Cutaneous listeria, i.e. localized papulopustular lesions on the hands and arms occasionally seen among farmers and veterinarians, can result from contact with infective material (Godshall et al., 2013; Zelenik et al., 2014). In animals, infections with *L. monocytogenes* have been recorded in many domestic and wild animals, most commonly in ruminants (Quinn et al., 2002). In poultry, the acute disease is rare, but can be seen sporadically, especially among young birds. The infection occurs either in an encephalitic or septicemic form (Kurazono et al., 2003; Crespo et al., 2013).

1.2.4 **YERSINIA SPP.**

Yersinia spp. belong to the family *Enterobacteriaceae*. To date, 18 different *Yersinia* species exist but only three, *Y. pestis*, *Y. enterocolitica* and *Y. pseudotuberculosis*, are reported to be pathogens of animals and humans (Fredriksson-Ahomaa, 2015). The plasmid for *Yersinia* virulence (pYV) is common to all these pathogenic strains and is needed for bacterial replication in the host tissue (Portnoy and Falkov, 1981; Reuter et al., 2014). The *yadA* gene located on the pYV encodes the outer membrane protein YadA, which promotes the attachment of *Y. enterocolitica* and *Y. pseudotuberculosis* to the intestine (Fredriksson-Ahomaa, 2015).

Enteral yersiniosis is an inflammatory gastrointestinal disease caused by two enteropathogenic *Yersinia* species, *Y. enterocolitica* or *Y. pseudotuberculosis*, the former being the most commonly isolated (Bucher et al., 2008; Long et al., 2010; Fredriksson-Ahomaa, 2012). Today, yersiniosis is the third most frequently reported foodborne bacterial enteritis in the EU (EFSA, 2015). Non-enteral *Y. pestis* is transmitted by the flea and causes the systemic infection known as bubonic plague, as well as pneumonic and septicemic plague (Wren, 2003).

1.2.4.1 ***Yersinia enterocolitica***

Y. enterocolitica is a heterogeneous group of organisms classified into six biotypes and over 60 serotypes. Strains belonging to five of the biotypes (1B, 2 - 5) carry the pYV virulence plasmid and are considered to be pathogenic (Kapperud et al., 1984). The most common bioserotype associated with human disease is 4/O3, which has a ubiquitous distribution (Fredriksson-Ahomaa, 2015). The most virulent type is biotype 1B, which is highly pathogenic to

humans but is very seldom isolated in Europe (Robins-Browne et al., 1989). *Y. enterocolitica* is most commonly transmitted via the fecal-oral route after ingestion of contaminated food or water. The main sources of human infection are assumed to be contaminated, undercooked pork and pork products, pigs being a major reservoir of pathogenic *Y. enterocolitica* (Fredriksson-Ahomaa et al., 2006, Fredriksson-Ahomaa, 2015). However, *Y. enterocolitica* has also been isolated from contaminated blood products, vegetables, surface water, wild rodents and pets (Fukushima et al., 1993; Sandery et al., 1996; Bottone, 1999; Hayashidani et al., 2003; Lee et al., 2004). In addition, *Y. enterocolitica* has sporadically been isolated from chicken eggshell surfaces and also from chicken meat and carcasses (Floccari et al., 2000; Favier et al., 2005; Bonardi et al., 2010).

Gastroenteritis is the most frequent outcome of *Y. enterocolitica* infection, especially among young children. In older children and young adults, acute yersiniosis can be present as pseudoappendicular syndrome. Sometimes long-term sequelae, including reactive arthritis, *erythema nodosum*, uveitis, glomerulonephritis and myocarditis, can occur, and are mainly seen in young adults (Cover and Aber, 1989; Bottone, 1999). Though *Y. enterocolitica* is mainly a human enteric pathogen it has been implicated in sporadic ovine abortion (Corbel et al., 1990). It can also cause enteric disease, precipitated by stress, in pigs, farmed deer, goats, lambs, dogs and cats (Fredriksson-Ahomaa et al., 2001; Quinn et al., 2002). It is also a common cause of mortality in hares (Frölich et al., 2003).

1.2.4.2 *Yersinia pseudotuberculosis*

All *Y. pseudotuberculosis* strains are considered to be pathogenic, although pathogenicity varies (Carniel, 2001). The serotypes associated with human disease are O1 – O5 (Fredriksson-Ahomaa, 2015). The most common infection sources of *Y. pseudotuberculosis* have been reported to be carrots and lettuce (Nuorti et al., 2004; Jalava et al., 2006). In addition, *Y. pseudotuberculosis* has been found in the environment and water but also in various wild and domesticated animals (Fukushima et al., 1998; Hayashidani et al., 2002; Niskanen et al., 2002). Wild birds may be a source of infection of *Y. pseudotuberculosis* for backyard chickens, although it has only very rarely been isolated from wild birds (Fukushima et al., 1991).

In humans, *Y. pseudotuberculosis* causes mesenteric lymphadenitis, diarrhea, and septicemia (Ljungberg et al., 1995). Sporadic abortions caused by *Y. pseudotuberculosis* have been reported in cattle, sheep and goats (Witte et al, 1985; Jerret and Slee, 1989; Otter, 1995).

1.3 DETECTION, IDENTIFICATION AND TYPING OF PATHOGENS IN POULTRY

Accurate pathogen detection and identification is essential for correct disease diagnosis, selection of possible treatment options and also for the epidemiological evaluation of the infection. Detection of pathogens can be divided into direct methods, where the pathogen or part of it (antigen) is detected, and into indirect methods where pathogen-induced antibodies or pathogen-produced toxins are detected. Antibodies, also termed immunoglobulins, are proteins produced by B lymphocytes and are an essential component of adaptive immunity. One of the functions of antibodies is to detect foreign molecules, i.e. antigens, bind to them and neutralize the infective agent and they can generally be detected 1 - 3 weeks p.i. (Bermudez and Stewart-Brown, 2008).

Direct detection methods can further be divided into conventional and molecular methods. A conventional method, such as bacterial culturing, generally involves multiple steps, is time consuming but usually relatively inexpensive, and on many occasions is still the “gold standard” because of its reliability. The development of molecular methods, such as PCR, and especially real-time PCR, enables more rapid detection, identification and also quantification of pathogens. Molecular-based methods also detect non-culturable and non-viable cells (Bhunja, 2014; Fournier et al., 2014). Identification of the pathogens can be based on the phenotypic or genotypic characteristics of the microorganism. Conventional methods rely on phenotypic identification, such as Gram staining and biochemical reactions. Genotypic identification methods can be classified as sequence-based and non-sequence-based (Fournier et al., 2014).

1.3.1 BACTERIAL ISOLATION

In conventional culturing, the bacteria of interest are first isolated, i.e. either cultivated directly from a sample on selective medium or enriched in a special enrichment media, and sub-cultivated on selective culture media, which provides preliminary visual confirmation of the typical growth of certain pathogen. The pathogen is then further identified using biochemical methods, such as commercially available API (analytical profile index) tests, by serological methods, for example direct agglutination or by molecular methods such as PCR based on a specific gene only existing in the target bacterium (Fredriksson-Ahomaa, 2012). Increasingly also matrix assisted laser desorption/ionization mass spectrometry (Maldi TOF) is used for a rapid and sensitive microbial identification and typing (Singhal et al., 2015). A large number of molecular methods are available for bacterial subtyping, such as PFGE and MLST for epidemiological purposes (Hänninen et al., 2001; Mandal et al., 2011; Kovanen et al., 2016).

1.3.2 VIRUS ISOLATION

Virus isolation in specific pathogen-free embryonated chicken eggs is still the “gold standard” method, for example for isolating AIV, NDV and IBV, but it requires multiple steps and is time consuming. However, it is recommended to be used for diagnosis of the first clinical cases of a new viral disease and to obtain virus for further laboratory analysis (OIE, 2015). Viruses can be isolated in cell cultures and laboratory animals as well as in eggs.

1.3.3 POLYMERASE CHAIN REACTION (PCR)

Traditional polymerase chain reaction (PCR) is based on the amplification of a target gene in a thermocycler in the presence of thermoresistant DNA polymerase and target-gene-specific primers. PCR products are then separated by gel electrophoresis and usually visualized under ultraviolet light using ethidium bromide-stained DNA (Mandal et al., 2011). Multiplex PCR is a variation of traditional PCR, where several sets of specific primers are used in order to detect simultaneously multiple gene targets (Law et al., 2015). The use of reverse transcriptase as a first step enables amplification of RNA, which is converted to complementary DNA.

In real-time PCR, a specific detection chemistry based on fluorescence, is chosen for the reaction. Generally, either double-stranded DNA binding dye (such as SYBR® Green) or sequence-specific probes (such as TaqMan®) are used. The fluorescence is generated during the PCR reaction and the amount of the fluorescence released during amplification is directly proportional to the amount of amplified DNA. Thus, the higher the amount of studied DNA in the sample, the faster the fluorescence will increase during the reaction. (Law et al., 2015).

1.3.4 PULSED-FIELD GEL ELECTROPHORESIS (PFGE)

Pulsed-field gel electrophoresis (PFGE) is a non-sequence-based genotyping method that enables typing of bacterial isolates by digesting DNA with rare-cutting restriction enzymes tested to be suitable for each pathogen studied (Sabat et al., 2013). The restricted fragments are resolved into a pattern of discrete bands in gel by electrophoresis, which constantly changes the electric field to be able to separate large DNA fragments (Yan et al., 1991; Tenover et al., 1995). The DNA restriction patterns of different isolates can be compared and their relatedness evaluated. The drawback of the method is that for many zoonotic pathogens there are no standardized criteria for analyzing the fragment patterns and interpretation of the results may differ among laboratories (Tenover et al., 1995). However, it is frequently used in epidemiological studies, for example, for *C. jejuni* and *Y. enterocolitica* and is

extremely useful for differentiation of closely related strains (Hänninen et al., 2001; Fredriksson-Ahomaa, 2006).

1.3.5 MULTILOCUS SEQUENCE TYPING (MLST)

Multilocus sequence typing (MLST) is based on identifying combinations of nucleotide sequences (alleles) of 400 – 500 base pairs, obtained from several individual housekeeping genes. After sequencing, the alleles are assigned a random integer and the combination of alleles at each locus specifies the sequence type. MLST is frequently used for genotyping bacteria such as *C. jejuni* (Dingle et al., 2001). Currently, whole-genome-MLST is used to identify bacteria at the clone level (Maiden et al., 2013; Kovanen et al., 2016). MLST is an effective tool to detect groups of related isolates in population genetics but lacks the resolution to differentiate between very closely related isolates for epidemiological purposes (Colles et al., 2012).

1.3.6 SEQUENCING OF DNA

Different DNA sequencing methods have been developed. However, the Sanger method, where oligonucleotides are sequenced by using fluorescence-labeled chain-terminating dideoxynucleotides, has been that most frequently used during recent decades (Sanger et al., 1977). Next-generation sequencing now enables faster, inexpensive and more accurate sequencing using several different currently available technologies (Illumina, Roche 454, Ion torrent, Solid).

1.3.7 AGGLUTINATION TEST

Agglutination involves specific antigen-antibody binding, which results in measurable agglutination. Many different tests have been developed, such as the whole blood plate agglutination test, the tube agglutination test, the slide agglutination test, the rapid serum agglutination test and the microagglutination test. The agglutination test is not very sensitive, but as a simple, inexpensive, specific and rapid immunoassay it is still commonly used for poultry flock level screening of, for example, *Salmonella* spp. and *Mycoplasma* spp. (OIE, 2012a).

