Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
Thesis 2023:48

Neonatal colisepticemia in broiler chickens: -Avian pathogenic *Escherichia coli* and risk factors for high first week mortality

Neonatal coliseptikemi i slaktekylling: -Aviærpatogene *Escherichia coli* og risikofaktorer for høy første-uke dødelighet

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Ås, April 2023
Inger Helene Kravik

Table of Contents

1 Abbreviations and definitions

APEC	Avian pathogenic *E. coli*
cc	Clonal complex
cgMLST	Core genome multi locus sequence typing
ELA	Embryo lethality assay
EPEC	Enteropathogenic *E. coli*
E. coli	*Escherichia coli*
ExPec	Extra intestinal pathogenic *E. coli*
FWM	First week mortality
MLST	Multi locus sequence typing
NC	Neonatal colisepticemia
NMEC	Neonatal meningitis causing *E. coli*
SNP	Single nucleotide polymorphism
UPEC	Uropathogenic *E. coli*
VAG	Virulence associated genes
WGS	Whole genome sequencing

Pullet:
A young hen, before reaching maturity

Cockerel:
A young cock or rooster, before reaching maturity

Parent rearing flock:
A parent breeding flock before reaching maturity.

Parent laying flock:
A parent breeding flock which has reached maturity and lays fertilized eggs which hatch to become a commercial broiler flock.

Broiler fattening flock:
A flock that is raised for meat production, and which is part of the food production chain, the commercial broiler chicken flock.

2 List of papers

Paper I

High sequence similarity between avian pathogenic E. coli *isolates from individual birds and within broiler chicken flocks during colibacillosis outbreaks*

I. H. Kravik, H. Kaspersen, S. K. Sjurseth, M. Jonsson, B. David, M. Aspholm, et al.

Vet Microbiol 2022 Vol. 267 Pages 109378,

DOI: 10.1016/j.vetmic.2022.109378

Paper II

A molecular epidemiological study on Escherichia coli in young chicks with colibacillosis identified two possible outbreaks across farms

I. H. Kravik, H. Kaspersen, S. K. Sjurseth, K. R. Dean, B. David, M. Aspholm, et al.

Vet Res 2023 Vol. 54 Issue 1 Pages 10,

DOI: 10.1186/s13567-023-01140-6

Paper III

Flock Size and High Previous First Week Mortality as risk factors for neonatal colisepticemia in broiler chicken flocks

I. H. Kravik, K. R. Dean, C. Sekse, B. David, S. K. Sjurseth, M. Aspholm, H. C. Bakka

3 Abstract

Broiler chicken meat is regarded lean and more sustainable than meat from other terrestrial production animals. Because of this, the industry is continuing to grow globally. To maintain animal welfare, production sustainability and prevent antibiotic resistance in a growing industry, it is crucial to keep disease levels at a low.

Colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC), is one of the most frequently reported bacterial diseases in broiler chicken, and it comprises several local and systemic diseases, such as: salpingitis, cellulitis, colisepticemia and neonatal colisepticemia.

Neonatal colisepticemia (NC) typically affects broiler chicken flocks within the first week post hatching, resulting in high mortality and morbidity. The mortality in a flock increases from one to five days post hatching, resulting in first week mortality (FWM) > 1.5 % and with some reports reaching 20 %.

During 2014 – 2016, an increasing number of NC cases were reported in the Nordic countries. APEC (ST117, O78:H4) was later identified as the causative agent, but several other APECs were also identified. The reports revealed a lack of knowledge on the epidemiology of APEC within the Nordic broiler chicken industry. In addition, there was little knowledge of risk factors for high FWM due to NC.

The main aim of the PhD project was to generate in-depth knowledge on the diversity of APECs causing NC in Norwegian broiler chicken flocks and their genetic traits. Further, the aim was to identify the risk factors for high FWM due to NC.

This PhD project used whole genome sequencing (WGS) and bioinformatic analysis to map and type confirmed APEC isolates that were collected from Norwegian broiler chicken flocks with high FWM in 2018 to 2021. Multi locus sequence- and serotyping identified high sequence similarity between isolates from the same bird and identified that one to five APECs may be identified within affected flocks. Close to half of the distinct STs were only identified once. Core gene phylogenetic analysis of a total of 219 APEC isolates showed that two distinct sequence types (ST) sharing the same serotype profile may be genetically distant. The analysis, together with flock related

metadata, also identified two distinct, single-strain outbreaks of APEC across farms: ST23, O78:H4 and ST429, O2/O50:H1. All 219 APEC isolates from this study were screened for virulence-associated genes (VAGs) using an updated and extended database covering *E. coli* and APEC related VAGs. The screening showed what VAGs the outbreak-isolates carry and compared these to the other APEC isolates included in the analysis. Finally, a matched case-control study, including the same flocks as the molecular study, identified *high FWM in the previous flock* (OR = 3.33) and *flock size* (OR = 1.7) as risk factors for high FWM due to NC.

Altogether, this PhD study presents novel information on the molecular epidemiology of APEC in the Norwegian broiler chicken industry. The analyses illustrated the need to sample multiple birds to identify the disease-causing APEC within a flock and the importance of combining two methods (e.g. MLST and serotyping) for the precise typing of APEC. This work identified two highly virulent strains of APEC, which were alone capable of causing disease in more than one flock within a few months. The high resolution, core genome phylogenetic analysis of the outbreak isolates was shown to be useful for APEC outbreak investigations and indicated that the SNP-distances might be somewhat different from what is reported for outbreaks of zoonotic agents. The screening for VAGs also confirmed the carriage of *hlyF* and *ompT*, two genes known to be important for virulence, in APEC. Furthermore, the risk factor study identified the need for more research on the survival of APEC in the poultry house between flocks. To avoid rapid spread of disease within the flock, producers of large flocks should increase the management capacity to remove and cull dead and morbid birds when an outbreak of NC is suspected.

4 Norsk sammendrag

Kyllingkjøtt er et magert og bærekraftig produkt sammenliknet med annet kjøtt fra landbruket. Dette fører til økt etterspørsel og produksjon. For å vedlikeholde dyrevelferden, den bærekraftige produksjonen og holde antibiotika forbruket på et lavt nivå i en voksende industri, er det viktig å holde sykdommer på et lavt nivå.

Colibacillose er en fellesbetegnelse på lokale eller systemiske infeksjoner, forårsaket av Aviær patogene *E. coli* (APEC), og inkluderer, blant annet: eggleder betennelse, navle- og plommesekk betennelse, cellullitt, hjarre's sykdom, colispetikemi og neonatal coliseptikemi. Det er ansett som en av de vanligste bakterielle sykdommene hos fjørfe på verdensbasis.

Neonatal coliseptikemi (NC) forårsaker høy førsteuke-dødelighet blant slaktekylling. I en flokk med NC øker dødeligheten typisk fra dag én til fem etter klekking: førsteuke-dødeligheten i flokken overstiger ofte 1,5 %, og internasjonalt har det vært rapportert flokker med førsteuke-dødelighet opp mot 20%.

Mellom 2014 og 2016 ble det observert en økning av NC blant slaktekylling i de nordiske landene. Analyser av prøver viste at sekvenstype (ST) 117, serotype O78:H4 var hovedårsaken til de overnevnte problemene, men analysene identifiserte også flere ulike typer APEC. Det ble klart at det manglet kunnskap om hvilke APEC som sirkulerer i den nordiske slaktekylling populasjonen. Det manglet også kunnskap om hvilke risikofaktorer i kyllingens miljø som kunne potensielt bidra til den økte dødeligheten i flokken.

Ved hjelp av moderne sekvenserings teknologi og bioinformatiske analyser var målet med dette PhD-prosjektet å bidra med økt kunnskap om APEC som forårsaker NC blant norsk slaktekylling. Videre var det et ønske om å tilegne seg kunnskap om hvilke risikofaktorer som kunne bidra til den høye førsteuke-dødeligheten i flokker med NC.

I prosjektet har vi helgenomsekvensert 219 APEC isolater fra til sammen 49 slaktekylling og oppalsflokker med NC og høy førsteuke-dødelighet. Isolatene har blitt typet ved hjelp av multi locus sequence typing (MLST) og serotyping. Analysene viste høy ST-likhet innad i en fugl, mens innad i en flokk ble det identifisert opp til fem

ulike STer. Halvparten av STene presentert i studiet ble kun identifisert i ett enkelt isolat. Fylogenetiske analyser av alle isolatene viste hvordan to ulike STer kan ha samme serotype-profil, men samtidig være svært genetisk ulike. Sammen med metadata fra flokkene, ble det videre identifisert to større utbrudd forårsaket av to ulike APEC-varianter: ST23, O78:H4 og ST429, O2/O50:H1. Fylogenetiske kjernegenom analyser av utbrudds-isolatene viste SNP (single nucleotide polymorphism)-distansene mellom isolater prøvetatt innad i en flokk og mellom flokker diagnosert med NC. Ved hjelp av en utvidet gen-database ble alle de 219 APEC isolatene undersøkt for virulens-assosierte gener (VAGer). Resultatene sammenliknet forekomsten av de ulike VAGene i utbruddsisolatene, opp mot de øvrige isolatene inkludert i analysen. Tilslutt, ved hjelp av en «matched case-control» studie, ble det indentifisert to risikofaktorer for høy første-uke dødelighet grunnet NC: *høy førsteuke-dødelighet i forrige flokk* (OR = 3,33) og *flokkstørrelse* (OR = 1,7).

Resultatene fra dette doktorgradsstudiet har gitt viktig og utdypende informasjon om epidemiologien av APEC innad i norsk slaktekylling produksjon. Det ble vist at det er nødvendig å ta prøver fra mer enn en kylling i en flokk for å identifisere hvilke(n) type(r) APEC som var årsaken til NC i flokken. Analysene viste også viktigheten av at to ulike typings-metoder blir brukt (f.eks MLST og serotyping) for å presist kunne type APEC. Det ble videre identifisert to høy-virulente typer APEC som enkeltvis forårsaket utbrudd av NC på tvers av gårder i Norge. De fylogenetiske analysene ga et viktig innblikk i hva vi kan forvente av likheter mellom APEC isolater fra slike utbrudd. Analysene som undersøkte for VAGer viste hvilke VAGer vi kan forvente blant APEC, og i særs hvilke VAGer de høy-virulente utbrudds-isolatene bar. Analysene bekreftet at tilnærmet alle APEC isolatene var bærere av *hlyF* og *ompT* genene, også kjent som viktige for virulens. Risikofaktor-studiet viste at det er behov for videre undersøkelser rundt hvordan APEC overlever i fjørfehuset mellom flokker. Resultatene indikerte også at produsenter med store flokker bør vurdere å be om hjelp til å fjerne døde og morbide fugler ved mistanke om NC, for å unngå rask spredning av sykdommen i flokken.

5 Synopsis

5.1 Introduction

5.1.1 Broiler chicken production

The poultry meat industry is growing globally. Chicken meat production is thought to be one of the most sustainable of the terrestrial animal meat productions: broiler chickens have high feed conversion rates, low water consumption and low carbon footprint. Further, the high demand for lean meat, considered healthier than other terrestrial animal protein sources, might explain why the chicken meat production continues to rise.

Worldwide, the Americas accounts for the largest production in broiler chicken meat, followed by China. The European chicken meat production is less than half that of China. Within Europe, Poland is one of the largest producers of chicken meat, followed by France, Spain and Germany (Figure 1, Table 1). Broiler chicken production continues to increase, and the global poultry meat production has doubled from 2000 to 2020. In Norway alone, the consumption of poultry meat has increased from 1 kg/inhabitant in 1960 to 3.2 kg in 1982 and 19.8 kg in 2014 (Figure 2) (Bagley, 2016; Shahbandeh, 2023).

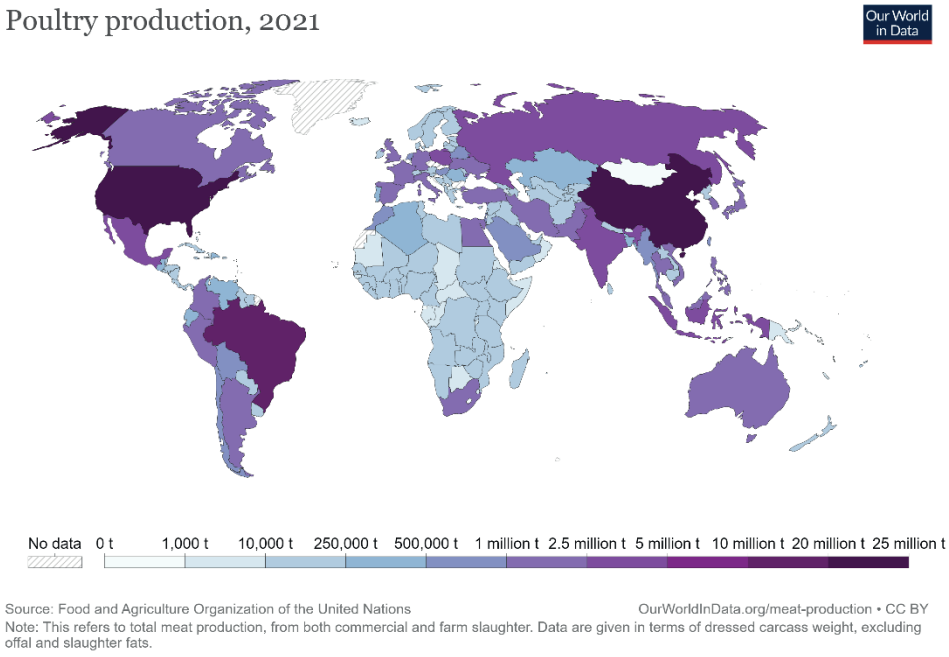

Figure 1: Distribution of poultry meat production in the world in 2021. Based on data from the Food and Agriculture Organization (FAO) database (Ritchie et al. 2017).