1.3.8 HEMAGGLUTINATION TEST (HA) AND HEMAGGLUTINATION INHIBITION (HI) TEST

Hemagglutination (HA) and hemagglutination inhibition (HI) tests are based on the ability of certain important avian viruses to agglutinate chicken red blood cells. In addition to NDV and AIV, other viruses, such as adenovirus causing egg drop syndrome, have hemagglutinating activity (Adair et al.,

1979). The HA test is used to test the presence of hemagglutinating viruses in samples and a positive result is revealed as hemagglutination. The HA test should be used only as a preliminary screening test and positive findings should be always confirmed with other laboratory tests. The HI test is based on the ability of specific antibodies to inhibit hemagglutination and a positive outcome (presence of antibodies) is when no hemagglutination results (FAO, <http://www.fao.org/docrep/005/ac802e/ac802eod.htm>).

1.3.9 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Enzyme-linked immunosorbent assay (ELISA) is a plate-based immunoassay technique used for detecting antibodies, other proteins or peptides such as antigens or toxins. Different ELISA formats have been developed – direct, indirect, sandwich and competitive assays – and are commercially available for the detection of common poultry diseases and foodborne pathogens (Zhao et al., 2014). Depending on the assay, a specific antigen or antibody is bound on to the walls of microtiter plate well and the presence of antibodies/antigens in the tested sample is detected using an enzyme-conjugated (secondary) antibody and substrate reaction. Generally, ELISA tests are sensitive and specific tests. However, false-positive results can result because of cross-reaction with other antigens/antibodies (Schrijver and Kramps, 1998).

1.4 ANTIMICROBIAL RESISTANCE

Antimicrobials have played an important role in the treatment of various diseases both in humans and animals. The effective control of infections caused by Gram-positive bacteria was achieved after the discovery of penicillin in 1929 and control of *Mycobacterium tuberculosis* after the isolation of streptomycin in 1943 (Fleming, 1929; Comroe, 1978). In the 1940s it was discovered that the use of sub-therapeutic levels of antimicrobials could improve growth rates and feed conversion of production animals, which rapidly led to the use of various antimicrobials in animal feed (Bird, 1968; Khachatourians, 1998). However, it was also soon evident that some bacterial pathogens rapidly developed resistance to many of the first effective drugs and, for example, *Staphylococcus aureus* quickly developed resistance to penicillin by production of beta-lactamases (Murray and Moellering, 1978). The use of large quantities of antimicrobials to prevent and/or control human and animal infectious diseases and in agriculture created favorable conditions for the mobilization and change of resistance elements in bacterial populations (Brown and Wright, 2016).

Already in 1969, the Swann Committee in the UK for the first time recommended that antimicrobials used for the treatment of animal and human infectious diseases should not simultaneously be used as growth

promoters (Swann et al., 1969). Seventeen years later, in 1986, Sweden was the first country to ban completely the use of antimicrobial growth promoters. In 1997, The World Health Organization (WHO) gave a recommendation that antimicrobials used for therapy should not be used for growth promotion (WHO, 1997). Several years later, in 2000, global principles for the containment of antimicrobial resistance in food animals were adopted (WHO, 2001). In January 2006, the EU completely banned all non-medicinal antibiotics in animals (European Union, 2003). However, it is estimated that the global antimicrobial consumption by livestock will continue to rise from the current level, by 67 % by 2030 (van Boeckel et al., 2015).

1.4.1 RESISTANCE MECHANISMS

Bacteria can be resistant to certain antimicrobials innately, but they can also acquire resistance via spontaneous mutations in chromosomal genes and by horizontal gene transfer (Khachatourians, 1998). The evolution of resistant strains is a natural phenomenon but the use and misuse of antimicrobial drugs has markedly accelerated the emergence of drug-resistant strains (Fair and Tor, 2014).

Antibiotics can be categorized according to their principal mechanism of action. Beta-lactam antibiotics, i.e. penicillins, cephalosporins, monobactams and karpabenems, bind to the penicillin binding proteins that normally catalyze the bacterial transpeptidase reaction, thus interfering in bacterial cell wall synthesis (Hooper, 2001). Aminoglycosides (streptomycin, gentamicin) and tetracyclines inhibit bacterial protein synthesis by binding to the ribosomal 30S subunit and macrolides (erythromycin, tylosin) by binding to the ribosomal 50S subunit (Brodersen et al., 2000; Kotra et al., 2000; Tenson et al., 2003). Fluoroquinolones (nalidixic acid, ciprofloxacin) interference with bacterial replication by binding either with DNA gyrase (topoisomerase II) or topoisomerase IV (Hooper, 2001). Rifamycins block the bacterial transcription by binding to the DNA-dependent RNA polymerase (Floss and Yu, 2005). Sulfonamides are inhibitors of bacterial enzymes required for the synthesis of tetrahydrofolic acid (Huovinen et al., 1995). Resistance genes enable the bacteria to survive in the presence of antibiotics, for example by enzymatic destruction, i.e. producing enzymes that can inactivate the antibiotic drug (beta-lactamases) or by producing an alternative metabolic pathway that bypasses the action of the antibiotic. No single mechanism is considered responsible for the resistance in a bacterial organism and several different mechanisms may work together to confer resistance to a single antimicrobial agent (Khachatourians, 1998; Tenover et al., 2006).

Horizontal evolution of resistance, i.e. the acquisition of new genetic material from other bacteria, can occur between the same species or between different species and genera. Mechanisms include conjugation,

transformation and transduction. Conjugation is the direct transfer of DNA, in the form of a plasmid, from one bacterial cell to another. In transformation, free DNA fragments from the environment are taken up by the bacterial cell. In transduction, bacteriophages transfer DNA between bacterial cells (Huddlestone, 2014).

1.4.2 EXTENDED SPECTRUM BETA-LACTAMASE (ESBL) AND AMPC

ESBL/AmpC-producing bacteria are able to hydrolyze penicillin, 2nd and 3rd generation cephalosporins and monobactams (Cantón et al., 2008). Hundreds of different beta-lactamase genes have been detected, the most common ESBL genes being *bla_{SHV}*, *bla_{TEM}* and *bla_{CTX-M}* and the most frequently detected AmpC genes being *bla_{CMY}* and *bla_{DHA}* (Pfeifer et al., 2010). Most of the genes are plasmid-mediated but *E. coli* isolates also carry a chromosomally located *ampC* gene (Jacoby, 2009; Pfeifer et al., 2010).

Extended-spectrum cephalosporins are listed by the WHO as being critically useful antimicrobial drugs in human medicine, and it is now estimated that a risk of transmission of ESBL/AmpC-producing *Enterobacteriaceae* from farm animals to humans exists through the food chain (WHO, 2007; Seiffert et al., 2013). ESBL/AmpC-producing *E. coli* are prevalent in poultry (Pitout and Laupland, 2008; Seiffert et al., 2013). In 2009 – 2011, in the Netherlands, resistant bacteria were detected on 100 % of broiler farms studied (Dierikx et al., 2013; Huijbers et al., 2014). A study from Germany found 73 % and 57 % ESBL and AmpC-producers in cecal samples of healthy broilers, respectively (Reich et al., 2013). In Sweden, in 2010, 34 % of broilers were found to carry ESBL/AmpC-producing *E. coli* (Bengtsson et al., 2011). A recent study from Finland found ESBL/AmpC-producing *E. coli* from 25 % of broiler farms and 8 % of the samples studied (Päivärinta et al., 2016). In those samples, 33 % carried *bla_{CTX-M-1}* and 55 % carried *bla_{CMY-2}* genes. The high prevalence of ESBL-producing *E. coli* in poultry has raised the question of whether meat could be a source of resistant bacteria in humans. A recent study used whole-genome sequencing to study the relatedness of cephalosporin-resistant *E. coli* from humans and chicken meat, poultry and pigs and demonstrated no evidence of clonal transmission from poultry to humans (de Been et al., 2014). However, more studies are needed to answer this question unequivocally.

1.5 BACKYARD POULTRY PRODUCTION

Backyard poultry production has traditionally meant raising domesticated birds such as chickens, ducks and turkeys in close proximity to the house for the purpose of producing meat or eggs for the family. In developing countries,

so-called village chickens have an important role in alleviation of poverty and improvement of food security (Copland and Alders, 2005; Mack et al., 2005). In many instances, chicken products are the only source of high-quality animal protein for such households. Chickens are easily accessible and do not compete for human food resources because they can scavenge for most of their nutritional needs. They are thought to be among the most adaptable domestic animals, being able to survive in variable and harsh environmental conditions (Nhleko et al., 2003). In addition, poultry products can be sold to provide a source of income (Kitalyi, 2007).

In industrialized countries the production of eggs and poultry meat is based on commercial large-scale poultry production systems where biosecurity measures are at a high level, flock health is regularly monitored and certain common diseases are prevented by vaccinations (Hafez and Hauck, 2015). However, at the same time, keeping backyard poultry has become increasingly popular. In addition to keeping chickens for eggs and meat, owners often treat the birds as pets (Garber et al., 2007; Karabozhilova et al., 2012; Smith et al., 2012; Yendell et al., 2012; Elkhoraibi et al., 2014). The animal welfare aspect, as well as favoring local food production and a more sustainable food source, has an important role in the popularity of backyard chickens in industrialized countries (Pollock et al., 2011; Karabozhilova et al., 2012; Elkhoraibi et al., 2014).

The number and variety of different indigenous chicken breeds and lines in the world is vast. In 1998, the Natural Resources Institute Finland (Luke), in cooperation with the Ministry of Agriculture and Forestry, founded a program for the conservation of genetic diversity among twelve Finnish landrace chicken lines. At the end of 2011, there were 285 indigenous flocks registered in the conservation program, containing a total of 4788 chickens (Luke, personal communication).

The registration of backyard poultry flocks in the Finnish National Poultry Register has been obligatory since May 2011, even for the owners of one or two chickens. In May 2012, when our study began, there was a total of 365 small (< 500 birds), non-commercial chicken flocks in the register. These backyard poultry flocks consisted mostly of landrace layer hen lines, but also included other domestic gallinaceous birds such as turkeys, quails and geese (Tike, personal communication). It is important to emphasize that these numbers are probably a huge underestimation of the actual number of flocks.

1.5.1 MANAGEMENT AND BIOSECURITY

The interest in management and biosecurity practices for backyard poultry flocks in industrialized countries is increasing and during the last ten years several questionnaire studies have been conducted among backyard poultry

owners (Table 2). Knowledge of the key characteristics of backyard poultry hygiene and husbandry practices is important when estimating the possible health risk backyard poultry flocks could pose to the commercial poultry industry and/or humans.

Backyard poultry flocks are typically small (< 50 birds) (McBride et al., 1991; Garber et al., 2007; Lockhart et al., 2010; Zheng et al., 2010; Burns et al., 2011; Van Steenwinkel et al., 2011; Beam et al., 2012; Karabozhilova et al., 2012; Smith et al., 2012; Madsen et al., 2013a; Elkhoraibi et al., 2014; Kauber et al., 2016). The chickens are kept for personal consumption of eggs (Zheng et al., 2010; Burns et al., 2011; Van Steenwinkel et al., 2011; Karabozhilova et al., 2012; Smith et al., 2012; Yendell et al., 2012; Madsen et al., 2013a; Elkhoraibi et al., 2014) and as pets (Garber et al., 2007; Karabozhilova et al., 2012; Smith et al., 2012; Yendell et al., 2012; Elkhoraibi et al., 2014). There is also lack of biosecurity practices associated with backyard flocks (McBride et al., 1991; Garber et al., 2007; Zheng et al., 2010; Burns et al., 2011; Van Steenwinkel et al., 2011; Beam et al., 2012; Karabozhilova et al., 2012; Smith et al., 2012; Yendell et al., 2012; Madsen et al., 2013a; Kauber et al., 2016).

Table 2. Questionnaire/interview based studies of backyard poultry flocks in industrialized countries.

Title	Survey method and the number of respondents	Reference
Health survey of backyard poultry and other avian species located within one mile of commercial California meat-turkey flocks.	Interview: 62 respondents	McBride et al., 1991
Epidemiological study of Newcastle disease in backyard poultry and wild bird populations in Switzerland.	Interview: 169 respondents	Schelling et al., 1999
Non-commercial poultry industries: Surveys of backyard and gamefowl breeder flocks in the United States.	Questionnaire: 540 respondents	Garber et al., 2007
A cross-sectional study of ownership of backyard poultry in two areas of Palmerston North, New Zealand.	Questionnaire: 20 respondents	Lockhart et al., 2010
A cross-sectional survey of influenza A infection and management practices in small rural backyard poultry flocks in New Zealand	Questionnaire: 54 respondents.	Zheng et al., 2010
Preliminary investigation of bird and human movements and disease-management practices in noncommercial poultry flocks in southwestern British Columbia.	Interview: 18 respondents	Burns et al., 2011
Assessing biosecurity practices, movements and densities of poultry sites across Belgium, resulting in different farm risk-groups for infectious disease introduction and spread.	Questionnaire: 286 respondents	Van Steenwinkel et al., 2011
Backyard chicken keeping in the Greater London Urban Area: welfare status, biosecurity and disease control issues.	Questionnaire: 30 respondents.	Karabozhilova et al., 2012
Epidemiologic characterization of Colorado backyard bird flocks.	Questionnaire: 317 respondents	Smith et al., 2012
Antibody prevalence of low-pathogenicity avian influenza and evaluation of management practices in Minnesota backyard poultry flocks.	Interview: 150 respondents	Yendell et al., 2012
Salmonella awareness and related management practices in U.S. urban backyard chicken flocks.	Questionnaire: 382 respondents	Beam et al., 2013
Evaluation of Maryland backyard flocks and biosecurity practices	Questionnaire: 41 respondents.	Madsen et al., 2013a
Backyard chickens in the United States: a survey of flock owners	Questionnaire: 1487 respondents	Elkhorraibi et al., 2014
Salmonella knowledge, attitudes and practices: A survey of backyard poultry owners residing in Seattle, Washington and the surrounding metropolitan area.	Interview and videotaping: 50 respondents	Kauber et al., 2016

1.5.2 HEALTH, DISEASES AND MORTALITY

Infectious diseases are recognized as a major limitation to backyard poultry production (Guèye, 1998; Mack et al., 2005). Chickens raised under backyard production systems are exposed to a wide variety of pathogens, and infections can predispose them to secondary infections by other pathogens (Guèye, 1998; Bettridge, 2014). In developing countries, the epidemiology and prevalence of important notifiable viral avian diseases (ND and AI) in the village production systems are quite commonly studied due to the serious outbreaks during recent years (for example: Otim et al., 2007; Harrison and Alders, 2010; Serrão et al., 2012; Rasamoelina Andriamanivo et al., 2012). In contrast, the health of backyard poultry flocks in industrialized countries is still scarcely studied, though interest has increased recently along with the popularity of the hobby.

According to responses to the questionnaires, flock owners subjectively stated health of the birds to be generally good (McBride et al., 1991; Garber et al., 2007; Burns et al., 2011; Elkhoraibi et al., 2014) but only little veterinary/diagnostic laboratory expertise was used (Garber et al., 2007; Zheng et al., 2010; Karabozhilova et al., 2012; Smith et al., 2012; Yendell et al., 2012; Madsen et al., 2013a; Elkhoraibi et al., 2014). According to serological studies done in the USA, Switzerland and New Zealand, occurrence of NDV and AIV is relatively low (0 – 30 %) (McBride et al., 1991; Schelling et al., 1999; Wunderwald and Hoop, 2002; Zheng et al., 2010; Yendell et al., 2012; Madsen et al., 2013b; Madsen et al., 2013c) (Table 3). However, in recent times, backyard flocks have been involved in several disease outbreaks. For example, in 1998, ND was diagnosed in a backyard flock of 48 gamefowl in California (Crespo et al., 1999). In Italy, in 2000, an outbreak of ND was registered and 219 of the 254 infected premises involved backyard flocks (Capua et al., 2002). Also in 2002 – 2003 in an ND outbreak in California, there was involvement of backyard premises (Whiteford and Shere, 2004). In the study of Terregino et al. (2007) it was found that 12 % of backyard flocks tested positive for AIV by PCR and Dimitrov et al. (2016) reported the circulation of highly related NDV in Ukraine and Bulgaria backyard poultry farms from 2002 until 2013. Table 3 summarizes the results of serological studies of infectious diseases among backyard poultry flocks in industrialized countries.

The seroprevalence of respiratory pathogens (aMPV, IBV, ILTV, *M. gallisepticum* and *M. synoviae*) in backyard poultry flocks is commonly high (Wunderwald and Hoop, 2002; de Wit et al., 2004; Madsen et al., 2013b; Haesendonck et al., 2014) (Table 3). Many of these diseases, such as aMPV, IB, and ILT are often controlled in commercial flocks using vaccines, but backyard flocks are vaccinated only very rarely (Wunderwald and Hoop, 2002; Haesendonck et al., 2014). Studies concerning the seroprevalence of the common avian pathogens AEV, CIAV and IBDV, against which commercial poultry are routinely vaccinated, are scarce (Millar and Naqi, 1980; McBride

et al., 2002; Wunderwald and Hoop 2002) (Table 3). Antibodies against these diseases are detected frequently, but many times the role of vaccine-induced antibodies cannot be accurately confirmed.

Table 3. Serological studies of occurrence of selected avian diseases in backyard poultry flocks in industrialized countries. Results show the percentage of seropositive birds/total birds.

	The occurrence (%) of											Reference
	AI	ND	IB	ILT	AE	CIA	IBD	MG	MS	aMPV		
Texas, USA	N/A	N/A	N/A	N/A	N/A	N/A	100	N/A	N/A	N/A		Millar and Naqi,1980
California, USA	0	4	22	N/A	21	N/A	23	12	27	N/A		McBride et al.,1991
Switzerland	N/A	1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		Schelling et al., 1999
Switzerland	3	2	73	28	57	80	65	69	69	N/A		Wunderwald and Hoop, 2002
New Zealand	4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		Zheng et al., 2010
Minnesota, USA	0.1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		Yendell et al., 2012
New Zealand	N/A	30	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		Dunowska et al., 2013
Maryland, USA	N/A	12	N/A	49	N/A	N/A	N/A	7	N/A	N/A		Madsen et al., 2013b*
Maryland, USA	4	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A		Madsen et al., 2013c
Belgium	N/A	N/A	76	30	N/A	N/A	N/A	37	76	64		Haesendonck et al., 2014*

*Vaccination status could not be confirmed in these studies.

MG: *M. gallisepticum*, MS: *M. synoviae*, N/A: not available

The most common diseases and actual causes of mortality among backyard poultry are not known. The owners of backyard chickens use veterinary diagnostic laboratory services only very rarely, which makes estimation of diseases and mortality causes very challenging (Garber et al., 2007; Zheng et al., 2010; Karabozhilova et al., 2012; Smith et al., 2012; Yendell et al., 2012; Madsen et al., 2013a; Elkhoraibi et al., 2014). In California, Mete et al. (2013) performed a retrospective study of the reasons for backyard chicken mortality between 2007 and 2011. Infectious diseases were diagnosed as most important (60 %), MD and *E. coli* bacteria being the most common causes. The

questionnaire studies also provide some data about the common disease signs seen among backyard poultry and the results are summarized in Table 4. However, the reported proportions of health events should be interpreted with caution because they are based on owner observations.

Table 4. The owner-reported signs of diseases among backyard poultry.

	Karabozhilova et al., 2012	Smith et al., 2012	Yendell et al., 2012	Madsen et al., 2013a	Elkhoraihi et al., 2014
Country	UK	USA	USA	USA	USA
Signs of disease	The occurrence (%) of				
Unexplained death	N/A	24	N/A	N/A	N/A
Ectoparasites	N/A	19	N/A	N/A	11
Endoparasites	N/A	N/A	N/A	N/A	N/A
Parasites	91	N/A	N/A	N/A	N/A
Diarrhea	N/A	12	N/A	29	11
Respiratory signs	N/A	13	7	10	N/A
Egg-related problems	23	N/A	N/A	29	10
Neurological signs	N/A	N/A	N/A	5	N/A
Injuries	N/A	N/A	N/A	N/A	8
Weight loss	N/A	N/A	N/A	7	N/A
Lameness	N/A	N/A	21	N/A	N/A

N/A: not available

1.5.3 ZOOBOTIC BACTERIAL DISEASES

Very limited information is available about the prevalence of *Salmonella* and *Campylobacter* in backyard poultry (Table 5). However, a little more prevalence data are available from free-range and organic poultry production, which can be compared with backyard poultry farms, at least regarding their outdoor access. Sulonen et al. (2007) studied 642 fecal samples from 20 Finnish organic laying hen farms in autumn and spring and 84 % and 76 % of the farms were positive for *Campylobacter*, respectively. In northern Spain Esteban et al. (2008) performed a survey on the occurrence of *Campylobacter*, *Salmonella* and *Listeria* in 60 free-range chicken flocks from 34 farms and the results were 71 %, 27 % and 3 %, respectively. A study from Egypt indicated that exposure of children to *Campylobacter* infected backyard poultry may represent a route for transmission of *Campylobacter* infection (El-Tras, 2015).

No prevalence data on *L. monocytogenes* or *Yersinia* spp. in backyard poultry are available. However, Crespo et al. (2013) reported an outbreak of *L. monocytogenes* in an urban poultry flock and in the 5-year retrospective

study of Mete et al. (2013) three listeriosis cases were found from backyard chicken necropsies.

Table 5. Prevalence of certain zoonotic bacterial diseases in backyard poultry flocks in industrialized countries. Results show the percentage of seropositive flocks.

Country	No. of farms	<i>Salmonella</i> spp. %	<i>Campylobacter</i> spp. %	<i>L. monocytogenes</i> %	<i>Yersinia</i> spp. %	
New Zealand	35	N/A	86	N/A	N/A	Anderson et al., 2011
USA	39	0	N/A	N/A	N/A	Madsen et al., 2013b
Australia	30	13	N/A	N/A	N/A	Manning et al., 2015

N/A: not available

1.6 COMMERCIAL POULTRY INDUSTRY IN FINLAND

In Finland, in 2014, an average citizen consumed 77 kg of meat and a quarter (20 kg) of this was from poultry, mostly broiler meat (Luke, 2014). Finland's broiler farms (~230, including parent stock farms) are located mainly in the southwestern and western part of the country and produce 65 million broilers annually (108 million kg meat). The same southwestern area is home to most of Finland's 3.6 million laying hens in approximately 270 farms, making the area high in poultry density. The turkey meat farms (~40) that produce approximately 800 000 turkeys for slaughter every year are more spread out (Luke, 2015).

The health status of Finnish commercial poultry is good (Table 6). Commercial flocks are kept mainly in-housed under strict biosecurity and are vaccinated against a limited range of common infectious poultry diseases (Tables 6 and 7). Efficacy of vaccinations and prevalence of certain pathogens are frequently monitored through a voluntary national disease control program of Evira. The use of live vaccines is strictly controlled by the poultry industry itself and, for example, live IBV, aMPV and ILTV vaccinations are not used in Finland (Table 7). In addition, Finland has an official ND vaccination-free status and ND vaccinations are prohibited by law.

Table 6. Current disease status and vaccinations of commercial poultry in Finland.

Disease/pathogen	Status in commercial poultry in Finland (last finding)	Vaccination in commercial poultry in Finland
Avian encephalomyelitis	Active	Vaccination of breeders/layers
Avian Influenza	Free	No vaccinations
Avian metapneumovirus	Free (1999, broiler breeder flock)	No vaccinations
Chicken infectious anemia	Active	Vaccination of breeders
Infectious bronchitis	Active	Live vaccines not used
Infectious bursal disease	Active	Vaccination of breeders/layers
Infectious laryngotracheitis	Free	No vaccinations
Marek's disease	Active	Vaccination of breeders/layers
Newcastle disease	Free (2004, commercial turkey flock)	Official vaccination-free status
<i>Mycoplasma gallisepticum</i>	Free	No vaccinations
<i>Mycoplasma synoviae</i>	Free (2015, 1 layer farm)	No vaccinations

Table 7. The current recommended vaccination program for commercial poultry in Finland. Modified from Finnish Food Safety Authority Evira.