Table 1: Slaughter weight in million tonnes of poultry meat in 2021. Included are data from each continent in the world, from the five largest producing countries in Europe and from the Nordic countries. The data are in descending order within each category. Based on data from the Food and Agriculture Organization (FAO) database (Ritchie et al. 2017).

Geographical region	**Production of poultry meat in million tonnes**
Continents	
Americas	53.40
Asia	53.15
Europe	20.96
Australia	1.33
Africa	7.65
Selected European countries	
Poland	2.52
France	1.62
Spain	1.56
Germany	1.55
Italy	1.37
Nordic countries	
Sweden	0.18
Denmark	0.16
Finland	0.15
Norway	0.12
Iceland	0.01

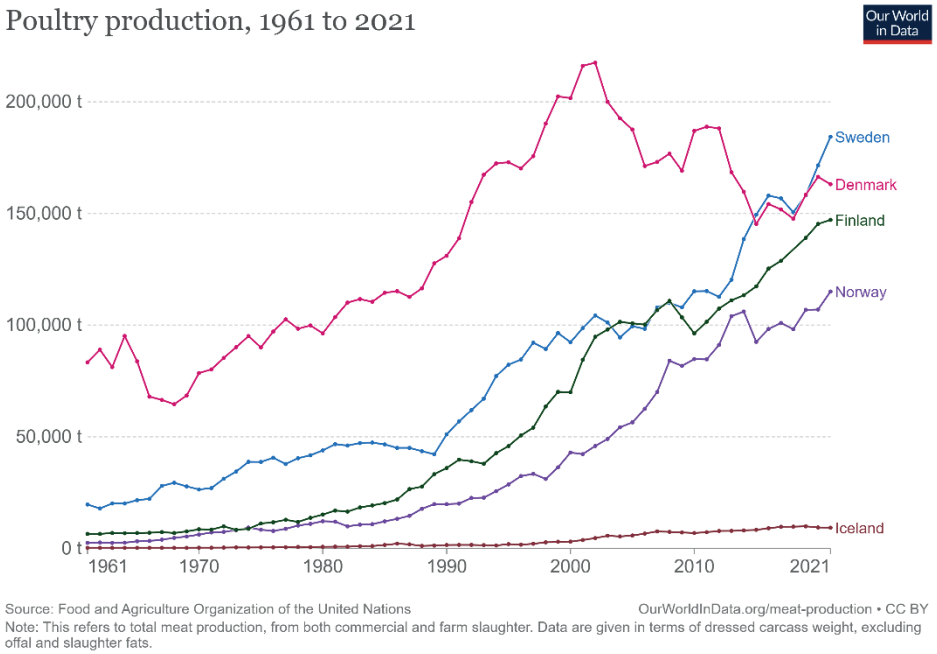

Figure 2: Poultry meat production in tonnes in Scandinavia from 1961 to 2022. Based on data from the Food and Agriculture Organization (FAO) database (Ritchie et al. 2017).

Production systems worldwide vary greatly depending on climate and economy. Several systems exist for keeping broiler chicken, such as open housing, semi open housing and closed housing. Furthermore, the chickens may be kept in floor housing on litter, in multiple level cage systems, mesh floor systems or mixed systems. In colder climates, such as in the Nordic countries, closed housing systems are the most common, whereas in warmer climates open or semi-open housing may be employed. In Norway, most broiler chicken producers have closed floor-housing systems, although some organic chicken farms include outdoor access (Bagley, 2016; Gussem, 2018; Falk et al., 2021).

This thesis will present results from studies conducted under Norwegian production conditions and parallels from these studies may be drawn to countries with similar production systems.

5.1.2 Broiler chicken production in Norway

Key numbers

As of 2021, there were 531 broiler chicken fattening farms (size above 1000 chicken) and 92 farms keeping broiler parents (rearing and laying flocks) registered in Norway. The most common hybrid in Norway is Ross 308 and the second most common is Hubbard JA 787. Other types of slow-growing hybrids are used to a lesser extent, but they are increasing in number. The Ross 308 broiler chickens are slaughtered between 29-35 days and reach an average dressed weight between 1-1.5 kg. Hubbard JA 787s live slightly longer, 45-48 days, with an average dressed weight of 1700 grams (Aviagen, 2023, Norsk Kylling, 2018, Kjos et al., 2019).

In 2020, approximately 67 million broiler chickens were slaughtered in Norway and the number increased to 72 million in 2021. Chicken meat accounts for approximately 30 % of the total meat production in Norway and Norway is self-sufficient with retail poultry meat, having produced 105 943 tonnes of chicken meat in 2021 (Falk et al., 2021; Kjos et al., 2022).

Breeding structure

The breeding of broiler chickens follows a strict pyramidal structure. Norway has not bred its own poultry for several decades, therefore, broiler chicken production is dependent on imports from other countries. The parent generation is imported as eggs from Sweden, Great Britain and France. The imported eggs are hatched in parent-hatcheries before being distributed to parent rearing farms.

At the rearing farm the pullets are sexed and kept separate, but in the same house. At 17 weeks of age, the cockerels are transported to the parent laying farms, closely followed by the pullets at 18 weeks of age. The cocks and hens are kept mixed and at approximately 20 weeks the hens start to lay eggs. The fertilized eggs are collected and transported to broiler chicken hatcheries on a weekly basis, where the chicks are hatched. Day-old broiler chicks are then distributed to fattening farms (Falk et al., 2021; Kjos et al., 2021) (Figure 3).

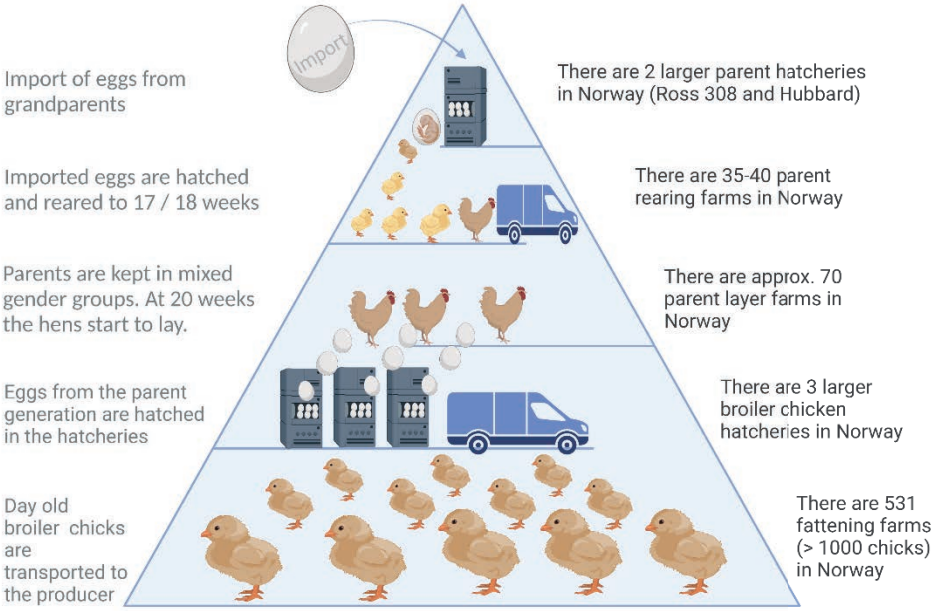

Figure 3: The broiler chicken breeding pyramid in Norway, including the number of broiler chicken farms and hatcheries in each level of the pyramid as of 2022. (Created with BioRender.com)

Welfare program for broiler chicken

All farms that produce broiler chicken meat with an animal density >25kg/m^2 are obliged to follow the animal welfare program for broiler chickens stated in the Norwegian regulations for the keeping of chickens and turkey (Animal welfare regulation, 2017). With fulfilment of the welfare parameters, the maximal density allowed for a broiler chicken flock in Norway is 36 kg/m^2. In comparison, EU legislation allows a maximum density of up to 42 kg/m^2 provided that certain regulations are met (Council directive, 2007). Footpad lesions are used as an indicator of welfare in broiler chicken production, and are actively used to regulate animal density on the farm. If a high number of footpad lesions are registered within a flock at slaughter, the producer is obliged to decrease animal density in the upcoming flock. Further, the welfare program requires daily registrations of welfare and production data (number of housed chicks, daily mortality, cause of mortality, air quality etc.). Data from catching and transport, as well as from the slaughtering process are also registered (dead on arrival (DOA), wing fractures, condemnations

etc.) The animal welfare program also requires a minimum of two veterinary visits per year and one external audit every three years (Animal welfare regulation, 2017).

Health status 2021

The Norwegian broiler production has a rather unique position in the world with very few WOAH (World organization for animal health, previously OIE)-listed diseases reported in 2021: One parent layer flock was seropositive for avian paramyxovirus-1, the causative agent of Newcastle disease (ND). Moreover, the Salmonella surveillance program in 2021 found no Salmonella amongst commercial broiler chicken flocks in Norway. The most frequently reported health challenges in 2021 amongst commercial broiler chicken flocks included gizzard erosion and ulcerations, coccidiosis with necrotic enteritis and colibacillosis (Falk et al., 2021).

5.1.3 Colibacillosis

Avian pathogenic *Escherichia coli* (APEC) are responsible for a wide range of local (omphalitis/yolk sac infection, cellulitis, swollen head syndrome, vaginitis, salpingitis, orchitis) and systemic diseases (colisepticemia, septicemia of respiratory origin, neonatal colisepticemia and coligranuloma (Hjarre's disease)) in birds. The various forms of disease caused by APEC are commonly referred to as colibacillosis (Nolan et al., 2020). Colibacillosis is therefore considered either a local or systemic disease caused by APEC, where the various manifestations depend on, amongst others, age and production form.

Production-related stress, immunosuppressive viral disease or poor management conditions are thought to precede the development of colibacillosis. Stressors from various origins compromise the immune barrier, facilitating the extra-intestinal entry of opportunistic *E. coli*. Some examples include the increase of salpingitis at the onset of egg production in layer hens, or cellulitis occurring secondary to abrasions of the skin. Factors that might compromise the integrity of the skin include: straw bedding, fast-growing hybrids and mixed-gender groups (wounds inflicted by cocks). Swollen head syndrome has been associated with compromised respiratory epithelial cells of the upper respiratory tract due to avian metapneumovirus, infectious bronchitis virus or high levels of ammonia (Nolan et al., 2020).

Colisepticemia develops frequently in broiler chicken flocks already within the first few days post-hatching. Many affected birds show retention of an inflamed yolk sac and an open umbilicus. This suggests that the navel could be the portal of entry.

Vertical dissemination of *E. coli* has been identified throughout the breeding pyramid. However, day-old chicks in the modern broiler production, have a very low microbial load, as commensal gut bacteria are only partially acquired within the embryo. Transmission of APEC giving rise to colisepticemia in young chicks, is thought to be vertical as well as horizontal at the hatchery (Nolan et al., 2020; Ding et al., 2017; Poulsen et al., 2017; Oikarainen et al., 2019; Mehat et al., 2021; Christensen et al., 2021; Kaspersen et al., 2020; Giovanardi et al., 2005). Horizontal spread of APEC is primarily thought to occur via the respiratory tract, from fecally contaminated dust-particles. In the newly hatched chicks, however, the fecal dust contamination is low, and an increased pathogenic pressure of certain strains of *E. coli*, potentially from vertical transmission, together with individual or external stressors, may lead to the development of local outbreaks of colisepticemia. However, the modes of transmission and spread of APEC, resulting in high mortality due to colisepticemia, remain vague (Mehat et al., 2021; Kathayat et al., 2021).

This thesis will focus on colisepticemia in newly hatched chicks which results in high first week mortality (FWM) within the broiler chicken flock and will from this point on be referred to as neonatal colisepticemia (NC).

Neonatal colisepticemia (NC)

Symptoms

Symptoms of NC include apathy, anorexia, dyspnea and death. Within an affected flock, daily mortality typically increases from one-five days post-hatching, after this, mortality generally decreases (Nolan et al., 2020).

Diagnosis

Diagnosis of NC is based on a combination of clinical signs in the flock, macro-pathological findings during necropsy and bacteriological examination.

Post mortem examination of NC includes lesions associated with a per-acute to acute fibrinous polyserositis. Typical macroscopic lesions include enlarged spleen, edematous serous membranes, with or without fibrin (Figure 4a). Commonly the umbilicus is affected with hemorrhage and edema (omphalitis), and/or with retention of an inflamed yolk sac (Nolan et al., 2020; Christensen et al., 2021; Kromann et al., 2022a).

Bacteriological examination shows growth of *E. coli* from associated lesions or well perfused-organs such as the liver, spleen or bone marrow. On blood agar, *E. coli* colonies are typically medium large, greyish-white and smooth. The colonies may or may not have a mucoid consistency with or without a weak, underlying, greenish discoloration (Figure 4b). On rare occasions, *E. coli* may show β-hemolytic properties. Care should be taken in the diagnosis that the dominant bacteria is *E. coli*, and that there is no evidence of contamination during sampling. A mixed culture of bacteria including *E. coli* does not qualify for a colibacillosis diagnosis (Nolan et al., 2020; Kromann et al., 2022a; Scheutz & Strockbine, 2001).

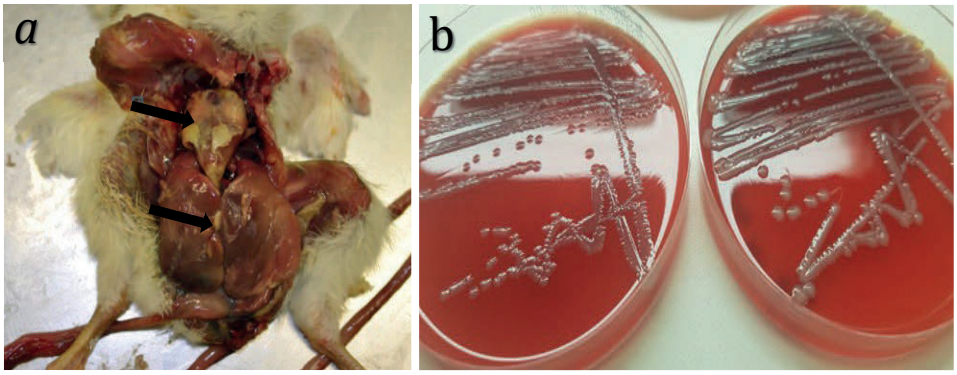

Figure 4: a) Four-day old chick during necropsy. Fibrin is seen over the pericardium and sparsely on the liver. b) Growth of Escherichia coli on blood agar. (Photo by: Øyvor Kolbjørnsen[a] and Inger H. Kravik[b])

Treatment and prevention

The use of antimicrobials is not recommended for the treatment of NC due to the acute nature, short longevity of the disease and the large impact on human health with regards to the global rise of antibiotic resistance. Even though antibiotics still may be used in the treatment of NC worldwide, no broiler chicken flocks in Norway were treated with antibiotics in 2021 (Kjos et al., 2022; Christensen et al., 2021; NORM NORM-VET, 2021).

At present, no commercially available alternatives to cure NC exists. Testing of various alternatives for treatment and prevention is ongoing. These alternatives include immune-modulatory strategies such as: probiotics, bacteriophages, essential oils or novel small molecules. Recent studies have also looked at hybrids with better

genetic resistance to APEC (Kathayat et al., 2021; Swelum et al., 2021; Kathayat et al., 2018; Kazibwe et al., 2020; Huff et al., 2005; Monson et al., 2021).

Vaccination for the prevention of colisepticemia is practiced to a varying degree in Norway, but more commonly in other European countries (Kromann et al., 2021; Lozica et al., 2021a). If vaccination is practiced, the parent-stock is most commonly vaccinated. Within the grandparent-stock vaccination is practiced (Nortura SA, personal communication, March 2023; Norsk Kylling, personal communication, September 2022).

There are several types of vaccines against APEC: live attenuated, inactivated, recombinant or subunit vaccines. Three examples are: (1) Nobilis *E. coli* (MSD Animal Health), an inactivated subunit vaccine which contains the fimbrial antigen F11 as well as a flagellar toxin, (2) Poulvac *E. coli* (Zoetis) is a live attenuated vaccine of serogroup O78 with a deleted *aroA* gene and (3) Nisseigen Avian Colibacillosis Vaccine CBL (Nisseiken Co., Ltd.) which consist of an *E. coli* strain of serogroup O78 with a deleted *crp* gene (Swelum et al., 2021).

To date, live attenuated vaccines have provided the best protection against homologous strains, but to a lesser extent cross protection. The commercial live vaccines most commonly include the serogroups O1, O2 and/or O78 (Nolan et al., 2020; Koutsianos et al., 2020).

Autogenous vaccines that target specific, relevant isolates of APEC are developed to target the APEC identified on a farm, or within a region, and can be produced upon demand. These autogenous vaccines have shown to have a protective effect, eliminating bacteria amongst the vaccinated group and reducing macro-pathological lesions (Kromann et al., 2021). However, the positive effect of vaccination is thought to be short-lived. Vaccination of certain strains may lead to a shift in the strain of APEC causing problems on the farm (Christensen et al., 2021; Lozica et al., 2021b; Landmann et al., 2017).

Current knowledge maintains that the best prevention against outbreaks of colisepticemia is good management and stringent biosecurity measures. High biosecurity includes closed production systems with “all in all out” policies and strict routines for disinfection between flocks. This also involves the use of disinfection sluices and protective clothing (Swelum et al., 2021; Refsum et al., 2014). Cleaning of

water lines have also proven important for the prevention of colisepticemia outbreaks (Vandekerchove et al., 2004; Awawdeh, 2004). In an outbreak situation, the quick removal of moribund or dead birds to prevent the horizontal spread of the disease has also been recommended (Christensen et al., 2021).

5.1.4 *Escherichia coli* and avian pathogenic *E. coli* (APEC)

Escherichia coli

Escherichia coli, commonly known as *E. coli,* is a bacterium which belongs to the taxonomical order *Enterobacteriales* and family *Enterobacteriaceae* (Adeolu, 2016). *Enterobacteriaceae* is a diverse family including several well-known genera, amongst others, *Proteus*, *Salmonella*, *Citrobacter*, *Yersinia*, *Shigella*, *Enterobacter*, *Klebsiella*, as well as *Escherichia.* Bacteria belonging to this family are facultative anaerobic, gram-negative rods. All genera are catalase positive and oxidase negative. *Enterobacter*, *Klebsiella* and *Escherichia* also have the ability to ferment sugars to acid and reduce nitrate to nitrite (Adeolu, 2016; Timoney et al., 1988).

Within the genus *Escherichia* there are five species: *E. hermannii*, *E. fergusonii*, *E. vulneris*, *E. blattae* and *E. coli* (Scheutz & Strockbine, 2001).

Escherichia coli is a promiscuous bacterium with the ability to rearrange chromosomal segments and incorporate horizontally transferred genes.
The pangenome of *E. coli* is considered open, indicating the potential to evolve by gene acquisition. This ability has led to the development of numerous different *E. coli* types, all likely derived from the Salmonella lineage some 100 million years ago. Most *E. coli* are gut-commensals of warm-blooded animals and in healthy poultry, approximately 10^6 colony forming units of *E. coli* are found in one gram of feces (Nolan et al., 2020; Mehat et al., 2021; Scheutz & Strockbine, 2001; Chaudhuri et al., 2012; David et al., 2008).

The divergence of the *E. coli* genome has led to the development of pathogenic strains of *E. coli*. Through deletion, recombination or acquisition of DNA fragments as a response to selective pressure, specific virulence attributes have been acquired to adapt to specific niches. Some virulence attributes may be encoded on mobile genetic elements, while others have evolved and been integrated into the genome. The most successful combinations of virulence factors have persisted, producing pathotypes of *E. coli* that are capable of invading and causing disease in healthy individuals. These

pathotypes, with their subdivisions, are often referred to as enteropathogenic *E. coli* (EPEC), which cause disease within the intestinal tract, or extra-intestinal pathogenic *E. coli* (ExPEC), which may cause disease in extra-intestinal sites (Kaper et al., 2004).

In humans, the ExPECs include neonatal meningitis causing *E. coli* (NMEC) and uropathogenic *E. coli* (UPEC). The *E. coli* pathotypes associated with disease in poultry appear genetically related to the human ExPECs and are referred to as avian pathogenic *E. coli* (APEC) (Nolan et al., 2020; Mehat et al., 2021; Poolman & Wacker, 2016; Ewing, 1986; Ewers et al., 2007; Stromberg et al., 2017; Jørgensen et al., 2019).

On the surface of *E. coli,* several antigens are expressed, commonly referred to as O (somatic, n=173), K (capsular, n= 60), H (flagellar, n=56) and F (fimbrial) antigens. These antigens enable *E. coli* to be motile, adhere to the mucosal surface and in some instances invade the host. Pathogenic *E. coli* cause disease by colonization of a mucosal site, evasion of host defenses, multiplication and host damage. Specific adherence factors such as fimbriae allow pathogenic *E. coli* to colonize sites that they normally do not inhabit (Scheutz & Strockbine, 2001; Kaper et al., 2004).

Typing *E. coli*

Eschericia coli, including APEC, have traditionally been typed with the use of laboratory serological tests. The serological tests used for *E. coli* have focused on the identification of the various surface antigens O, K and H (see above), which together comprise a serotype. It is most common to type the O-group and occasionally the H-antigens, the F-antigen is rarely typed. Today serological typing may also be performed *in silico* based on data from WGS (Joensen et al., 2015; Uelze et al., 2020).

The serogroups O1, O2, O18, O35, O36, O78 and O111 are frequently reported in association with the various forms of colibacillosis in birds, while O1, O2 and O78 are reported with the highest prevalence in association with NC (Nolan et al., 2020; Mehat et al., 2021; Huja et al., 2015; Johnson et al., 2022).

With the development of laboratory techniques, methods for the characterization of *E. coli* have also developed (Figure 5).

Assigning *E. coli* to phylo-groups was introduced by Clermont and coworkers at the beginning of the millennium. With a triplex polymerase chain reaction (PCR) method, the presence or absence of three genes (*chuA*, *yjaA* and *TspE4.C2*) grouped *E.coli* into

four phylo-groups A, B1, B2 and D (Clermont et al., 2000). Later, the method was revised to include a fourth gene sequence: *arpA*, resulting in a quadruplex PCR. The latter method now groups *E. coli* into eight phylo-groups: A, B1, B2, C, D, E, F and G (Mehat et al., 2021; Clermont et al., 2013; Clermont et al., 2019).

Avian pathogenic *E. coli* have previously been associated with phylo-group B2 and F, however several reports group *E. coli* from poultry samples to other phylo-groups as well (Nolan et al., 2020; Mehat et al., 2021; Newman et al., 2018; Delannoy et al., 2020; Mageiros et al., 2021; Denamur et al., 2021). Recently the phylo-group G was introduced as a poultry-associated phylo-group (Clermont et al., 2019).

Another method for the molecular typing of *E. coli* is multi locus sequence typing (MLST), which groups *E. coli* according to seven housekeeping genes: *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *recA*, and *purA*. Based on the allele-variants of these seven genes, each unique gene profile is assigned a sequence type (ST) number. With whole genome sequencing (WGS) becoming readily available, presenting the ST of *E. coli* strains has become the standard way to present the *E. coli* in question (Denamur et al., 2021; Wirth et al., 2006; Shaik et al., 2022; Maiden et al., 1998).

The most common STs identified as high virulent APEC are to date: ST23, ST117 and ST428/429. Other STs identified with high prevalence from colibacillosis lesions include ST10, ST69, ST95, ST131, ST140 and ST354 (Mehat et al., 2021; Kromann et al., 2022b; Papouskova et al., 2020; Apostolakos et al., 2021).

Numerous STs for *E. coli* are reported, but the ST-numbers do not illustrate if the STs are closely related or not. To understand the genetic relatedness between the numerous STs reported, clonal complexes (cc) of highly prevalent STs may be presented. The STs belonging to the same cc often differ by only one of the seven gene-alleles used for ST-identification. The ST is therefore a single locus variant (SLV) of other STs within the same cc. (Denamur et al., 2021).

Some cc recognized in APEC include: ccST10 (ST10, ST44, ST48, ST178), ccST23 (ST23, ST88, ST90), ccST350 (ST350, ST371) (Apostolakos et al., 2021).