	MD	Coccidiosis	IBD (live)	CIA (water)*	AE	IBD (Inactivated)	CIA (im)*
	At the hatchery	5-9 days of age	2-8 weeks of age	10 weeks of age	10-16 weeks of age	16-20 weeks of age	16 weeks of age
Egg production							
GPS	X	X	X	X	X	X	X
PS	X	X	X	X	X	X	X
Layers	X	X	X		X		
Meat production (broiler)							
GPS	X	X	X	X	X	X	X
PS	X	X	X	X	X	X	X
Broilers		X**	(X)				

* Alternative methods; ** Only organic broilers are vaccinated (1-day-old)

(X) 10-14 days of age if there is a problem on the farm

GPS: greatparent stock; PS: parent stock; im: intra muscularis

Because of the exceptionally low *Salmonella* prevalence among food-producing animals, Finland has been granted permission to run its own food safety policy concerning *Salmonella* control in the EU. The policy is called the Finnish Salmonella Control Program and it covers pork, beef, poultry, and also the products thereof. The objective of the control program is to maintain the annual prevalence of *Salmonella* below 1 % at the national level. It is also important to emphasize that the program covers meat and egg production for all the serotypes of *Salmonella*, not only *S. Enteritidis* and *S. Typhimurium* (Maijala et al., 2005).

2 AIMS OF THE STUDY

The major aim of the work comprising this thesis was to assess the role of backyard flocks as potential reservoirs for particular zoonotic bacterial agents and infectious avian viruses in Finland.

The specific aims were:

- I. To survey backyard poultry flock owners about their key management and biosecurity practices, and to establish the most common causes of mortality among these birds.
- II. To investigate the occurrence of common zoonotic bacterial pathogens among backyard poultry flocks and their environment.
- III. To investigate the occurrence of common avian virus antibodies among backyard poultry flocks.
- IV. To investigate the genotypes of recent avian infectious bronchitis outbreaks in commercial and backyard poultry flocks.

3 MATERIALS AND METHODS

3.1 QUESTIONNAIRE (I)

Between May and July 2012 a questionnaire was sent to all backyard flock owners that were registered either with the voluntary Luke chicken conservation program or the voluntary Finnish Poultry Association hobbyist register, or both. These registers are the only voluntary registers for backyard poultry owners in Finland. The national poultry register could not be used because of privacy regulations. The questionnaire contained 35 questions, including both binary and open-ended questions and focused on general flock parameters, bird health, bird movement and biosecurity practices. It was possible to respond to the questionnaire anonymously. At the end of the questionnaire, the owner was able to sign up for the voluntary farm visit where blood and cloacal samples were taken.

3.1.1 SAMPLING (II – IV)

3.1.1.1 Samples collected from backyard poultry flocks (II, III)

Blood samples, cloacal swabs and environmental boot swabs were collected from 457 chickens from 51 voluntary backyard poultry farms during October 2012 and January 2013. Animal test approval was not required because the sampling was part of an animal disease-monitoring program carried out with the Evira. Backyard poultry flocks were defined as flocks where the birds were kept for eggs or other products consumed mainly by the owners, and for which the overall number of birds was fewer than 500. The total number of chickens on the 51 farms was 1121, indicating that 41 % of chickens were sampled. The flock size varied from 3 to 80 birds. In very small farms (< 20 chickens), 10 of the chickens were sampled (or fewer if the number of chickens was less). In larger farms (> 20 chickens) 20 chickens were tested. One pair of boot sock samples was collected from each farm. In addition, the owners were asked to retake one boot sock sample between May and June 2013.

Blood samples (III)

The blood samples were obtained from the wing vein (*vena brachialis*) in Venosafe® evacuated blood collection tubes (Terumo Europe, Leuven, Belgium), chilled to 4 °C and sent immediately to the Evira veterinary virology

laboratory, where the samples were stored as sera at – 20 °C until further analysis.

Cloacal swabs (II, III)

Three cloacal swabs were taken from each bird. The aim was to collect approximately 1 gram of feces on each swab, but this was not always achieved. Two of the swabs were collected using Proback Transport Swabs (Technical Service Consultant, Heywood, UK). The samples were sent immediately to Helsinki University, Department of Food Hygiene and Environmental Health laboratory and analyses were started within 48 h of sampling. In the laboratory, one cotton wool stick was transferred into 10 ml of BPW (Buffered Peptone Water (ISO), LAB M, Kerava, Finland) and the other stick into 5 ml of Bolton selective enrichment broth (Oxoid, Basingstoke, UK). The third cloacal swab was collected using a dry brush swab, which was then placed in UTM-RT MINI transport medium (Copan Italia, Brescia, Italy) and sent to the Evira Veterinary Virology laboratory.

Environmental boot swabs (II)

One boot sock sample containing one pair of disposable thin cotton was collected per farm by walking on the bedding of the chicken house. Each boot sock sample was transported to Helsinki University, Department of Food Hygiene and Environmental Health laboratory in a sterile plastic bag. The analyses were started within 48 h of the sampling. The boot sock was transferred into 90 ml of buffered peptone water (BPW) broth and 20 ml of BPW was then added to 80 ml of Bolton selective enrichment broth (Oxoid).

3.1.1.2 Samples for the infectious bronchitis virus study (IV)

Samples were submitted to Evira from different regions of Finland during 2011 – 2013. The samples were:

- Blood samples and dead birds from commercial table egg farm experiencing egg drop and mild signs of respiratory infection (case 1).
- Dead hen from a backyard poultry flock (case 2).
- Blood samples from 45 backyard flocks.
- Blood samples and cloacal swabs from commercial parent stock breeder and layer flocks experiencing egg drop and mild signs of respiratory infection (case 3 and 4).
- Cloacal swabs from broiler flock with signs of mild respiratory infection, slightly increased mortality and decreased growth (case 5).

3.1.2 NECROPSY DATA (I)

The postmortem findings for non-commercial chickens were estimated through a retrospective study of results from necropsies submitted to Evira (Helsinki) during 2000 – 2011. The study included all dead/euthanized chickens that came from flocks < 500 birds. The owners met the costs of the necropsies, except in 2011 when the necropsies were free during the national IBV study to encourage owners to send samples. A poultry pathologist performed the necropsies in Evira. Macroscopically changed tissues were further studied histologically. If there were no clear causes of death, the following tissues were studied microscopically: BF, brain, lungs, heart, liver, spleen, kidneys and thigh muscle. The tissue specimens were fixed with formalin, embedded in paraffin and stained with hematoxylin and eosin. The endo- and ectoparasites were investigated from all the necropsied birds and parasites were microscopically examined and identified to species level.

3.1.3 ANTIBODY TESTING (III – IV)

3.1.3.1 Enzyme-linked immunosorbent assay (ELISA) (III, IV)

Antibodies against IBV, ILTV, CIAV, IBDV and AEV in the sera samples were tested with commercial ELISA kits: Infectious Bronchitis Virus Antibody Test Kit, Chicken Anemia Virus Antibody Test Kit, Infectious Bursal Disease Virus Antibody Test Kit and Avian Encephalomyelitis Virus Antibody Test Kit by IDEXX (IDEXX Corporation, Westbrook, Maine, USA) and Fowl Laryngotracheitis Virus Antibody Test Kit by Synbiotics (Synbiotics Corporation, San Diego, CA, USA). All tests are indirect ELISA tests except Chicken Anemia Virus Antibody Test Kit which is blocking ELISA test. The kits were used according to the manufacturer's instructions. Antibodies against AIV were tested with the commercial competitive ELISA test (ID Screen® Influenza A Antibody Competition Multi-species ID Vet, Grabels, France) according to the manufacturer's instructions.

3.1.3.2 Hemagglutination inhibition (HI) test (III, IV)

NDV antibodies were tested for using an HI test according to the Council Directive 92/66/EEC (Community measures for the control of Newcastle Disease). In addition, positive AI ELISA samples were tested for H5 and H7 antibodies (WHO, 2002).

3.1.4 VIRUS ISOLATION FROM EMBRYONATED EGGS (IV)

Embryonated eggs were inoculated with sample suspensions from cases 1 - 4 via the allantoic cavity route. Briefly, 200 µl of the sample suspension was

injected into the allantoic cavity of 9 - 11 day-old embryonated eggs obtained from breeder farms tested to be free of IBV, NDV and AIV and the eggs were placed back into the incubator. Eggs that died during 24 hours post inoculation were discarded. The allantoic fluid was collected daily from the eggs that had died. The remaining eggs were killed after 6 days and the allantoic fluids were collected. In addition, eggs that received the same inoculum were incubated through 6 days p.i. and then examined for characteristic lesions to verify IBV infection and RT-PCR. In case 2, the allantoic fluid collected was inoculated again into the embryonated eggs for a second passage.

3.1.5 ISOLATION OF *SALMONELLA*, *LISTERIA*, *YERSINIA* AND *CAMPYLOBACTER* (II)

Salmonella was isolated directly from the BPW by inoculating 100 µl on XLD (xylose lysine deoxycholate) agar (LAB M). In addition, after overnight (16 - 18 hours) enrichment at 37 °C, 100 µl of the BPW was inoculated on to a selected MSRV (modified semi-solid Rappaport-Vassiliadis) agar (LAB M). After 24 hour incubation at 42 °C, spreading growth, if present on MSRV agar, was cultivated on XLD agar. Typical colonies were identified using API 20E strips (bioMerieux, Marcy l'Etoile, France).

Listeria was isolated directly from the BPW by inoculating 100 µl on to Oxford agar (LAB M). In addition, after BPW overnight (16 - 18 hours) enrichment at 37 °C, 100 µl was also inoculated into 10 ml of Fraser broth (LAB M) and after a 2-day incubation at 37 °C, 10 µl was cultured on Oxford agar. Also cold enrichment was done: after 21 days at 4 °C, 10 µl of the BPW broth was inoculated on to Oxford agar plates. Typical colonies on Oxford plates were identified with API Listeria (bioMerieux).

Yersinia was isolated directly from the BPW by inoculating 100 µl on to CIN (cefsulodin– irgasan– novobiocin) agar (LAB M). In addition, after BPW overnight (16 - 18 hours) enrichment at 37 °C, 100 µl of the BPW was inoculated on to CIN agar. Also cold enrichment was done: after 21 days at 4 °C, 10 µl of the BPW broth was inoculated on to CIN plates. Typical colonies on CIN plates were identified using API 20E strips (bioMerieux).

Campylobacter was identified by using selective enrichment in Bolton broth (Oxoid). After 48 hours incubation in a microaerobic atmosphere (5 % O₂, 10 % CO₂ and 85 % N₂), 10 µl of the enrichment was cultivated on mCCDA (modified charcoal cefoperazone deoxycholate) plates (Oxoid). All incubations were performed at 37 °C for 2 days. Typical *Campylobacter* colonies were Gram-stained and after cultivation on nutrient blood agar plates, pure cultures were stored at -70 °C in skimmed milk containing 15 % glycerol.

3.1.6 PCR (II – IV)

3.1.6.1 RNA extraction (III, IV)

The RNA of coronavirus, NDV and AIV was extracted for the RT-PCR from organ/swab suspensions or allantoic fluids using the QIAamp Viral Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

3.1.6.2 RT-PCR and sequencing of coronaviruses (III, IV)

The primary coronavirus detection was performed with OneStep RT-PCR kit (Qiagen, Hilden, Germany) and spike gene specific primers published by Keeler et al. (1998) [IV] or polymerase gene-specific primers published by Muradratsoli et al. (2010) [III]. In III an additional spike-gene-specific RT-PCR with primers designed in this study was carried out. The partial spike gene sequencing was done with the primers used in the PCR, BigDye Terminator Cycle sequencing kit v3.1 and ABI3130 automatic sequencer (Applied Biosystems). The sequences were edited and the nucleotide identities calculated using the EMBOSS package (Rice et al., 2000). The sequences chosen for the phylogenetic analysis were aligned with the ClustalW program (Thompson et al., 2002) and the neighboring joining phylogenetic tree was created with the MEGA 5.05 (I) (Tamura et al., 2011) or MEGA 6 (IV) program (Tamura et al., 2013).