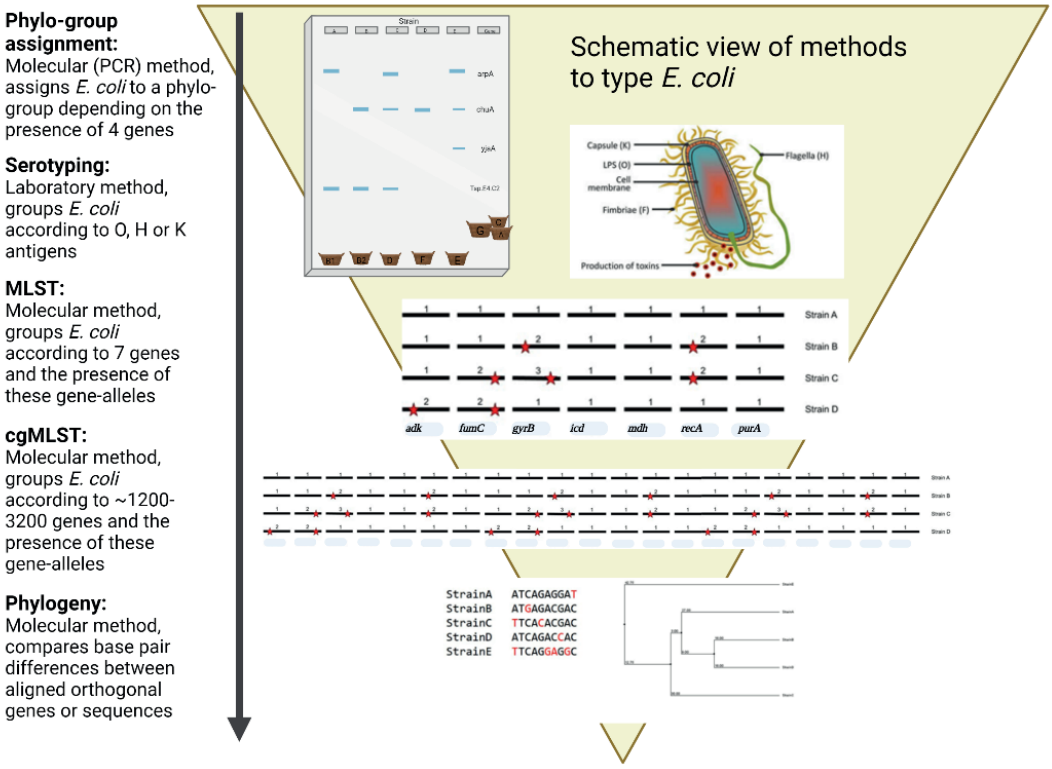

Figure 5: Methods used to type E. coli *in ascending order according to resolution: The methods with the highest resolution are depicted at the bottom, narrow end of the triangle. (Created with BioRender.com)*

Whole genome sequencing as a tool for typing *E. coli*

Whole genome sequencing has become a readily available tool for investigating molecular characteristics. High throughput sequencing is still considered an expensive technique compared to other molecular techniques such as PCR or pulsed-field gel electrophoresis (PFGE). However, WGS and bioinformatic analyses enable a myriad of analyses to be performed on the same sample. From one sample one may gain insight into information, such as: genes associated with antimicrobial resistance (AMR), genes associated with fitness, survival and virulence and detection of specific gene markers for typing, e.g.: serotyping, phylo-grouping, MLST or core gene MLST (cgMLST). By combining more than one typing method, for example MLST combined with serotyping, a more accurate typing of the *E. coli* strain may be performed (Bogaerts et al., 2021; Egli et al., 2019). Furthermore, WGS has a higher resolution compared to other molecular typing methods:

Core gene MLST (cgMLST) is a high-resolution typing method which is based on the same principle as MLST (gene by gene approach), but screens for a considerably larger number of genes, e.g. over 2000 genes in the cgMLST scheme for *E. coli* (Zhou et al., 2020). The method compares allele variants in the draft genome of a strain against the reference scheme alleles for each locus. The predetermined loci used in the comparison are typically present in 95-99% of the analyzed strains (Silva et al., 2018).

Phylogenetic analyses are other high-resolution methods enabling us to compare the relatedness between different *E. coli* on a base pair level. Phylogenetic analyses identify base pair differences in an alignment of orthologous genes (core gene), or sets of orthologous sequences (core genome) between all the aligned genomes included in the analysis. The differences in base pairs are referred to as single nucleotide polymorphisms (SNPs). The size of the orthologous sequences in relation to the total size of each genome included in the analysis is referred to as the genome coverage. The number of SNPs identified, together with the genome coverage, enables us to evaluate the relatedness of bacterial isolates. The core gene analysis enables comparison of genetically distant isolates while the core genome analysis should only be used when comparing highly similar isolates (Schürch et al., 2018; Kaspersen & Zeyl, 2022). Highly related isolates may be considered the same clone, depending on the date and geographical distribution. Isolates are considered part of an outbreak if epidemiological data support this (Egli et al., 2019; Schürch et al., 2018; Nouws et al., 2020).

Whole genome sequencing is important, not only for typing *E .coli*, but in outbreak investigations, including tracing APEC in outbreaks of NC (Christensen et al., 2021).

Avian pathogenic *Escherichia coli*- defining APEC

In a broader sense, an APEC is referred to as an *E. coli* causing disease in an extra-intestinal location in a bird. The definition may be sufficient for the farmer or veterinarian for diagnostic purposes, but it is too broad a definition to allow a further unravelling and understanding of the pathogenesis and epidemiology behind colibacillosis and APEC in a research setting or in outbreak investigations (Mehat et al., 2021; Collingwood et al., 2014).

Historically, to assess the virulence and differentiate an APEC from a non-APEC, avian *E. coli* were inoculated into chickens in a chicken lethality test (Cloud et al., 1985). Later this method was replaced by inoculation into eggs: embryo lethality assay (ELA) (Nolan et al., 1992; Wooley et al., 2000; Gibbs & Wooley, 2003; Barnes et al., 2008).

The past decades have produced numerous studies in the genomic characteristics of *E. coli*, moving the field forward to define APEC with the use of molecular techniques. Amongst others, several studies have attempted to differentiate APEC from non-pathogenic *E. coli* with the use of PCR screening methods for the presence of virulence-associated genes (VAGs) (David et al., 2008; Rodriguez-Siek et al., 2005; Kemmet et al., 2013). Many different VAGs have been identified in APEC, but the importance of all these genes have not necessarily been verified (Table 2).

Table 2: Virulence associated genes (VAGs) associated with E. coli *and belonging to the group of ExPEC (Apostolakos et al., 2021; Barbieri et al., 2013; Ewers et al., 2005).*

Category	VAG	gene
Adhesins	csg cluster, curly fibers	*csgA-F*
	e.coli common pilus	*ecpA*
	e.coli common pilus	*ecpR*
	fimbrial adhesin	*fimC*
	fimbrial adhesin	*fimH*
	heat resistant agglutinin	*hra*
	long polar fimbriae	*lpfA*
	p-fimbriae, major structural subunit	*papA_F11*
	p-fimbriae, pilus assembly	*papC*
	p-fimbriae, encodes pilus tip adhesin	*papG*
	Temperature-sensitive hemagglutinine gene	*tsh*
	Afambrial adhesin AFA -I, Dr family of adhesins	*afaD*
	Iron-regulated-gene homologue adhesin	*iha*
	E. coli attaching and effacing gene that encodes intimin	*eae*
	fimbrial protein	*yfcV*
Invasins	Promotes invasion of brain microvascular endothelial cells	*ibeA*
	Promotes invasion of brain microvascular endothelial cells	*ibeB*
	Promotes invasion of brain microvascular endothelial cells	*ibeC*
	Type V secretion system, protease involved in intestinal colonisation	*pic*
Iron acquisition	hemin receptor	*chuA*
	heme uptake	*chuS-W*
	heme uptake	*chuX*
	heme uptake	*chuY*
	Encodes ferric yersinibactin uptake receptor, siderophore receptor	*fyuA*
	Encodes iron repressible gene associated with yersiniabactin synthesis	*irp2*
	Iron regulated, siderophore receptor, outer membrane protein	*ireA*
	Aerobactin operon, involved in iron uptake and transport	*iucA*
	Aerobactin operon, involved in iron uptake and transport	*iucB*
	Involved in aerobactin synthesis	*iucC*
	Aerobactin operon, involved in iron uptake and transport	*iucD*
	Ferric aerobactin outer membrane receptor gene	*iutA*
	Putative iron transport operon	*sitA*
	iron transport protein	*sitB*
	iron transport protein	*sitC*
	iron transport protein	*sitD*
	Outer mebrane protein for serum resistance	*traT*
	outer membrane protein for increased serum survival	*iss*
Protectins	Polysialic acid capsule biosynthesis protein	*neuC*
	Encodes a protease able to cleave colicin	*ompT*
	kps cluster, involved in encoding capsular (K) antigens	*kpsE*
	kps cluster, involved in encoding capsular (K) antigens	*kpsMII_K1*
	kps cluster, involved in encoding capsular (K) antigens	*kpsM_K15*

	kps cluster, involved in encoding capsular (K) antigens	*kpsMII*
	kps cluster, involved in encoding capsular (K) antigens	*kpsMII_K4*
	kps cluster, involved in encoding capsular (K) antigens	*kpsMII_K5*
	kps cluster, involved in encoding capsular (K) antigens	*kpsMIII_K10*
	kps cluster, involved in encoding capsular (K) antigens	*kpsMIII_K96*
[6, 48]Toxins	Avian *E. coli* hemolysin	*hlyF*
	Hemolysin	*hlyE*
	Uropathogenic-specific protein (bacteriocin)	*usp*
	Vacoulating autotransporter toxin	*vat*
	cytolethal distending toxin, blocks mitosis	*cdtA*
	cytolethal distending toxin, blocks mitosis	*cdtC*
	heat stabile enterotoxin	*astA*
Miscellaneous	ets operon, Encodes ABC transporter, efflux pump protein	*etsC*
	Structural gene of the ColV operon	*cvaC*
	Structural gene for ColM activity	*cma*
	ter gene cluster, tellurite resistance gene*	*terC*
	microcin	*mchF*
	microcin	*mchB*
	microcin	*mchC*
	dispersin transporter protein	*upaG*
	Enteroaggregative immunoglobulin repeat protein	*air*
	LEE-encoded TTSS effectors	*espF*
	cell-cycle-inhibitor factor	*cif*
	colicin	*cba*
	translocated intimin receptor	*tir*
	glutamate decarboxylase gene**	*gad*
	carcinoembryonic antigen, family of cell adhesion	*cea*
	endonuclease colicin E2	*celb*
	colicin	*cia*
	Hybrid non-ribosomal peptide, polypeptide megasynthase	*clbB*
	hexosyl transferase homologue	*capU*
	bacteriocin microcin B17	*mcbA*
	microcin M, part of colicin H	*mcmA*
	non - LEE encoded effector A and B	*nleA,B*
	Salmonella HilA homologue	*eilA*
	Tir domain-containing protein	*tcpC*

In 2008, Johsnon *et al.* suggested a set of VAGs to be associated with highly pathogenic APEC: *iutA*, *hlyF*, *iss*, *iroN* and *ompT* (Johnson et al., 2008). Tivendale *et al*. recognized that the majority of the VAGs identified with APEC were somewhat conserved and could be located on a single plasmid, the colV plasmid. According to Tivendale *et al.* less virulent APEC could carry some of these genes, either on a plasmid or chromosomally, but non-pathogenic *E. coli* would neither carry the colV plasmid nor the associated VAGs (Tivendale et al., 2009). In 2014, Collingwood *et al.* presented the defining trait for APEC as the presence of the ColV and colBM plasmids (Collingwood et al., 2014). Later this definition has been questioned, as several studies have identified the same virulence associated plasmids and genes in *E. coli* from cecal samples collected from healthy birds (Johnson et al., 2022; Mageiros et al., 2021).

In 2022, Johnson *et al.* proposed screening for high virulence APEC to include the VAGs *hlyF* and *ompT* as well as specific gene markers from what is referred to as dominant APEC variants of specific STs and the serogroup O78 (Johnson et al., 2022), (Figure 6). Depending on the specific *E. coli*, the theory is that certain types of *E. coli* may acquire certain VAGs and develop into virulent APEC (David et al., 2008; Mageiros et al., 2021; Johnson et al., 2008). However, a clear definition of an APEC in *sensu stricto,* beyond the use of ELA, does not exist, and the APEC-type is still considered poorly defined (Mehat et al., 2021).