3.1.7 NEWCASTLE DISEASE AND AVIAN INFLUENZA REAL-TIME RT-PCR (III)

In NDV real-time RT-PCR a OneStep RT-PCR kit (QIAGEN, Hilden, Germany) was used and the genetically conserved NDV polymerase (*L*) gene real-time RT-PCR was carried out with a TaqMan® Chemistry and Bio-Rad CFX96 machine according to the protocol published by Fuller et al. (2010).

In AIV real-time RT-PCR the OneStep RT-PCR kit (QIAGEN, Hilden, Germany) was used and the AIV *m*-gene real-time RT-PCR was carried out according to the EU Diagnostic Manual for Avian Influenza (Spackman et al., 2002).

3.1.8 SALMONELLA AND YADA-POSITIVE YERSINIA REAL-TIME PCR (II)

DNA was extracted from all overnight enrichments of BPW using a Chelex® 100 resin (Bio-Rad, Hercules, California). From the overnight enrichment, 100 µl was centrifuged at full speed (13000 x rpm) for 1 min. The supernatant was removed and the pellet was resuspended in 100 µl of a 5 % suspension of

Chelex® 100. The suspension was incubated at 56 °C for 20 min and then at 99 °C for 10 min (Fenicia et al., 2007). After 3 min centrifugation at full speed, 50 µl of the supernatant was transferred to a new tube and stored at -20 °C.

For real-time PCR screening, *ttr* of *Salmonella* spp. and *yadA* located on the virulence plasmid of pathogenic *Yersinia* spp. were amplified (Fukushima et al., 2003; Malorny et al., 2004). The total reaction volume for PCR was 25 µl, containing 1x ready-to-use mix (iQ™SYBRGreen Supermix, Bio-Rad, Hercules, CA, USA), 200 nM of primers (Oligomer, Helsinki, Finland) and 2 µl of template. A three-step protocol with annealing at 58 °C and 40 cycles followed by melting curve analysis was used. The fluorescence intensity of the SYBR®Green was studied using the CFX96™ Real-Time PCR Detection System (Bio-Rad). The sample was considered positive when the threshold cycle was below 38 and a specific melting temperature was observed.

3.1.9 **CAMPYLOBACTER SPECIES CONFIRMATION BY MULTIPLEX PCR (II)**

Species confirmation of *Campylobacter* (*C. jejuni*/*C. coli*) was performed using species-specific multiplex PCR (Denis et al., 1999). DNA was isolated from subcultures cultivated on nutrient agar containing 5 % blood using a commercial DNA extraction Kit (Wizard Genomic Purification Kit, Promega, Madison, WI, USA). For visualization of PCR products, 10 ml aliquots were subjected to electrophoresis in a 1.5 % agarose gel stained with ethidium bromide for 2 hours at 100 V and viewed under ultraviolet light.

3.1.10 **ESBL/AMPC-PRODUCING E.COLI PCR (II)**

One milliliter of overnight BPW of boot sock samples and a maximum of ten cloacal swabs taken from individual chickens at the same farm were pooled in 10 ml BPW with 1 mg/l cefotaxime (Sigma-Aldrich, Munich, Germany) and incubated overnight at 37 °C. Subsequently, 10 µl of the selective enrichment broth was spread on MacConkey agar plates (Becton, Dickinson and Company, France) containing 1 mg/l cefotaxime and incubated overnight at 37 °C. Typical lactose-fermenting pink colonies were picked and presumptive *E. coli* isolates were confirmed with the API 20E test (bioMérieux SA, France). *E. coli* isolates expressing ESBL and/or AmpC phenotype with the AmpC and ESBL ID Set (D68C, Mast Diagnostics, UK) were tested for the presence of the ESBL or the plasmid-borne AmpC genes as described previously by Dallenne et al. (2010). If no ESBL/AmpC genes were detected, mutations in the promoter region of the chromosomal *ampC* gene were examined: the 343-bp region was amplified using the primers described previously: 50-GTTGTTTCCGGGTGA TGC-30 (Hasman et al., 2005) and 50-TGGAGCAAGAGG CGGTA-30 (Nelson and Elisha, 1999). Obtained PCR products were confirmed with sequencing.

The sequencing was performed in the Institute of Biotechnology (Helsinki) with the same primers used in the PCR. Sequences were analyzed with CLC Main Workbench software (version 6.6.2, CLCbio, Denmark).

3.1.11 PULSED-FIELD ELECTROPHORESIS (PFGE) TYPING OF *CAMPYLOBACTER* (II)

For the PFGE analysis, the isolates were grown on nutrient blood agar for 2 days at 37 °C in a microaerobic atmosphere. The bacterial cells were harvested and treated with formaldehyde to inactivate endogenous nucleases (Gibson et al., 1994). The bacteria were embedded in 1 % low melting-point agarose plugs (SeaPlaque GTG; FMC Bioproducts, Rockland, Maine). After DNA purification 2 mm slices of the agar plugs were digested using KpnI (New England Biolabs Inc.; 20 U per sample) restriction enzyme (Maslow et al., 1993). The restriction fragments were separated with ramped pulses of 1 – 25 s for 19 hours on a Gene Navigator (Pharmacia LKB Biotechnology AB, Uppsala, Sweden). The results were analyzed with BioNumerics V. 5.10 software (Applied Maths, Kortrijk, Belgium) using the Dice similarity coefficient with 0.5 % optimization and 1 % tolerance. Clustering was performed with the unweighted pair group method using arithmetic averages.

3.1.12 ANTIMICROBIAL SUSCEPTIBILITY TESTING (II)

3.1.12.1 *C. jejuni* and *C. coli*

In total, 57 *C. jejuni* isolates (up to nine isolates per *Campylobacter*-positive farm) and the only *C. coli* isolate were tested for ciprofloxacin, erythromycin and tetracycline susceptibility (MIC lg/ml) using the agar dilution method on Mueller-Hinton blood agar plates according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2008). Epidemiological cut-off values were used for *C. jejuni* isolates to consider them to be resistant to ciprofloxacin, erythromycin and tetracycline with MICs of >0.5, >4 and >2 µg/ml, respectively (EUCAST, 2015). The MIC of 8 µg/ml to erythromycin was used for *C. coli*. *C. jejuni* ATCC 33560 was used as a quality control strain.

3.1.12.2 *L. monocytogenes*, *S. enterica*, *Y. enterocolitica* and *Y. pseudotuberculosis*

Antimicrobial susceptibility was tested according to CLSI guidelines by using the broth microdilution method (VetMICTM, National Veterinary Institute SVA, Uppsala, Sweden) (CLSI, 2008). The panel of antimicrobial agents included in the VetMIC GP-mo (version 2) was used for *Listeria* and Vet- MIC GN-mo (version 4) was used for *Yersinia* and *Salmonella*. For *L.*

monocytogenes, the *Staphylococcus* breakpoints determined according to EUCAST (2015) criteria were used for chloramphenicol, ciprofloxacin, clindamycin, erythromycin, fusidic acid, gentamicin, penicillin, oxacillin, tetracycline and trimethoprim and for cephalothin and kanamycin breakpoints according to CLSI (2014) were used. The species-specific breakpoints for penicillin and erythromycin were used according to EUCAST (2015). For *Y. enterocolitica* and *Y. pseudotuberculosis*, breakpoints set to *Enterobacteriaceae* were used for ampicillin, cefotaxime, ceftazidime, chloramphenicol, ciprofloxacin, colistin, gentamicin and trimethoprim and breakpoints set to *E. coli* were used for nalidixic acid, sulfamethoxazole, streptomycin and tetracycline according to EUCAST (2015). The breakpoint for kanamycin was obtained from CLSI (2014) guidelines. *Streptococcus pneumoniae* ATCC 49619, *Staphylococcus aureus* ATCC 29213 and *E. coli* ATCC 25922 were used as quality control strains.

3.1.13 STATISTICAL ANALYSIS (II)

Statistical analysis was performed using SPSS 23 (IBM SPSS Software, New York, USA). The sizes of the 51 flocks were not normally distributed (Kolmogorov–Smirnov test of normality $P < 0.05$). A Mann–Whitney U-test was used to analyze the significance between the mean number of animals in the positive and negative flocks. A confidence level of 95 % was applied.

4 RESULTS

4.1 BACKYARD POULTRY FLOCKS IN FINLAND (I)

4.1.1 GENERAL CHARACTERISTICS

Of the 378 questionnaires sent, 181 were completed and returned and 178 were accepted for the study (response rate 48 %). The returned questionnaires came from all regions of Finland. All flocks included chickens and in 35 % of the flocks there was at least one other gallinaceous bird, turkey being the most common. The majority (71 %) of flocks had 11 – 50 birds, only 9 % were flocks of more than 50 birds. Most of the birds were kept for eggs (79 %) and as pets (72 %) and almost all flocks (98 %) were kept outdoors at least part of the year. Most owners (83 %) had registered the flock on the national poultry register. Bird movement was frequent: most of the participants had purchased (82 %) or sold (76 %) birds during the previous 5 years.

4.1.2 BIOSECURITY PRACTICES

Only 13 % of the respondents reported that they changed shoes when entering the poultry premises and only 35 % had the possibility to wash hands when leaving the premises. In one third (36 %) of the farms the chickens had a possibility to be in a contact with wild birds, though the majority (77 %) of respondents reported that they complied with the national legislation that requires keeping poultry inside during the spring migration of wild birds (March 1st to May 31st). The backyard poultry farmers commonly had other farm animals (55 %) and pets (90 %) and they also allowed visitors to visit poultry premises (84 %). Only 6 % of the farms were located less than three kilometers from the commercial farm and connections between backyard and commercial poultry farms were rare (6 %).

4.1.3 FLOCK HEALTH

The owner-reported flock health was excellent or good (96 %) and mortality of the birds was low. The most common health issues encountered were ectoparasites (31 %), sudden deaths (30 %) and diarrhea (18 %). Only one owner once vaccinated the chickens against Marek's disease. In one quarter (24 %) of the flocks at least one bird had been medicated during the previous year. The medications were usually routine treatments against parasites. Of the 178 respondents, 169 (95 %) reported no veterinary contact during the last year.

4.1.4 POSTMORTEM FINDINGS

Necropsy was performed on a total of 132 non-commercial chickens at Evira (Helsinki) during 2000 - 2011. The chickens examined were either spontaneously dead or euthanized at the farm. The most common postmortem findings were MD (27 %) and colibacillosis (17 %). All chickens examined tested negative for *Salmonella* spp. One or more ectoparasite species was found from 19 % of the chickens, *Menacanthus stramineus* being the most common. Endoparasites were found from 40 % of the chickens, *Heterakis gallinarum* being the most common.

4.2 ZONOTIC BACTERIA IN BACKYARD POULTRY FLOCKS (II)

Table 8 summaries the methods and results of bacteriological studies of *Salmonella*, *Campylobacter*, *Yersinia* and *Listeria*.

4.2.1 SALMONELLA SPP.

Salmonella Typhimurium phage type U277 was isolated from one farm from a boot sock sample taken by the owner (spring sampling). The boot sock sample was also positive according to PCR. The cloacal samples and boot sock samples taken 6 months earlier (winter sampling) from the same farm were negative both by culturing and PCR. *S. Typhimurium* was susceptible to all tested antimicrobials.

In addition to isolation, all samples were studied by PCR for *Salmonella* carrying the *ttr* gene (Malorny et al., 2004). *Salmonella* was detected from two cloacal swabs (0.4 %) and three boot sock samples (3 %).

4.2.2 CAMPYLOBACTER SPP.

C. jejuni was the most common zoonotic pathogen isolated from the backyard poultry farms; 45 % of the farms were positive either for individual cloacal sampling or for environmental boot sock samples (winter/spring). *C. coli* was isolated only once from a boot sock sample in winter sampling. *Campylobacter* was isolated more often from the boot sock samples (22 %) than from cloacal samples (13 %) and also more often in winter than in spring (14 %). However, no significant difference was recorded ($P > 0.05$).

In total, 31 different PFGE types (genotypes 1 - 31) were obtained among *C. jejuni* isolates from 57 cloacal swabs (up to nine isolates per *Campylobacter*-positive farm) and 15 boot sock samples using KpnI restriction enzyme. On six farms (27 %), isolates with several different PFGE types were detected. Only

two PFGE types (genotypes 3 and 18) were found to overlap between two farms and genotype 9 was the only type that was isolated from the same farm in winter and spring.

Most *C. jejuni* isolates were susceptible to ciprofloxacin and tetracycline (both 79 %) but isolates simultaneously resistant to ciprofloxacin and tetracycline were detected at six farms (27 %). In addition, isolates either resistant to ciprofloxacin or tetracycline were detected from two farms. All isolates of PFGE type 3, were resistant to ciprofloxacin and tetracycline. On two farms, isolates with same PFGE type showed different susceptibilities to ciprofloxacin and tetracycline. All isolates were susceptible to erythromycin.

4.2.3 *LISTERIA MONOCYTOGENES*

L. monocytogenes was recovered from 33 % of the farms but it was found only rarely (1 %) from individual cloacal samples. It was significantly more frequently isolated from the environmental boot sock samples taken in the winter (26 %) than in spring (5 %) (Fisher's exact test, $P = 0.013$). Serotypes 1/2a and 4b were identified in both cloacal and boot sock samples (not published data). Serotype 1/2a was found on most (88 %) of the positive farms and serotype 4 on only two (12 %) positive farms.

Antimicrobial susceptibility of 19 *L. monocytogenes* isolates from 16 farms (6 cloacal swabs, 13 boot sock samples) was tested against 12 antimicrobial agents. All *L. monocytogenes* isolates were susceptible to cephalothin, chloramphenicol, erythromycin, gentamicin, kanamycin, penicillin, tetracycline and trimethoprim. None of the isolates showed susceptibility to clindamycin, fucidin or oxacillin and one isolate was resistant to ciprofloxacin.

4.2.4 *YERSINIA SPP.*

Y. enterocolitica was frequently isolated from the farms (31 %), and it was more frequently isolated from environmental boot sock samples (10 %) than from cloacal samples (2 %), and from spring sampling (22 %) than from winter sampling (10 %). All isolates were *yadA* negative belonging to non-pathogenic biotype 1A. *Y. pseudotuberculosis* was rare, it was isolated from only one farm and from a single chicken's cloacal swab. The *yadA* gene was also confirmed by PCR and the serotype was O:1.

Additionally, all samples were studied using PCR for *Yersinia* carrying the *yadA* gene. In total, *yadA* positive *Yersinia* was detected in 13 cloacal swabs (3 %) and five boot sock samples (6 %), but only the previously mentioned *Y. pseudotuberculosis* O:1 was confirmed by cultivation.

Antimicrobial susceptibility of 15 *Y. enterocolitica* isolates (10 cloacal swabs and 5 boot sock samples) from nine farms and one *Y. pseudotuberculosis* isolated from a cloacal swab, was tested against 13 antimicrobial agents. All *Y. enterocolitica* isolates were susceptible to cefotaxime, chloramphenicol, ciprofloxacin, colistin, gentamicin, kanamycin, nalidixic acid, streptomycin and tetracycline. Some isolates showed resistance to ceftazidime, sulfamethoxazole and trimethoprim. Three of the isolates were also susceptible to ampicillin. The *Y. pseudotuberculosis* isolate was resistant to colistin, but susceptible to ampicillin.

Table 8. The summary of methods and results of bacteriological studies of *Salmonella*, *Campylobacter*, *Yersinia* and *Listeria*.

BACTERIOLOGICAL STUDIES							
Sampling in 51 backyard flocks							
51 boot sock samples from bedding (winter)							
457 cloacal samples							
BPW						Bolton broth 48 h, 37 °C	
Oxford 37 °C 24–48 h	BPW 37 °C 16–18 h	BPW 4 °C 20 d	XLD: 42 °C 24 h	BPW 37 °C 16–18 h	CIN 30 °C 24 h	BPW 37 °C 16–18 h	mCCDA agar 48 h, 37 °C 5% O ₂ , 10% CO ₂ , 85% N ₂
	Fraser 37 °C 48 h	Oxford 37 °C 24–48 h		MSRV 42 °C 24 h		BPW 4 °C 20 d	Cloacal samples: 13 % <i>C. jejuni</i> 0 % <i>C. coli</i>
	Oxford 37 °C, 24–48 h		XLD 42 °C 24 h		CIN: 30 °C 24 h		Boot sock samples: 22 % <i>C. jejuni</i> 2 % <i>C. coli</i>
Cloacal samples: 1 % <i>L. monocytogenes</i> Boot sock samples: 26 % <i>L. monocytogenes</i>		Cloacal samples and boot sock samples: 0 % <i>Salmonella</i>		Cloacal samples: <i>Y. enterocolitica</i> 2 % <i>Y. pseudotuberculosis</i> 0.2 % Boot sock samples: <i>Y. enterocolitica</i> 10 % <i>Y. pseudotuberculosis</i> 0 %			

4.2.5 ESBL/AMPC *E. COLI*

Lactose-fermenting *E. coli* was isolated from MacConkey-cefotaxime agar plates from nine samples. In six samples (three farms) AmpC-positive *E. coli* isolates, which phenotypically were confirmed as AmpC-producers, were detected by PCR. On one farm, AmpC-*E. coli* was isolated only from the boot sock sample. On two farms, AmpC-*E. coli* was isolated from both the boot sock and pooled cloacal swab samples. Plasmid-borne AmpC gene (*bla_{CMY-2}*) was found on two farms as well as mutations in the promoter region of chromosomal *ampC*. No ESBL genes were detected.

4.3 ANTIBODIES AGAINST AVIAN VIRUSES IN BACKYARD POULTRY FLOCKS (III)

In total, 457 samples were tested for antibodies against AEV, CIAV, IBV, IBDV and ILTV. In addition, 298 samples were tested for antibodies against NDV and AIV. AEV and CIAV were both common findings (both 86 %) at farm level. Only 0.7 % of the 298 chickens studied were positive for AIV antibodies and in further studies they were found to be negative for H5 and H7 antibodies. The results are summarized in Table 9.

Table 9. The methods and results of selected avian viral diseases in backyard poultry flocks in Finland.

VIROLOGICAL STUDIES												
Sampling in 51 backyard flocks												
	457 blood samples						457 cloacal samples					
	ELISA test					HI test			RT-PCR (number of samples)			
	AE, CIA, IB, IBD, ILT 457 samples					AI 298	ND 298	AI- ELISA pos tested for H5/H7	ND real- time RT- PCR 298	AI real- time RT- PCR 298	polymerase gene specific RT- PCR and spike gene specific RT- PCR 457	
Virus	CIA	AE	IB	ILT	IBD	AI	ND	AI	ND	AI	IB	
Positive chickens %	53	34	21	4	3	0.7	0	0	0	0	2	
Positive farms %	86	86	47	12	20	5	0	0	0	0	10	

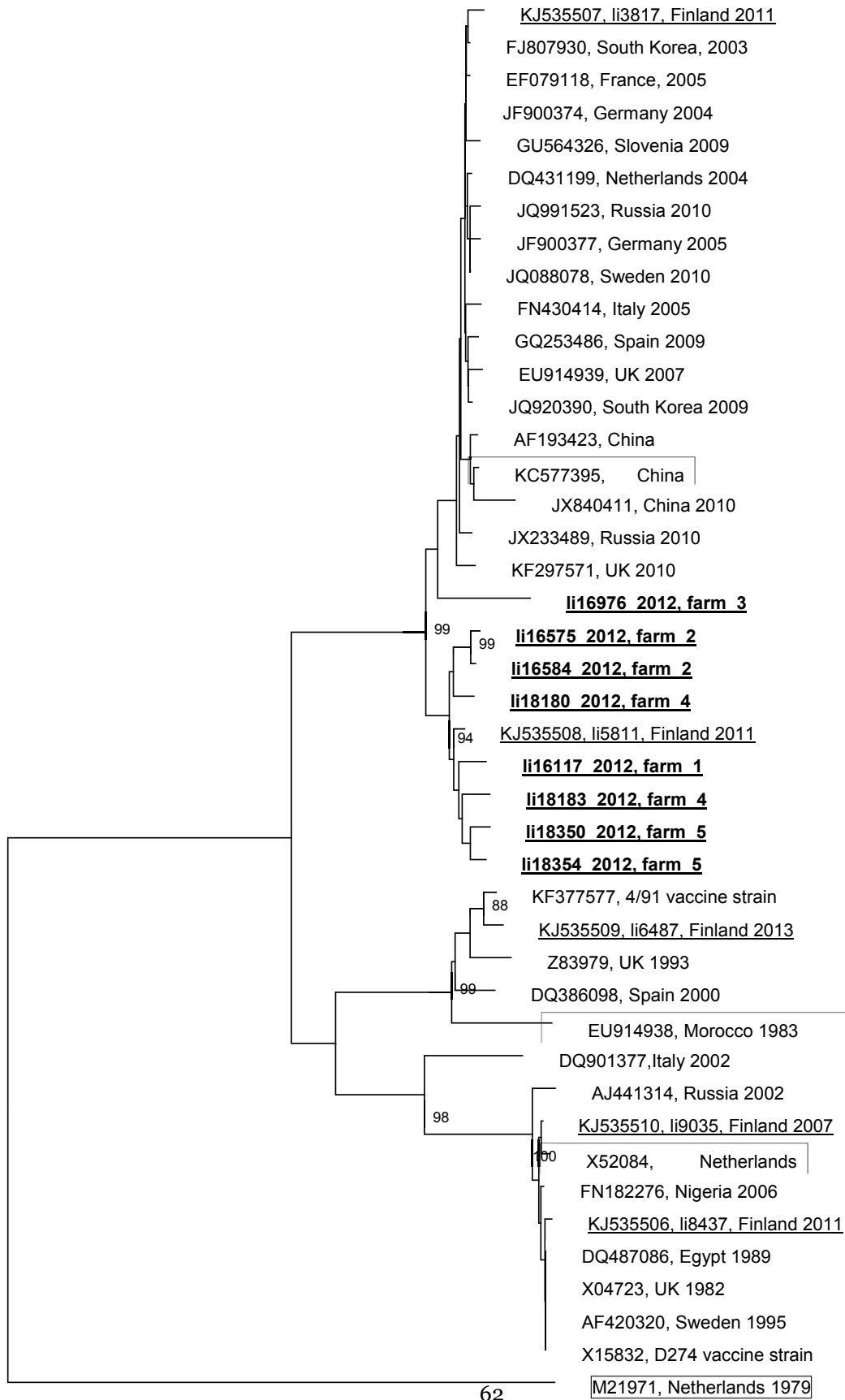
4.4 INFECTIOUS BRONCHITIS VIRUS GENOTYPES IN FINLAND (III, IV)

In 2011 - 2013 several outbreaks of IBV were detected in Finland. The first case was from a commercial table egg-producing farm and the QX genotype (li3817/2011) was detected from Finland for the first time (Figure 1). Soon another QX genotype (li5811/2011) was found from a dead backyard poultry hen, but it shared only 91.5 % nucleotide identity with the first case (li3817/2011). After that, several cases from commercial breeder, layer and broiler farms were detected and vaccine-like genotypes D274 (li18437/2011) and 4/91 (li6487/13) were detected.