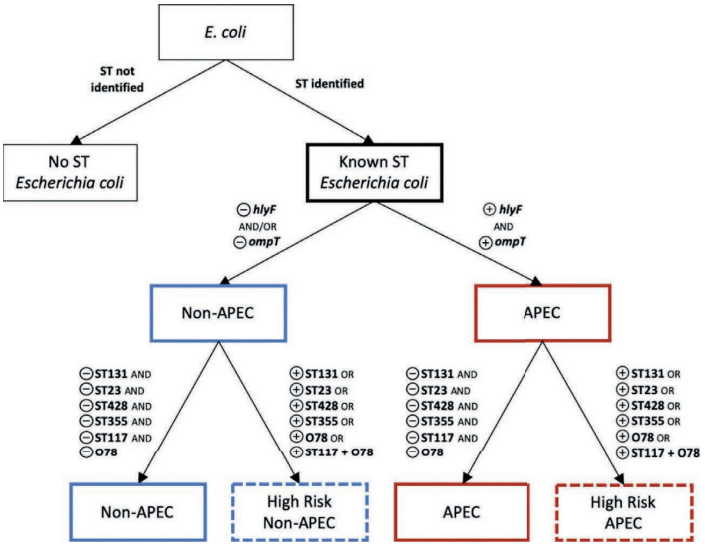

Figure 6: Scheme describing how to screen for high virulence APEC as described by T. Johnson. The figure is retrieved from the publication by Johnson et al. (Johnson et al., 2022)

5.1.5 Knowledge gaps

The knowledge of *E. coli* causing disease in poultry is well documented and widely distributed. There are quite a few research papers presenting the various types of colibacillosis, associated macroscopic lesions and the types of APEC identified from these flocks. Further, research to define APEC and its virulence potential are numerous.

As such, what has changed to indicate the need for further studies on APEC?

With WGS becoming readily available, in-depth knowledge of the bacterial genomic traits on large sample collections may be acquired without exponentially increasing the labor and cost. Further, the high resolution of sequenced data enables accurate comparisons of APEC strains and their genetic relatedness. This opens for more extensive, in-depth research on APEC.

In 2014-2016, a wave of NC-cases was identified within the Nordic countries. A genomic study on isolates from these cases revealed 47 closely related APEC of

ST117, serotype O78:H4, but also a genetically diverse population (Ronco et al., 2017). The study also revealed that knowledge on the epidemiology of APEC within the Nordic broiler chicken production was lacking. Further, there was no knowledge of risk factors associated with outbreaks of NC within a generally well-managed (FWM <0.8%) broiler chicken industry. The need to identify the epidemiological aspects of APEC within the Norwegian broiler chicken production and identify the risk factors contributing to NC resulting in high FWM, was recognized.

Gaining specific and in-depth knowledge on the diversity and types of APEC, as well as risk factors for NC, will move the field forward to improve the current preventive strategies against outbreaks of NC.

5.1.6 Aims of the study

The main aim of this project was to generate in-depth, research-based knowledge on APEC causing NC in Norwegian broiler chicken flocks.

The following research questions were to be answered within my PhD studies:

1. How diverse is APEC within a broiler chicken flock diagnosed with NC?
2. Is there one, or more, strain(s) of APEC causing local outbreaks of NC in Norwegian broiler chicken flocks and can we rank APEC according to virulence?
3. How genetically related are the APEC isolates identified within a flock and between flocks in an outbreak setting?
4. Assuming the presence of individual cases of NC in most flocks, what risk factors are associated with local outbreaks of NC in broiler chicken flocks?

5.2 Material and Methods

This chapter will give a short summary of the methods used in this PhD project. For further details, see papers I-III.

5.2.1 Study design

The PhD project was designed to gain knowledge on the diversity and prevalence of the different APECs circulating the Norwegian broiler chicken production. High-resolution sequencing techniques and bioinformatics analysis would increase our knowledge on the genetic relatedness between APECs within and between flocks with NC, including their carriage of VAGs. Further, the project aimed to identify farm- and production-related risk factors associated with high FWM due to NC. Sampling for APEC and the collection of data for the risk factor study was, therefore, directed to include flocks within the broiler breeding pyramid which were experiencing high FWM due to NC. This included parent rearing flocks and broiler chicken flocks, but only if less than 14 days of age (Figure 7).

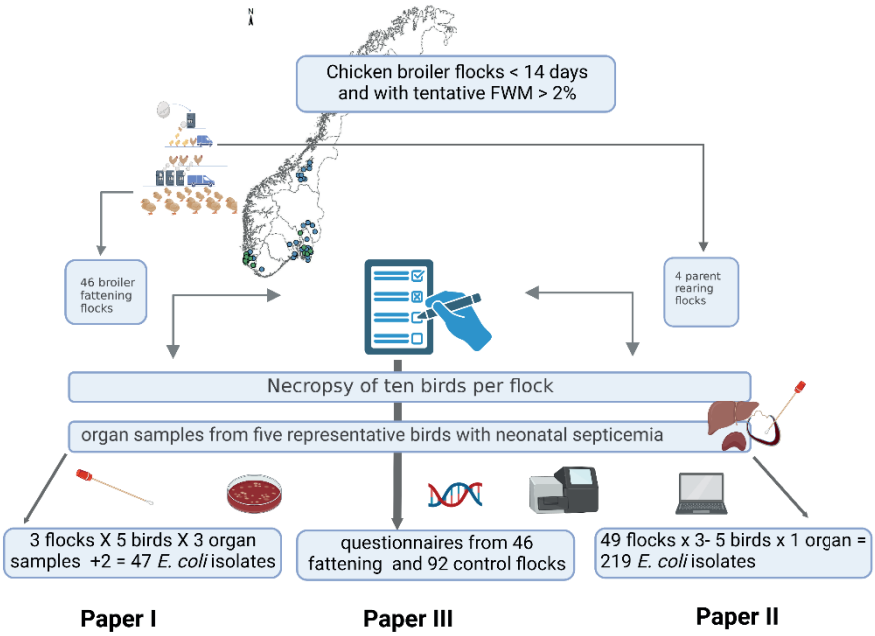

Figure 7: The study design, including the study population, methods for collecting data and samples, laboratory methods and the datasets used for each of the three papers included in this PhD thesis. The samples were from the Norwegian broiler chicken population. All samples are taken from chicks < 14 days of age and from flocks with tentative first week mortality (FWM) > 2%. Three organs were sampled for bacterial examination from five birds/flock. The samples were bacteriologically examined before a selection of E. coli *isolates underwent DNA extraction, whole genome sequencing (WGS) and bioinformatics analyses. (Created with BioRender.com)*

We wanted data and bacterial samples from typical flocks with outbreaks of NC (case flocks). The case flocks were defined as flocks where FWM was expected to reach >2%, and where at least five of ten necropsied birds showed typical macro-pathological lesions associated with NC.

From September 2018 to July 2021, collaborating veterinarians visited broiler chicken farms where a sudden, unexpected increase in mortality within a flock of newly placed chicks had occurred. The veterinarian would perform necropsy on ten birds recently found dead or euthanized for animal welfare reasons. Samples were collected for bacteriological examination from five of the ten macroscopically examined birds and flock data and pathological findings were recorded by filling out an extensive questionnaire.

To identify the potential vertical transmission of APEC, samples from the associated parent flocks, as well as the hatchery, were included, but only if two broiler progeny flocks from the same parent flock were diagnosed with NC. Parent layer flocks and the hatchery would also be sampled at onset of lay, if the parent flock had been diagnosed with NC during the first 14 days of rearing.

Samples were also collected from control flocks to compare APEC causing disease in individual birds in flocks where FWM remained low, to APEC causing outbreaks of NC . Control flocks were defined as flocks situated within the same county as the case flocks, they were of the same age (hatching date +/- 3 days) as the case flocks, but FWM had to be ≤0.8%. Sampling of the control flocks was performed within two weeks of a confirmed NC diagnosis of the case flock and using the same procedure as in the case flocks.

Samples collected from the parent layer flocks, hatcheries and control flocks have later been excluded from the PhD studies. This is further discussed in chapter 5.4 under Methodological considerations.

5.2.2 Sampling procedure

Field veterinarians performed necropsies on ten birds in each case flock. The five birds with the most typical macro-pathological lesions consistent with NC were further selected for bacteriological sampling. The five birds were sampled from the spleen, liver and one other organ with typical lesions of NC, such as the umbilicus, body cavity, pericardium or bone marrow. Individuals from the parent layer flocks, recently succumbed or euthanized for welfare reasons, were sampled from the liver, spleen and oviduct. Swabs with Amies transport medium with charcoal were used for bacteriological sampling (Copan© gel amies with charcoal).

From the hatchery, dust cloths were used to sample the hatchery trays and pieces of eggshell together with five chicks (succumbed or weak and euthanized) were collected. All samples (swabs, dust-cloths, eggshells and chicks), together with a completed questionnaire, were sent to the Norwegian Veterinary Institute by express post (within 24 hours). Swabs were immediately cultured, while the chicks and dust-cloths were frozen at -20 ^{O}C for later analysis.

Altogether, samples were collected from case flocks (49), control flocks (21), parent rearing flocks (5), parent laying flocks (20) and hatchery (7).

5.2.3 Bacteriological examination

Samples from up to three organs per animal were plated onto two 5 % bovine blood agar (BA) plates (Oxoid, Basingstoke, UK) and one Heart Infusion Agar (HIA) plate with 6 % lactose saccharose solution. The samples were then incubated at 37 °C ± 1 °C for 16–24 hours; one BA plate under anaerobic conditions, the other in a 5 % CO_2 atmosphere and the HIA agar was incubated under normal atmospheric conditions (Figure 8). Following incubation, each sample was registered, and the colony phenotypes and bacterial growth was described.

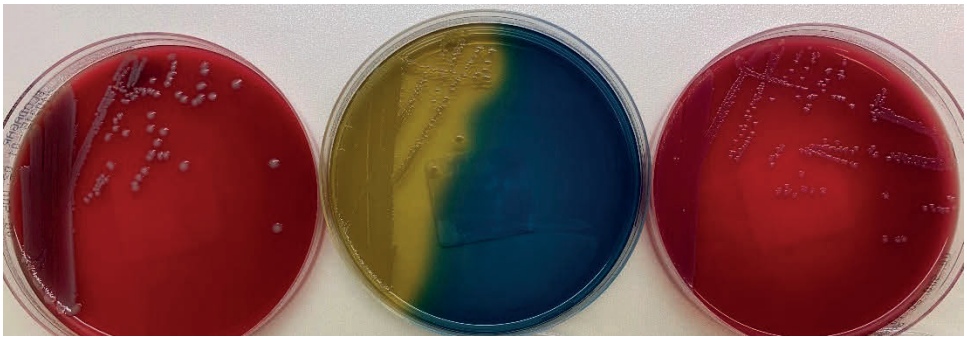

Figure 8: Growth of E. coli *colonies on blood agar incubated either anaerobically (far right), in CO_2 chamber (far left) and on heart infusion agar (HAI) under normal atmospheric pressure (middle). The sample was seeded out on the previous day and shows pure culture of* E. coli *(Photo by: Inger Helene Kravik)*

Bacterial growth was graded according to purity on BA: (1) pure culture: Pure growth of *E.coli*, no other bacteria were identified with a minimum of five colonies of *E. coli* present. (2) Almost pure culture: Close to pure culture of a medium-rich growth of *E. coli*, but with a sparse presence of a "foreign" bacterium and/or with minimal growth of *Enterococcus spp*. or *Proteus spp.* on top of the *E.coli* colonies. (3) Dominating growth of *E.coli*: Medium to rich growth of *E. coli* including a sparse growth of one other bacterial type. Mixed culture (4): *E.coli* and a minimum of two other types of bacteria present in equal amounts.

A minimum of one colony with typical *E. coli* colony morphology was picked from each positive sample and re-plated onto BA and incubated in a 5 % CO_2 atmosphere at 37 °C ± 1 °C for 16–24 hours. Cultures of *E. coli* were confirmed using the MALDI–TOF MS (Bruker Daltonics, Germany) and further frozen at – 80 °C in a medium containing 85 % glycerol and Heart Infusion Broth (for further details see materials and methods paper I).

5.2.4 Selection of APEC-isolates

To ensure a correct selection of APEC isolates to be included in the study, a stepwise diagnostic procedure was performed: (1) an individual diagnosis of NC was given based on bacteriological examination (grade 1-3) and the presence of a minimum of two macro-pathological lesions associated with NC. (2) A flock was diagnosed with NC if FWM > 1.5 % and a minimum of three birds from the flock were diagnosed with NC. Only APEC-isolates from flocks diagnosed with NC were selected for WGS.

5.2.5 High throughput sequencing and bioinformatic analyses

DNA extraction was performed using the QIAmp® DNA Mini Kit (Qiagen) according to manufacturer's protocol with minor modifications (see paper I for details).