Cloacal samples were obtained from 51 voluntary backyard poultry farms between October 2012 and January 2013. Five of the 51 farms (10 %) had chickens that tested positive for coronaviruses by RT-PCR. In total, coronavirus was detected in nine birds, but only eight viruses could be partially

sequenced for phylogenetic analysis (li16976/2012, li16575/2012, li16584/2012, li18180/2012, li18350/2012, li18354/2012, li16117/2012, li18183/2012). The phylogenetic analysis based on 436 nucleotides of the spike gene showed that all coronaviruses from backyard chickens collected in this study were QX type IBV, grouping together with GI-19 lineage (Valastro et al., 2016). They shared 86 % nucleotide identity at a minimum. The previously found QX strains in Finland (li5811/2011 and li3817/2011) grouped tightly with them (Figure 1).

Figure 1. Phylogenetic analysis of Finnish backyard poultry IBV strains based on 436 nucleotides of the spike gene. Only bootstrap values higher than 85 % are shown. The Finnish 2012 backyard poultry virus strains are in bold and underlined and the Finnish 2007, 2011 and 2013 strains are underlined. The prototype strains of lineages GI-12, GI-13, GI-19 and GII-1 according to Valastro et al. (2016) are boxed



0,05

5 DISCUSSION

5.1 BACKYARD POULTRY FLOCKS IN FINLAND

The general characteristics and management of backyard poultry flocks in Finland were studied for the first time using a questionnaire. The questionnaire response rate, 48 %, was fairly good compared with that for other similar studies, such as those of Smith et al. (2012) (39 %) and Madsen et al. (2013a) (4 %). It was not possible to use the national poultry register as the source of the backyard poultry owners for privacy reasons, which may have resulted in biases in the study. As we used the voluntary Luke conservation program register and the Finnish Poultry Association's hobbyist register, it may be that we contacted the owners most active and interested in the hobby and they already had more knowledge about chicken health than the average owner. Additionally, because all owners in the Luke register raise mainly Finnish landrace chicken lines, it may be that the health status of those chickens is better than it is among pure-bred show chicken breeds that may even be imported.

There is no common definition for backyard poultry flocks. In our study, we used the definition of the Dutch Ministry of Agriculture, Nature and Food Quality, in which backyard poultry flocks comprise fewer than 500 birds (Bavinck et al., 2009). Two of the farms were excluded from our study because they each had more than 500 birds but, at the same time, only 9 % of the flocks in our study had birds more than 50. Many other studies have used the size definition of fewer than 1000 birds (Garber et al., 2007; Smith et al., 2012; Yendell et al., 2012; Madsen et al., 2013). However, the flock sizes in previous studies from others were similarly small (mainly < 50 birds), which means that the results from different studies are probably well comparable (McBride et al., 1991; Garber et al., 2007; Lockhart et al., 2010; Zheng et al., 2010; Burns et al., 2011; Van Steenwinkel et al., 2011; Beam et al., 2012; Karabozhilova et al., 2012, Smith et al., 2012; Madsen et al., 2013; Elkhoraibi et al., 2014).

The general characteristics of backyard poultry flocks in our study were surprisingly similar to those from other industrialized countries. In addition to the small flock size, the chickens were kept mainly for personal consumption of eggs (Zheng et al., 2010; Burns et al., 2011; Van Steenwinkel et al., 2011; Karabozhilova et al., 2012; Smith et al., 2012; Yendell et al., 2012; Madsen et al., 2013; Elkhoraibi et al., 2014), were also seen as pets (Garber et al., 2007; Karabozhilova et al., 2012; Smith et al., 2012, Yendell et al., 2012; Elkhoraibi et al., 2014) and the chickens had access to outdoors for at least part of the year (Lockhart et al., 2010; Smith et al., 2012; Yendell et al., 2012; Elkhoraibi et al., 2014).

One common finding for our and several other studies was the need for improvement in biosecurity practices (McBride et al., 1991; Garber et al., 2007; Zheng et al., 2010; Burns et al., 2011; Van Steenwinkel et al., 2011; Beam et al., 2012; Karabozhilova et al., 2012; Smith et al., 2012; Yendell et al., 2012; Madsen et al., 2013a). Biosecurity practices can roughly be divided into those that protect the birds and those that protect the owners. Typically on Finnish backyard poultry farms, visitors were allowed into the poultry premises (84 %), the owners did not change shoes when going to the premises (87 %), birds had access to outdoor (98 %), and they were also in a close contact with other farm animals (55 %), wild birds (36 %) and pets (90 %), all factors which could increase the risk of the birds contracting contagious diseases. On the other hand, owners rarely had a possibility to wash hands when leaving the poultry premises (35 %) and also quite often the chickens were free ranging in the yard (44 %), which meant close contact between the birds and owners and predisposition of the owners to zoonotic pathogens carried by the birds. The outdoor access is an intriguing issue because it clearly predisposes the birds to pathogens but at the same time reflects well the perception about what good animal welfare means to us today (Terragni and Torjusen, 2007).

Frequent bird movement is a known risk for contagious avian diseases such as AI (Sims et al., 2005; Wang et al., 2006). The movement (selling/purchasing/exhibition) of birds varied among studies. The studies of Garber et al. (2007) and Van Steenwinkel et al. (2011) found that birds in backyard flocks were moved only rarely. In Colorado and British Columbia, as well as in our study, birds were moved frequently from the home premises, although the different time lines make comparison difficult (Burns et al., 2011; Smith et al., 2012). In the Colorado study, the movement also impacted negatively on the health (respiratory disorders) of the birds (Smith et al., 2012).

Almost all owners in our study (96 %) reported that the health of their birds was good or excellent, a finding that is similar to that for previous studies (McBride et al., 1991; Garber et al., 2007; Burns et al., 2011; Elkhoraibi et al., 2014). The most frequently reported health issues in our study were ectoparasites (31 %), sudden, unexplained deaths (30 %) and diarrhea (18 %). These three issues are quite visible and easy to recognize by the owners. It can be speculated that mild respiratory and neurological signs are much more difficult for owners to detect and can be a reason for the low numbers. However, the owner-stated results correspond quite well with the necropsy findings in which MD and colibacillosis were the most common infectious reasons for mortality. MD and colibacillosis can both cause diarrhea and sudden death without clear signs and MD also predisposes birds to parasites (Schat and Nair, 2008). Moreover, the low seroprevalence of serious avian diseases among these birds supports their good health status and reflects low

occurrence of these diseases among commercial poultry flocks. In California, Mete et al. (2013) conducted a similar five-year retrospective study where MD was the most common viral disease and *E. coli* was the most common bacterial infection causing mortality among backyard chickens. Our results support those and are no surprise because colibacillosis is the most common infectious bacterial disease in commercial poultry (Barnes et al., 2008) and MD is known to cause serious problems, especially in multi-age poultry farms (Heier and Jarp, 2000).

In our study, of the 51 sampled flocks, in 20 (39 %) one or more of the chickens had been medicated during the previous year. Most of the medications were against internal or external parasites. It is somewhat worrying that veterinarians seem to prescribe medications for backyard chickens that are not licensed to use in production animals (such as selamectin, Stronghold®), i.e. the medicines have no indicated withdrawal time for eggs or meat. It was common to almost all studies, including ours, that veterinary/diagnostic laboratory services were used among these flocks only infrequently (Garber et al., 2007; Zheng et al., 2010; Karabozhilova et al., 2012; Smith et al., 2012; Yendell et al., 2012; Madsen et al., 2013a; Elkhoraibi et al., 2014). This can seriously delay detection of certain notifiable diseases and possibly also aid the further spread of the disease, including to commercial farms.

5.2 COULD BACKYARD POULTRY POSE A HEALTH RISK TO HUMANS?

Contact with live poultry is a known risk factor for human infection with zoonotic pathogens such as *Salmonella* and *Campylobacter* (Pollock et al., 2012; Tobin et al., 2015). Also some avian viral diseases, such as AI and ND, are of zoonotic potential, although cases of human infection are rare (Swayne and King, 2003). However, many backyard poultry owners seem to be unaware of such risks and do not engage in appropriate hygiene practices while caring for their birds (Beam et al., 2012; Karabozhilova et al., 2012; Kauber et al., 2016). Despite the growing popularity of backyard poultry, little research has been done to establish the levels of occurrence of zoonotic pathogens in backyard flocks.

In our study, *C. jejuni* (45 %) and *L. monocytogenes* (33 %) were the most common pathogens detected in backyard poultry farms in Finland. Both these pathogens were more frequently found from the environmental boot sock samples than from individual cloacal samples and both were detected more often during winter sampling (October – January) than spring sampling (May – June). Also *Y. enterocolitica* was frequently isolated on the farms (31 %),

but all isolates were *yadA* negative, indicating that they were non-pathogenic (Tahir and Skurnik, 2001).

In Finnish commercial broiler flocks the annual *Campylobacter* prevalence is low (2 – 7 %) compared to other countries, and clear seasonal variation is apparent (EFSA, 2014; Llarena et al., 2015). A cold climate (< 6 °C), and snow cover in particular, does not promote the spread of *Campylobacter* in the environment, which could partly explain differences in the results between Finland and several other countries during the winter season (Patrick et al., 2004; Anderson et al., 2012; Jonsson, et al., 2012). A cold climate also requires that backyard chickens are housed during winter, thereby decreasing their contacts with the environment and disease reservoirs. Our finding that the occurrence of *Campylobacter* was higher in winter sampling, after an outdoor season, than in spring, after an indoor season, and a cold climate, supports this hypothesis. This could also at least partly explain the difference in occurrence between our results and results for backyard poultry from New Zealand (Anderson et al., 2012) (Table 5).

In Finnish organic laying hen farms with free access to the outdoors the *Campylobacter* prevalence in autumn was 84 % and in spring 76 % (Sulonen et al., 2007). The explanation for the lower *Campylobacter* prevalence in backyard poultry flocks is probably the smaller flock size and lower bird density. It is also known that chicken colonization of *Campylobacter* decreases as the birds get older (Genigeorgis et al., 1986).

Our PFGE results for *C. jejuni* showed a high level of genetic diversity, which confirm the findings of Anderson et al. (2012). Different *C. jejuni* genotypes were simultaneously found from the same farm and new types were detected in the successive sampling, indicating only transient colonization by *Campylobacter*. However, genotype 9, detected from one farm, was isolated both in winter and spring samples, indicating persistent colonization. This genotype was very similar in terms of restriction patterns to MLST type sequence 50, which is currently the second most common sequence type among human patients and the fourth commonest in poultry (Kärenlampi et al., 2007; de Haan et al., 2013; Kovanen et al., 2014). Additionally, several of the other *C. jejuni* PFGE genotypes detected from backyard poultry were very similar to genotypes previously identified in samples from organic laying hens and also human patients in Finland (Sulonen et al., 2007; Kärenlampi et al., unpublished results). This indicates that backyard chickens could be a potential source of *C. jejuni* infection for humans and represent a reservoir of pathogenic *C. jejuni* strains.

Children may be at particular risk of contracting enteric diseases because they are more likely to touch and handle the chickens and forget to their wash hands after the contact (Tobin et al., 2015; Kauber et al., 2016). A study from

Egypt established that children living in households that had *C. jejuni*-positive backyard poultry flock were almost four times more likely to be positive for *C. jejuni*, compared with those that did not live with infected poultry (El-Tras et al., 2015). Indeed, Centers for Disease Control and Prevention in the USA recommends that children under the age of 5 and immunocompromised people should not be in a close contact with poultry because of the increased susceptibility of such individuals to infectious diseases.