Selected *E. coli* isolates were prepared with Nextera DNA Flex library preparation kit (Illumina), and sequenced on an Illumina MiSeq instrument, resulting in 300 bp paired end reads. Initial quality control and assembly of samples were done using the Bifrost pipeline (Lagesen, 2020). This pipeline consists of read quality control, quality and adapter trimming, removal of PhiX and assembly. ARIBA (Hunt et al., 2017) was used to determine the ST according to the Achtman scheme (Wirth et al., 2006). Serotypes were identified using SerotypeFinder (Joensen et al., 2015).

For core gene and core genome analyses, the ALPPACA pipeline version 1.0.0 was used (Kaspersen & Zeyl, 2022), (for details see paper II).

A comprehensive screening of VAGs was performed using VirulenceFinder version 2.0.4 with and extended VirulenceFinder database. The database was extended with known APEC-associated genes found in the Virulence Factors of Pathogenic Bacteria database (Kathayat et al., 2021; Newman et al., 2018; Ewers et al., 2005; Liu et al., 2019).

5.2.6 Data collection

All data from the questionnaires, filled out at the time of sampling of the case flocks, were compiled in an excel spreadsheet. All pathological findings noted during necropsy in the same questionnaires, were added as present/absent and aggregated in the same excel sheet.

We chose a matched case-control study to evaluate potential risk factors for local outbreaks of NC in broiler chicken flocks. Data from defined control flocks for the risk factor study (paper III) were collected retrospectively. Control flocks were defined as flocks with FWM ≤0.8%, and were further matched to the case flocks based on: geographical location, hybrid and hatching date. For each case flock, two control flocks were selected. Individually, each company selected control flocks, based on the above mentioned criteria, with the help of a freeware randomization tool. Data from the control flocks were collected retrospectively from electronically stored production control databases or from manually filled-in "day lists". After interviewing

collaborating veterinarians, additional and missing data (not acquired through the questionnaires) were collected retrospectively for both case and control flocks as described above.

5.2.7 Data analysis and statistical methods

All data from the questionnaires, as well as data obtained from the WGS and bioninformatic analyses of the samples, were compiled in a single excel spreadsheet. Descriptive analysis was performed on pathological lesions, STs, serotypes and virulence associated genes. (Table 3, 4), (See paper I and II for detailed results)

Data that had been collected retrospectively from the case and control flocks, were merged and cleaned using R (R Core Team, 2021). The cleaned data was included in the risk factor study on local outbreaks of NC (paper III).

Variables for the risk factor analysis were evaluated, if data was missing from > 50% of the flocks, these variables were excluded from further analyses. Descriptive analyses, including mean, median, minimum and maximum values were performed on all remaining variables, before median imputation was performed on the remaining unreported data for continuous variables. Univariate analyses were used to evaluate which variables to include in a final multivariate logistic regression model.

The predictive ability of the final multivariate logistic regression model was assessed with a receiver-operating characteristics (ROC)-curve, and by calculating the area under the curve (AUC) using R programming, pROC package version 1.18.0 (Robin et al., 2011; R Core team, 2021). For details, see paper III.

5.3 Results- Summary of the articles

Paper I

In this paper, we aimed to identify the diversity of APEC from both within an individual bird suffering from NC and within a flock diagnosed with NC. The objective was to provide guidelines for a practical and economically efficient selection of isolates for genomic and epidemiological studies on NC, without affecting the reliability of the results.

Three case flocks (F1-F3) of the same broiler chicken hybrid, but differing geographical locations, were selected for the study.

Macro-pathological findings in the three flocks were consistent with previous descriptions of NC. From each flock, three organs (*spleen*, *liver* and one *other* organ with symptoms of colibacillosis), from each of the five birds (15 samples in total), were collected and bacteriologically examined. A minimum of one confirmed *E. coli* isolate from each organ was selected for WGS.

Altogether 47 *E. coli* isolates were sequenced, quality checked, assembled and further characterized *in silico* with regard to ST and serotype. Phylogenetic analysis (core genome analysis) was performed on identical STs if identified in > 5 isolates.

The results from the WGS and bioinformatic analyses showed high sequence similarity between APEC-isolates sampled from individual birds. In 12 out of the 15 birds included in the study, the same ST was identified in all three organs from an individual. Only one bird from each flock was identified with >1 ST from the sampled organs. In all instances, the isolates sampled from the liver corresponded with the dominant ST within the bird.

Flocks F1 and F2 were identified with ST429 in 15 of the 17 and in 14 of the 15 isolates sequenced within the flock, respectively. Flock F3 was identified with three STs: ST95, ST457 and ST10836. Sequence type 95 was identified in eight out of the 15 isolates sequenced from the flock.

In this study serotyping conformed with the identified STs: ST429 with O2/O50:H1, ST95 with O2/O50:H5, ST457 with O11:H25 and ST10836 with O41:H45.

Phylogenetic analysis of ST429 revealed two distinct clades (A and B) that were associated with flocks F1 and F2, respectively (Figure 1, paper I). Within clade A, the mean SNP- distance was 6.24 and the SNP-range was 0-21. Two isolates, one sampled from the *other* organ and one from the *liver,* formed a small cluster separate from the main cluster in clade A.

In clade B the mean SNP-distance was 4.45 with a SNP-range of 0-11.

The average genome coverage of the ST429 core genome alignment was 97.5%, and the mean SNP-distance across both clades was 35.97 with a SNP-range of 0-70.

Phylogenetic analysis of ST95 was performed on eight isolates from flock F3 (Figure 2, paper I). The mean SNP distance amongst all ST95 isolates was 18.35 with a SNP-range of 6-29. The average genome coverage was 96.40%.

Based on this study, a minimum of three birds from a flock should be sampled to increase the likelihood of recognizing the diversity of APEC within the flock, and identify the disease-causing APEC within the flock. One sample per bird may be enough to show the diversity of APEC within a flock, if several birds are sampled. In this study all samples from the liver corresponded to the dominating ST within a bird. Based on this study, the liver was considered a good choice to sample for these types of studies.

Paper II

In this paper, we present the results from a molecular epidemiological study on APEC associated with NC in broiler chickens in Norway. With the use of WGS and bioinformatci analyses, the aim of the study was to identify and compare APEC within- and between broiler chicken flocks experiencing local outbreaks of NC. Potential association between flocks and the identified APECs were recognized with the help of flock-related metadata, such as: sampling date, hybrid, hatchery and parent flock.

Systematic collection of APEC isolates was performed as described in materials and methods. An individual diagnosis of NC was based on the presence of macro-

pathological lesions associated with NC (Table 3), together with bacteriological examination showing pure to dominant growth of *E. coli.*

A flock was diagnosed with NC if FWM >1.5 % and if at least three out of five sampled birds from the flock were diagnosed with NC individually. Altogether 41 broiler chicken flocks and 4 broiler rearing flocks <14 days were diagnosed with NC and included in the study.

Table 3: Frequency of macro-pathological lesions reported (in descending order) during necropsy of flocks diagnosed with neonatal colisepticemia (NC) for the present PhD studies.

Macroscopic observation[1]	**Frequency in percent (%)[2]**	**Total (n)[3]**
Pericarditis	67.92	240
Enlarged spleen	61.4	236
Moist body cavity	59.2	240
Fibrinous peritonitis	53.3	240
Swollen liver	52.5	240
Umbilical haemorrhage	50.4	240
Perihepatitis	47.1	240
Femoral head and neck necrosis	33.8	240
Open umbilicus	28.8	240
Engorged spleen	20.9	235

[1]Macroscopic observations noted in a present/absent questionnaire during necropsy by the collaborating veterinarian.

[2]Frequency from which the observation was noted divided by the total number of birds examined and times 100.

[3]Total number of individual birds examined

From each flock, three - five birds were sampled and one isolate per bird, preferably from the liver, was included in the study. Altogether, 219 *E. coli* isolates were subjected to WGS and bioinformatic analyses for ST, serotype, presence of VAGs and phylogenetic analysis (core gene and core genome).

The results from the study showed that in more than half (26/45) of the flocks, one distinct ST was identified in all the sequenced samples from within the flock. In 9/45

flocks two distinct STs were identified and in 10/45 flocks ≥3 distinct STs were identified within a flock.

Altogether 32 different STs were identified. Of these, 15 were only identified once. The most common STs identified (≥5 isolates) in the study were (in descending order): ST23, ST429, ST95, ST117, ST371, ST69, ST101 and ST135 (Table 2, paper II).

The study further showed that ST23, ST117, ST371 and ST429 were more often found to be the sole cause of disease in a flock, while ST69, ST95 and ST101 were more often identified in combination with other STs within a flock.
In silico serotyping identified a total of 38 distinct serotypes in this study. Some serotype profiles were detected across several STs and some STs were identified with several serotype profiles (Table 4 and Figure 1, paper II).

Phylogenetic (core gene) analysis of all isolates showed that the isolates clustered according to their STs, and that isolates with the same serotype profile might be genetically distant. Some examples include serotype O78:H4 which was identified as ST23 and ST117and serotype O2/O50:H1 which was identified as both ST429 and ST135. Further, two STs were identified with more than one serotype profile: O1:H7 and O2/O5O:H5 (ST95) and O24:H4, O78:H4 and O161:H4 (ST117) (Table 4 and Figure 1, paper II).

The core gene analysis illustrated the need to present both ST and serotype when determining the APEC profile. Table 4 summarizes the individual APEC-profiles identified amongst 219 *E. coli* isolates, and how similar data may be presented in future molecular epidemiological studies on APEC.

Table 4: The number (N) of APEC-profiles (sequence- and serotypes) identified amongst the 219 E. coli *isolates sequenced. The STs identified with more than one serotype profile are highlighted in different colors.*

ST	serotype	N
10	O49:H12	1
10	O71:H40	1
10	O99:H33	2
23	O78:H4	81
69	O15:H6	1
69	O17/O44/O17/O77:H18	3
69	O23:H6	4
88	O8:H17	1
93	O5:H10	4
95	O1:H7	17
95	O2/O50:H5	7
101	O103:H21	2
101	O88:H8	5
117	O161:H4	1
117	O24:H4	5
117	O78:H4	5
135	O2/O50:H1	5
154	O134/O46:H38	1
155	O8:H20	2
191	O150:H20	1
349	O166:H15	1
371	O45:H19	9
428	O120:H4	2
429	O2/O50:H1	33
457	O11:H25	4
1112	O55:H27	1
1146	O103:H2	3
1170	O1:H4	2
1611	O125ab:H19	1
1640	O86:H27	1
1656	O8:H8	1
1684	O133:H4	1
1841	O103:H7	3
2040	O159:H20	2
2491	O4:H42	1
2690	O88:H16	1
2753	O171:H10	1
3006	O26:H32	1
5340	O112ac/O8:H46	1
10836	O41:H45	1

The two largest clusters in the core gene tree were represented by ST23 and ST429 (Figure 1, paper II). Both STs had one distinct serotype profile and all flocks, except one flock with ST429, clustered according to hybrid, sampling date and ST. The data suggested these two STs represented two individual outbreaks of NC across farms.

ST23 was identified in 81 isolates sampled from 17 flocks of the hybrid Ross 308, all sampled between March and May 2021. Core genome analyses of the ST23 isolates had an average genome coverage of 94.0%, a SNP range of 0–33 and a mean and median SNP-distance of 15. Within individual flocks, all but two flocks had mean SNP distances < 10. The SNP-range varied from 0 to 1 (smallest range) to 0–33 (highest range) within a flock (Figure 2, paper II).

ST429 was identified in 33 isolates from seven flocks: six flocks of the hybrid Ross 308 sampled between September 2018 and January 2019, and one flock of the hybrid Sasso sampled in August 2019.
The core genome analyses revealed an average genome coverage of 95.8% and the SNP range was 0–172 with a median SNP distance of 62. The tree diagram separated into three clades, and the isolates from the Sasso flock all clustered together in one clade. The Sasso flock originated from a parent layer flock imported from France as day-old chicks. The APEC isolates recovered from flocks of hybrid Ross 308 were dispersed within the remaining two clades. These flocks originated from different geographical locations within Norway and were from two different hatcheries, but the same parent hatchery. The mean SNP-distances within a flock were <10 SNPs in all but the Sasso flock (Figure 3, paper II).

When running the same analyses with only the Ross 308 flocks, the genome coverage was unchanged, but the SNP-range decreased to 0-76 with a median SNP distance of 62.

All isolates were screened for the presence of VAGs using an extensive database which included 629 entries of VAGs. Altogether, 112 VAG-variants were identified in at least one APEC isolate. Twenty-five VAGs were identified in all of the ST23 isolates, whereas 33 VAGs were identified in all of the ST429 isolates. The third group, consisting of the remaining STs, showed a higher diversity in the carriage of VAGs.

This study gave insight into the presence and distribution of APEC causing local outbreaks of NC across Norway during 2018-2021. The study identified how peaks of

NC resulting in high FWM across farms may be caused by a single strain of APEC of the same ST and serotype. Further, the core gene analyses illustrates the need to combine typing methods in order to better discriminate between APECs, as genetically distant STs may share the same serotype profile and one ST may be identified with several serotype profiles.