Although *L. monocytogenes* was frequently found from backyard poultry farms, it was only very rarely found from the cloacal samples of individual birds (1 %). Our finding supports those of previous studies and the conclusion that *L. monocytogenes* contamination of poultry meat occurs more often during slaughtering and further processing (Rørvik et al., 2003; Loura et al., 2005; Milillo et al., 2012; Sasaki et al., 2014). The finding that *L. monocytogenes* was significantly more frequently isolated in the winter boot sock sampling, after an outdoor season, also supports this hypothesis. Two serotypes, 1/2a and 4b, which have been responsible for most of the clinical cases worldwide, were found in our study, suggesting that they are widespread in the external environment (Lomonaco et al., 2015). Even though backyard chickens probably are not a frequent source of *L. monocytogenes*, according to this study this species is a common inhabitant of the backyard poultry house environment and thus can represent a potential risk for humans, especially the immune compromised, pregnant, neonate, and elderly (Vázquez-Boland et al., 2001).

S. enterica, *C. coli* and *Y. pseudotuberculosis* were only rarely isolated on the farms (2 % each). Thus, according to this study, they do not represent a common health risk to Finnish backyard poultry owners, although they can occasionally be shed by backyard poultry. The only *Salmonella* finding was from the owner-taken spring boot sock sampling, and it was an antimicrobial-sensitive *S. Typhimurium*, phage type U277 strain. Most probably the infection originated from wild birds, which have been shown to be an important source of phage type U277 in the Nordic countries (Kapperud et al., 1998; Refsum et al., 2002). The *Salmonella* infection was confirmed also from a subsequent sampling, and had been persistent on the farm for several years (unpublished results). Our study results confirm those of previous studies from other countries where the occurrence of *Salmonella* among backyard flocks has been low. In South Australia, 30 backyard flocks were screened for *Salmonella* spp. and the overall isolation rate was 10 % (Manning et al., 2015). In Maryland, USA, no *Salmonella* was found in cloacal swab samples or environmental drag swabs among 39 backyard flocks (Madsen et al., 2013b) (Table 5). In a retrospective study of Mete et al. (2013) *Salmonella* prevalence of necropsied backyard chickens was 2.7 %.

The *Y. pseudotuberculosis* strain in our study belonged to serotype O:1, which is the most common type found in human infections and wild animals, including birds (Le Guern et al., 2016). The same serotype has repeatedly been identified in Finnish outbreaks and it has been epidemiologically linked to fresh products (iceberg lettuce and carrots) and small wild mammals (Jalava et al., 2006). This indicates that wild animals may be an important infection source for backyard poultry.

Increasing antimicrobial resistance is a major threat to public health and the use and misuse of antibiotics has an important role in development of resistant bacteria (WHO, 2014). According to our study, backyard poultry flocks in Finland are treated with antimicrobials only rarely. Of the 51 sampled flocks, birds in only two flocks had been medicated with antibiotics during the previous year. However, wild birds and farm animals, with which backyard poultry commonly had contact, could act as reservoirs and potential spreaders of resistant bacterial isolates (Bonnedahl and Järhult, 2014). In our study, *Salmonella*, *Listeria*, *Yersinia* and *Campylobacter* isolates were tested for antimicrobial susceptibility and in general were susceptible to most of the antimicrobials tested. In addition, *E. coli* samples were tested for ESBL/AmpC-resistance. No ESBL genes were found, but from three farms *E. coli*-producing AmpC were detected. Our results suggest that the risk for transmission of resistant bacteria from backyard poultry to humans is low in Finland. Antimicrobial resistance testing among backyard poultry is very rare but recent studies show that resistance can be an issue on small-scale poultry farms, although it is usually less common than on commercial farms (Bertelloni et al., 2015; Braykov et al., 2016; Nguyen et al., 2016).

5.3 COULD BACKYARD POULTRY POSE AN INFECTION RISK TO COMMERCIAL POULTRY?

Despite the global distribution of IBV, no clinical cases were reported in Finland for almost three decades (personal communication with C. Ek-Kommonen). After April 2011, several distinct IBV outbreaks with signs of egg drop and mild respiratory infection were reported. The first outbreak occurred on a commercial layer farm producing table eggs and the virus was identified as a genotype similar to QX-IBV, a variant first discovered in China in 1996 that has now spread to most poultry producing areas (Wang et al., 1998; Worthington et al., 2008). The farm had several contacts with small backyard poultry flocks in different parts of Finland and the biosecurity practices on that farm were poor. All known contact farms, as well as surrounding farms, were traced and the birds were tested for IBV but were serologically negative. The source of the infection remains unknown and after this first outbreak QX-IBV has not been found from commercial farms (personal communication with A. Huovilainen).

Shortly after the first outbreak, another QX-IBV was found from a dead backyard poultry hen's organ suspension. It shared only 91.5 % nucleotide identity with the first outbreak strain, which means that the origin of the viruses was not the same. Subsequently, several cases from commercial breeder, layer and broiler farms were reported and vaccine-like genotypes D274 (li18437/2011) and 4/91 (li6487/13) were detected. One possible explanation for the fact that several different IBV were found during a very short period of time after a three-decade absence could be that after the first confirmed QX outbreak IBV was suspected and also tested for more readily. It is also possible that the vaccine-like viruses detected on commercial farms did not cause very clear disease signs. Because live IBV vaccines are not used in Finland, these viruses (D274 and 4/91) are probably at least partly spreading through importation of one day old breeder/layer parent stock chicks. Although the chicks are not vaccinated against IBV at the foreign hatchery, the vaccines are used and most probably easily found in the hatchery environment. In the future, closer comparison of specific vaccine strains and the field strains causing clinical disease in Finnish commercial farms, using whole-genome sequencing, would be interesting.

IBV had been circulating in backyard poultry flocks already before the first outbreak because after the QX-IBV isolation from the backyard hen, serological testing of 45 backyard flocks from different parts of Finland was performed and antibodies to IBV were detected in 73 % of them. But as we now know, backyard poultry owners contact veterinarians very rarely and most probably occasional mortalities and signs of respiratory infections went without a notification (Garber et al., 2007; Zheng et al., 2010; Karabozhilova et al., 2012; Smith et al., 2012; Yendell et al., 2012; Madsen et al., 2013a; Elkhoraibi et al., 2014). The transmission of QX-IBV has most probably happened through illegal imports of hobby chickens from other countries. Also wild birds may have a role as reservoirs and long-distance carriers of IBV (Chen et al., 2009; Hughes et al., 2009; Domanska-Blicharz et al., 2014). Though no evidence of transmission of QX-IBV from backyard poultry farms could be confirmed, it raised a question about the occurrence of IBV and other pathogens in backyard poultry flocks.

IBDV, CIAV and AEV are ubiquitous viral pathogens that cause clinical disease only in young chickens, but in addition, IBDV and CIAV can cause variable degrees of immunosuppression in older animals, rendering them susceptible to other infections. As expected, in our study, chickens on most farms were seropositive for CIAV and AEV, which is consistent with previous studies done among backyard poultry flocks (McBride et al., 1991; Wunderwald and Hoop, 2002; Hernandez-Divers et al., 2008). As the owners did not report any acute mortality among young chicks, nor was there an indication of these viruses in postmortem necropsy findings, it is probable that

the chicks were protected by maternal antibodies or the chickens encountered the viruses when older. A surprising finding was the scarce occurrence of antibodies against IBDV in the backyard chickens. In total, only 13 of the 457 sampled chickens had antibodies against IBDV and of the ten (20 %) farms associated with positive results, only one farm had several (4/10) seropositive chickens with high antibody titers. The remaining farms had only a single seropositive chicken each, and for three of those the antibody titer was low. Although the positive samples were retested and confirmed to be positive, this could indicate false positive results due to non-specific reactions in ELISA testing. This result supports our previous results from the necropsy data, where no clinical cases of IBDV were detected. These results together indicate that at the time of sampling IBDV was not common among backyard flocks.

Respiratory pathogens are a main cause of disease among commercial poultry (Jones, 2010). In our study, the seroprevalence of IBV and ILTV was low when compared with results from Belgium, Netherlands and Switzerland (Wunderwald and Hoop, 2002; de Wit et al., 2004, Haesendonck et al., 2014). However, in our study the ages of the sampled animals were not recorded and it is possible that most of the birds were young (< 4 years) which may have affected the results because ILTV has been found to be more seroprevalent among older birds (Haesendonck et al., 2014). The occurrence of coronaviruses was rare in cloacal samples. However, QX-IBV was found from 5 farms and it seems to be circulating among Finnish backyard poultry flocks and, surprisingly, no other variants were found among those flocks. Part of the reason may be that live IBV vaccinations are not performed in Finland, and no vaccine viruses are circulating in the field. When designing the study, the risk of other respiratory pathogens, aMPV and *Mycoplasma* spp., was estimated to be low and they were not included. Subsequently, this proved to be a mistake and currently *M. gallisepticum* and *M. synoviae* are known to be spreading uncontrolled among flocks (personal communication L. Rossow).

The occurrence of antibodies against NDV and AIV among backyard chickens was very low, 0 % and 0.7 %, respectively. No NDV or AIV were found from the cloacal swabs, either. However, although cloacal sampling is known to be more sensitive when detecting LPAI viruses by PCR, it would have been interesting to take oropharyngeal swabs. In surveillance studies, combined cloacal and oropharyngeal sampling would be optimal for the detection of both LPAI and HPAI viruses (Ellström et al., 2008). Waterfowl, especially ducks and geese, represent important risks for AIV transmission (Webster et al., 1992; Swayne and King, 2003; Olsen et al., 2006). In our study, it was not common to keep waterfowl in backyard poultry flocks. According to our questionnaire, ducks were housed only in 15 % and geese in 7 % of the flocks. This can lower the risk of transmission. To date, HPAI/LPAI viruses have not been detected in poultry in Finland.

According to our study, backyard poultry flocks in Finland are reservoirs of many viral diseases, some of which could cause serious problems and have economic impact if transmitted to commercial farms. However, most flocks appear to pose minimal risk for disease transmission because of the long distances between them and commercial flocks, small flock size and low seroprevalence of the important diseases. Any risk associated with backyard flocks can however be reduced by good farm management and employment of strict biosecurity measures on commercial farms.

6 CONCLUSIONS

Scant documented data exists on backyard poultry populations, their health status, reasons for mortality and zoonotic pathogens they carry. This is the first study to provide a detailed characterization of backyard poultry flocks in Finland.

The specific conclusions reached from the studies are:

1. The majority of backyard poultry farms in Finland were small (< 50 birds) and located distantly (> 3 km) from commercial poultry farms. Biosecurity practices on the farms were sub-optimal and the birds often had a possibility be in a contact with wild birds and other animals that could potentially predispose them to contagious pathogens. However, the owner-stated health of backyard poultry was good.
2. *C. jejuni* was commonly detected from the cloacal and environmental samples for backyard poultry farms and these birds could be a potential source of *C. jejuni* infection for humans, representing a reservoir of *C. jejuni* strains. *L. monocytogenes* was also a common finding on the farms. *Salmonella* spp. and *Y. pseudotuberculosis* were isolated from the backyard flocks rarely and no pathogenic *Y. enterocolitica* was found. Because of the lack of good hygienic practices after bird contact, the risk of transmission of bacterial pathogens from bird to human exists. Antimicrobial resistance of the zoonotic pathogens including AmpC/ESBL producing *E. coli* was not a problem in the backyard poultry flocks in Finland.
3. Backyard poultry flocks in Finland had antibodies against respiratory pathogens that are rare or non-existing on commercial farms. Although the seroprevalence of these pathogens among Finnish backyard poultry flocks was low compared with other countries, in certain situations they could pose a risk of contagious viral pathogens spreading to commercial poultry. However, because of the small size of the flocks and long distances between the farms, the risk is probably low.
4. QX-IBV was the only IBV type detected in backyard poultry flocks. Among commercial flocks, vaccine-like virus types D274 and 4/91 were the most commonly detected IBV types.

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