Phylogenetic analyses of the two outbreak isolates ST23 and ST429 gave further insight into the number of SNPs that one might expect in future, single-ST outbreaks of NC. Finally, an extensive screening of the presence of VAGs gave further insight into the prevalence of VAGs in APECs isolated from NC.

Paper III

The objective of this study was to identify risk factors for high FWM caused by NC in Norwegian broiler chicken flocks.

The study was designed as a matched case-control study. Case flocks were defined as broiler chicken flocks diagnosed with NC as described in paper II. Control flocks were defined as flocks with mortality <0.8%. We assumed a low grade of individual cases of NC within each control flock. The control flocks were matched to the case flocks by sampling date, hybrid and geographical location (see chapter 5.2.6 for details). Twice as many control flocks as case flocks were randomly selected based on the above-mentioned criteria. Data from the control flocks were retrospectively collected from electronically stored production control databases.

Altogether 46 case flocks and 92 control flocks were included in the study. Data from 91 variables were collected and additional variables were constructed, totalling 104 variables. Data cleaning removed unfit variables, and univariate and multivariate logistic regression was performed on a total of 37 variables.
The final logistic regression model resulted in two variables with *P*-values < 0.05. The two variables were FWM Previous Flock and Flock Size (both $P = 0.011$) with an estimated risk (OR) of 3.33 (CI = 1.45 – 9.16) and 1.07 (CI = 1.02 – 1.13), respectively. The AUC for the chosen model was calculated to be 0.747 (Table 4, paper III).

The results translate to a 233% increase in odds for high FWM in a flock if the previous flock also had high FWM. Further, by increasing the flock size by 1000 birds the odds for high FWM increases by 7%.

The study highlighted two farm management risk factors to consider when searching for preventive measures against local outbreaks of NC, in a generally well managed broiler chicken industry (FWM <0.8%) with high biosecurity.

5.4 Discussion

5.4.1 Material and methodological considerations

In this chapter I consider the methodological approaches used to answer the aims of my PhD studies. Further, I will discuss the problems we encountered and consider ways the studies could have been improved for future research on similar topics.

Study design

The PhD project was designed to focus on broiler chicken flocks with high FWM due to a systemic *E. coli* infection, in this thesis referred to as local outbreaks of neonatal colisepticemia (NC). The initial plan was, therefore, to collect samples from broiler chicken flocks and parent rearing flocks with NC, as well as hatcheries and parent layer flocks, if associations to the case flock diagnosed with NC were identified.

At the time of sampling, we were aware that one of the two parent hatcheries delivered eggs to two broiler fattening hatcheries in different parts of Norway, but we were not aware that one of the hatcheries changed the ID-number of the parent flock to correspond with their own system. The same parent flock could therefore, potentially have provided offspring to several of the sampled case flocks without the association being identified in the lab. Any connection between case- and parent flocks may therefore, have passed unnoticed. The problem was uncovered during a larger outbreak of colisepticemia in the spring of 2021 (ST23). Samples from associated parent flocks to this outbreak were collected, but not in time to be included in the PhD studies. Hence, the vertical connection between parent flocks and offspring were excluded from the molecular part of this PhD-study (paper II). A report on the outbreak of ST23, including a description of isolates from parent layer flocks and broiler fattening flocks will soon be published.

To identify any association between parent flocks and offspring suffering from NC, the deciphered parent flock IDs were included in the risk factor study (paper III). However, due to the sample size of the risk factor study and the numerous parent flocks contributing to the broiler flocks, we could not identify any statistical association.

Sampling

Sudden and unexpected increases in mortality during the first week of the broiler chicks' life, elicited the collection of samples for our studies. Any increase in FWM would initiate the chicken producer to contact a poultry veterinarian, who would then perform necropsy and sampling. This method of reporting and sampling flocks induces two levels of potential selection bias: (1) Individual broiler chicken producers may be more likely to call the veterinarian than other producers, (2) Some collaborating veterinarians may be more likely to sample for the project than others. This may be time, cost or location dependent.

From the fall of 2018 to the summer of 2021 altogether 18 different veterinarians from four broiler chicken companies collected samples for the project. Company A covers just above 50 % market share for broiler chicken production in Norway, company B and C account for the majority of the remaining market with company D accounting for an almost negligible share of the market (Kjos et al., 2022). There was an uneven distribution of samples received from the different companies: company A (47 %), B (39 %), C (12 %) and D (2 %). Company C started vaccination against *E. coli* in parent rearing flocks in 2019, and samples from this company declined in numbers after this.

During the sampling period of this thesis, two outbreaks of NC across farms resulted in clustering of samples: (1) six broiler chicken flocks of hybrid Ross 308 were sampled from mid-November 2018 to mid-January 2019, and (2) 17 broiler chicken flocks of hybrid Ross 308 were sampled from March 2021 to the end of June 2021. The remaining 26 flocks were more evenly distributed and included other chicken hybrids as well. A slight increase in samples received by our laboratory could be identified after a friendly reminder of the ongoing project.

The implications of an uneven sample distribution could be that some flocks, which should have been included in the project, were lost. My general opinion is that the loss of samples from a few flocks would not drastically change the results of the molecular epidemiological study resulting in paper II. The project, however, increased the awareness of an ongoing outbreak in the spring of 2021, which resulted in samples from 17 case flocks (ST23). In comparison, another single ST outbreak, identified as ST429, only included six flocks. One might hypothesize that one outbreak was greater than the other. However, the only difference may be the time of sampling. The outbreak with less cases occurred at the beginning of the project, where the

awareness of the project was low, while the other occurred late in the sampling period.

Other approaches to the selection of case flocks would be to sample all flocks the veterinarians visited and later select, based on FWM, a random number of sampled flocks from each month in the year. This presupposes either, that the veterinarian visits a chicken producer at regular intervals or, that the producer calls the veterinarian more than the two regulated visits during a year. If such an approach was to be chosen, the comprehensive necropsy and diagnostics performed by the veterinarians at each visit, would be too time consuming and expensive for non-diagnostic purposes. Further, a method where flocks are randomly sampled at regular intervals, independent of the FWM in the flock, would probably not illustrate the extent of the single strain outbreaks of NC, as was shown with the current method. This would also have resulted in fewer isolates to be included in the core genome analyses of ST23 (paper II).

To ensure the harmonization of macroscopic examination and evaluation, including procedures for collecting samples by collaborating veterinarians, instructions on how to perform necropsy and evaluate the macro-pathological lesions were presented at the Norwegian Veterinary Institute's pathology lab at the beginning of the project. Further, specific guidelines for necropsy, swabs for sampling and questionnaires were sent to the collaborating veterinarians.

Bacterial samples were collected in the field by local, collaborating veterinarians by swabbing organs during necropsy. Sampling under field conditions may be advantageous as samples are collected from fresh carcasses. However, the potential for contamination from the surroundings should, under these circumstances, be considered.

We chose to collect samples from the liver, spleen and one other organ with lesions associated with NC. The liver, a large organ, is readily available to sample. However, due to its connection to the intestine, potential contamination from the intestinal microbiome should be considered. The spleen or the bone marrow have both been suggested as more representative organs to sample for bacteremia. The spleen in newly hatched chicks is, however, only a few millimeters in diameter and recovering a representative sample from this organ may be difficult without contamination from the surroundings. Contamination may also occur when breaking the bone to retrieve

a representative sample from the bone marrow. The results presented in paper I showed high sequence similarity of *E. coli* isolates sampled from several organs within a bird, but also showed that sampling of the liver from fresh carcasses under field conditions was a sufficient method, or even a good choice, to identify the disease-causing APEC within a flock.

Bacteriological examination

Bacteriological examination was performed according to standard procedures for diagnostics at the bacteriology lab of the Norwegian Veterinary Institute. A selective medium for *E. coli*, such as MacConkey agar, has previously been used in studies on APEC (Johnson et al., 2022; Papouskova et al., 2020; Cummins et al., 2019). The choice to perform diagnostic examination using BA and HIA under anaerobic, CO_2 and aerobic conditions (see chapter 5.5.3. for details) was to offer collaborating veterinarians a diagnostic reply in favor of samples being sent to the project. The examination further enabled an overview of other potential causes for NC within a broiler chicken flock. Even though it was more laborious to include a full diagnostic examination of the bacterial samples, it ensured an accurate diagnosis of NC in the individual birds and flocks included in the study.

Selection of isolates

Selecting for APEC was done through a stepwise procedure including flock mortality, macro-pathological lesions and bacteriological examination.

Originally, pure growth of *E. coli* on BA was considered a requirement for the selection of APEC-isolates. However, this strict policy would have excluded many flocks from the study and given a narrow view of the problems seen in the field. The growth of *E. coli* on BA was therefore graded 1-4 (see material and methods for details). Finally, grades 1-3 were accepted to be included in the study, as long as the macro-pathological lesions associated with NC were present. The latter inclusion requirement was important to avoid the mis-diagnosis of colisepticemia caused by growth of intestinal bacteria.

Embryo lethality assay-testing is a method used to confirm that an *E. coli* isolate is an APEC (Nolan et al., 1992; Wooley et al., 2000). The stepwise selection for APEC, used in this PhD study, ensured a reliable inclusion of APEC isolates to the project, without the use of ELA. During the PhD study, we sequenced more than 219 *E. coli* isolates, identifying 32 distinct STs and 38 distinct serotypes: altogether 40 APEC-profiles (Table 3). Using ELA to test for pathogenicity amongst all APEC-profiles identified

would not be ethical from an animal welfare point of view, nor economically feasible. The systematic sampling and careful stepwise selection for APEC was therefore preferred.

Initially, in control flocks where FWM ≤0.8%, individual birds with symptoms of NC were sampled for *E. coli*. These isolates represented *E. coli* that had caused disease within an individual bird and were therefore, also considered APEC. The hypothesis was that an APEC causing NC resulting in high FWM would be different from APECs which presumably did not spread horizontally within the flock, and where the FWM remained low. Coinciding with the study by Kroman *et al.*, there was higher diversity of APEC within the control group (Kromann et al., 2022b). However, we did also identify the potentially more virulent APEC ST23 (O78:H4) amongst the isolates from the control flocks. This was not a casual finding, since the identification was evident in more than one flock. In fact, two of the control flocks identified with APEC ST23 (O78:H4) would have fulfilled the criteria for the case flocks had it not been for the low FWM. This led us to reconsider the definition of a control flock and samples from the control flocks were excluded from the molecular part of this PhD study.

Several studies have collected *E. coli* from the gut of healthy chickens to compare with APEC isolated from lesions associated with colibacillosis (Mageiros et al., 2021; Kemmet et al., 2013; Al-Kandari & Woodward, 2018). So far, a clear distinction between *E. coli* sampled from the chicken gut and APEC have not been identified. The theory is that an *E. coli* with the potential to cause extra-intestinal infections may reside in the gut (Mehat et al., 2021; David et al., 2008). A recent study by Thomson *et al*. reported a high diversity among *E. coli* isolates recovered from the gut of healthy chickens, encompassing almost the entire known phylogeny of *E. coli*, and that from a relatively small (n=81) set of isolates from within one flock (Thomson et al., 2022). Thomson identified both ST10 and ST23, which were also identified within this PhD study. This suggests that there is no simple way to define a control, as there is no simple way to define an APEC without animal trials. One way could be to use observed "singletons" as control isolates. These are distinct isolates only identified once within a large dataset, and therefore, are not considered the cause of disease. However, the ability of *E. coli* to acquire genetic elements from its surroundings and to develop potentially virulent properties complicates the discussion of a "true" control (Denamur et al., 2021, Feil et al., 2004).

Whole genome sequencing and bioinformatics analysis

The choice to use WGS was based on both the availability of WGS in house and because it at present is considered the reference standard for bacterial pathogen typing (Bogaerts et al., 2021). Whole genome sequencing enables several analyses to be performed on the same sample (Bogaerts et al., 2021; Egli et al., 2019). Combining more than one typing method on the same isolate enabled us to accurately type the *E. coli* isolates and gave in-depth insight to the genetic relations between the isolates sequenced. Further, WGS enables the uploading of raw reads to publicly available databases, ensuring reproducibility of the analyses performed. This includes control of the bioinformatic methods applied. Publicly available reads, together with associated metadata, enables comparative, in-depth analyses of large quantities of isolates e.g.: specific genes for virulence, genes for microbial resistance and relatedness between APEC-isolates. The need for a public database to map outbreaks of colibacillosis across national borders has been suggested (Christensen et al., 2021; Egli et al., 2019) and partially established (Kromann et al., 2022a), further supporting the need for publicly available genomic data on APEC.

The choice of programs used for the bioinformatic analyses was done by our team of bioinformaticians at the Norwegian Veterinary Institute. All software used in the genomic analyses are internationally recognized and well-documented for the use of the chosen analyses.

We chose in our studies to perform MLST to type APEC, supported by *in silico* serotyping. Multi locus sequence typing is considered the standard for presenting pathogenic bacteria at present (Bogaerts et al., 2021; Jolley et al., 2018). Serotyping, on the other hand, is considered the traditional way of typing *E. coli*, which improves comparability of our results to previous studies on APEC (Mehat et al., 2021). The two methods presented together ensure comparability of our results to historic as well as for future studies, and together the two methods present a more precise APEC-profile. Assigning APEC isolates to phylo-groups was never considered due to the low resolution of the method compared to MLST.

Another high-resolution typing technique is the cgMLST, which has a higher resolution than MLST, but lower resolution than phylogenetic analyses. We therefore, chose to type APEC using MLST and serotyping for the molecular epidemiological studies and perform phylogenetic analysis (core gene and genome) to gain knowledge on the relatedness between the APECs.

Data collection

Collection of data for the risk factor analysis (Paper III) was done through prewritten questionnaires. Any issues concerning handwritten and human imprecision in the writing or transfer of data digitally should be considered. However, such single errors are usually negligible in statistical studies above a certain sample size. Further, incorrect or nonsensical typing errors were noticed through quality checking and validating the data included in the study. One such example was the confusion of *hours* of darkness and *periods* of darkness during a 24-hour period .While one respondent describes the hours of continuous darkness, another might describe the number of times the chicks are exposed to darkness during 24h. It became evident that some answers were illogical and the reported data could not be trusted. We therefore decided to remove these two variables from the dataset.

Collecting data through electronic applications with multiple choice answers could be used to avoid mistyping and save time transferring data. However, developing an app or a digital form was considered too expensive for this project.

Another way to collect data would be to perform a complete retrospective analysis from electronically stored data. However, the data collected through questionnaires at the time of sampling the case flocks, were also useful for diagnostic purposes during the molecular studies (paper I and II). Flock related meta-data, together with the macro-pathological observations and bacteriological examination, were collectively important for the flock diagnosis.

Because of the above-mentioned reasons, the majority of variables included in the study were decided before sample-collection started. Retrospectively, the knowledge gained during the studies could have changed what questions were asked, and how they were worded. One example is the parent ID-number that differed depending on the hatchery, even though the eggs at the two hatcheries potentially could have been from the same parent flock. Another example was the question including use of disinfectants. The question included in the questionnaires was openly formulated with space for free text. This resulted in a collection of brand names without specifications of the methods or concentrations used, resulting in ambiguity in the answers. The variable was excluded from the analysis, even though several studies previously have identified the use of disinfection as important for the prevention of colibacillosis and persistence of *E. coli* in the poultry house (Awawdeh, 2004, Mo et al., 2016).

Study design for risk factor analyses

A matched case-control study was chosen for the risk factor analyses in paper III. In the "hierarchical pyramid of evidence" a case-control study lies just below a randomized control trial (RCT) and cohort studies, but above case reports (Miller & Wilbourne, 2002; Wilson, 2020).

The case-control study gives a good indication of what factors to further investigate from a wide selection of variables. Where RCTs are used to investigate the effect of an intervention, and the cohort study observes the differences between groups over time, case-control studies investigate the relative impact of several factors on the outcome, suggesting an association. By matching certain variables we might have missed some important associations such as *Seasonality (*Sample Date) or *Hybrid.*

To gain knowledge of what factors increase the odds for high FWM in flocks with NC we found the matched case-control study to be the most suited study design. This is because the study design requires less study time, is considered relatively cheap when compared to cohort or RCTs and is preferred when using a wide selection of variables.

5.4.2 Discussion of the main results

To our knowledge, no studies have previously sequenced and precisely profiled (sequence- and serotyped) APEC with a large set of systematically sampled *E. coli* isolates from multiple birds within a farm, and from two to three organs from each individual bird. In addition, this PhD project is unique in its collaboration with several broiler chicken companies, representing close to 100% of the market share of broiler chicken production in Norway. The collected dataset of systematically sampled APECs across farms diagnosed with NC in Norway gave a good insight into what to expect of APEC diversity in an individual bird, within a broiler chicken flock and within the Norwegian broiler chicken population between 2018 -2021. The state-of-the-art molecular techniques and bioinformatic analyses, gave in-depth knowledge of the relationship between APECs within and across farms. Finally, a survey of potential risk factors for increased flock mortality caused by APEC highlighted management factors of well-managed flocks with high biosecurity, to prevent outbreaks with NC.

In this part, I will discuss the main results achieved within the aims of my PhD studies.

High sequence similarity between APEC isolates within flocks with NC

The studies showed that identical APEC-profiles were identified in all three organs in 12 out of 15 individual birds examined (Paper I). More than half (26/45) of the broiler chicken flocks examined were identified with only one distinct APEC within the flock (paper II). Several flocks were identified with up to five different APECs within the flock. This shows the need to sample multiple birds from one broiler chicken flock to identify the disease-causing APEC(s) within the flock.

The reason why some flocks are affected by one APEC while others are affected by several, is not known. One hypothesis is that flocks affected by several APECs suffer from poorer individual health and management, and may thus be more prone to opportunistic *E. coli*. Another hypothesis is that certain APECs are more virulent and may alone cause disease in a flock. The latter theory has been supported by several publications (Mehat et al., 2021; Johnson et al., 2022; Cummins et al., 2019; Cordoni et al., 2016).

It is important to know what APEC(s) causes disease within the flock to know what preventive measures the farm should apply against future outbreaks of NC. A flock suffering from NC caused by several APECs might require better management, while a flock suffering from NC caused by a single, potentially more virulent APEC might require the production of an autogenous vaccine. The latter solution might be more applicable if infection persists in consecutive flocks in the same house.

Outbreaks of NC across farms are often caused by a single, potentially more virulent APEC.

To gain more knowledge on the true diversity of APECs causing NC in broiler chickens, we aimed to systematically sample and select for APEC-isolates from flocks with a confirmed diagnosis of NC. Multiple, (3-5) bacterial-samples were collected from up to five individual birds in each flock. Few studies have previously obtained APEC-isolates from several organs within the bird and from multiple birds within a flock to identify the diversity of APEC (Delannoy et al., 2020). This systematic collection of samples from flocks with NC enabled us to identify the disease-causing APEC within the flock, and rule out the single STs that are not considered to cause disease. Our studies showed that nearly half (15 out of 32) of the identified STs were only identified in a single isolate, and should therefore, not be considered amongst the disease-causing APECs in a flock. This corresponds with previous observations (Delannoy et al., 2020; Apostolakos et al., 2021; Cummins et al., 2019).

Our results identified two strains of APEC capable of causing larger outbreaks of NC across farms: ST429 (O2/O50:H1) and ST23 (O78:H4). These APECs can be considered potentially more virulent. The virulence potential of ST23 and ST429, together with ST117, is supported by several publications, which have amongst others, identified these STs with high prevalence in their studies on APEC (Mehat et al., 2021; Johnson et al., 2022; Papouskova et al., 2020; Apostolakos et al., 2021; Cummins et al., 2019). However, care should be taken when making this conclusion, as high prevalence of a ST does not necessarily mean it is the same APEC. Reporting only the STs is imprecise, as some STs, such as: ST95, ST117, ST69 and ST10 are associated with several serotype profiles (Table 3 and Figure 1, paper II) (Shaik et al., 2022). Sequence type 23 is also associated with several serotype profiles: O78:H9, O78:H17, O9:H12 and O8:H9 (Mehat et al., 2021; Papouskova et al., 2020; Cummins et al., 2019; Kromann et al., 2022). This shows that the distribution of APECs should, routinely, be more accurately reported, and that one single typing method is not sufficient when reporting APEC.

Grading the virulence potential of an APEC according to frequency is challenging. Historically, defining a true APEC and its virulence potential, without the use of animal trials, has been based on the carriage of VAGs. During the PhD studies we developed an extended database for the screening of VAGs. The intention was that not only would we acquire a greater understanding of what VAGs to expect in APEC, but we would also be able to better rank APEC according to virulence. The analysis compared the presence of VAGs in *E. coli* sampled from birds with NC, and all isolates are therefore, considered potential APECs.

Without a true control group, we decided to divide the isolates from the case flocks in three groups: (1) outbreak isolates of ST429, (2) outbreak isolates of ST23 and (3) the rest of the isolates. In this way we hoped to identify differences between the potentially more virulent outbreak strains, to the more sporadically identified STs (Figure 4, paper II).

We identified several VAGs which were present in all the outbreak isolates, but to a much lesser degree in the third group (for details see paper II). The carriage of VAGs identified in isolates of ST23 in our data-set corresponds with previous studies, except for the presence of *fyuA.* This VAG encodes a siderophore receptor that was identified in both the outbreak isolates from our study and in the outbreak isolates described by Kromann *et al.,* but not in the study by Thomson *et al.* which describes

E. coli from the gut of healthy chicken (Thomson et al., 2022; Kromann et al., 2023). The observations are interesting, but should be assessed with caution as the isolates from each individual outbreak are considered clonal, or close to clonal.

Another interesting observation was that the VAGs *hlyF* and *ompT,* which were present in all of the outbreak isolates, also were present in 89 % and 96 % of the group 3 isolates, respectively. The presence of these two VAGs have been suggested as a part of a screening tool to identify APEC (Johnson et al., 2022). Our results support the carriage of these two genes amongst APEC isolates. However, the presence of VAGs have been identified in *E. coli* sampled from the gut of healthy chicken as well as *E. coli* from lesions associated with colibacillosis. Although *E. coli* sampled from the healthy chicken gut are reported to carry fewer VAGs, the reports are contradictory. One example is ST58 from cecal samples from turkeys, which were identified as non-pathogenic when ELA-tested, despite the abundant carriage of VAGs (Johnson et al., 2022). Thomson *et al.*, (Thomson et al., 2022) reported similar results, where ST58 from the healthy chicken gut also carried abundant VAGs, including *hlyF* and *ompT*.

The screening tool introduced by Johnson *et al.* suggests including gene-markers for potentially *high risk* APEC: ST23, ST117, ST131, ST355 and ST428 as well as serogroup O78. Our results support that ST23 (O78:H4), that is highly virulent in chicken, carries *hlyF* and *ompT*. In addition, we show that ST429 (O2/O50:H1) also is highly virulent, while ST428 which is indicated by Johnson *et al.* as a *high risk* APEC, only was identified in two of the sequenced isolates within our study. The discrepancy may be due to geographical differences or, because the study by Johnson *et al.* mostly included clinical isolates from turkey, as well as cecal samples from turkey and chicken. Interestingly, both isolates identified as ST428 from our study carried the two genes *hlyF* and *ompT* (see paper II).

APEC isolates from outbreaks of NC show larger SNP ranges than what might be expected.

To see how similar isolates with the same APEC-profile were, we performed phylogenetic (core genome) analysis of ST95 (O2/O50:H5) (n=8), all from the same flock, and ST429 (O2/O50:H1) (n=33) and ST23 (O78:H4) (n=81), both from within and across flocks (Figure 2, paper I), (Figure 2 and 3, paper II).

Although relatively few isolates were sequenced in paper I, the results highlight the potential plasticity and rapid adaptation of *E. coli* when exposed to selection pressure.

For example, ST95 was one of three STs identified within one flock (paper I). The phylogenetic analysis of APEC ST95 (O2/O50:H5) showed a mean SNP-distance of 18.35 with a genome coverage of 96.4 %, while isolates of ST429 (O2/O50:H1) (paper I) and ST23 (O78:H4) (paper II) showed, for the most part, a mean SNP distance < 10 SNPs with a genome coverage ≥ 94 % within a flock. Increased selection pressure caused by the diversity of STs within this flock may
explain the larger SNP distance in the phylogenetic analysis of ST95 (O2/O50:H5). This is further supported when comparing the two flocks identified with ST429 (O2/O50:H1) from the first study (Figure I, paper I). In this analysis, one of the two flocks showed a SNP-range of 0-11, while the other flock showed a SNP-range of 0-21. In the latter flock, two isolates diverged from the main cluster representing this flock. These two isolates were, interestingly, both isolated from a bird where two other distinct STs were identified.

In paper II, isolates from several flocks were included in the phylogenetic analysis of ST429 (O2/O50:H1) and ST23 (O78:H4). In both analyses the mean, median and SNP-range increased when isolates from several flocks were added to the analysis. Within a flock, the SNP-range varied from 0-1 to 0-33 (Paper II).

Considering the fast adaptation of individual APECs, as described above, and how the pathogen circulates over the course of several months, through thousands of birds and potentially generations, encountering countless stressors, the variations in SNP-range are to be expected. The plasticity of the *E. coli* genome will continue to adapt in response to selective pressure, and the emergence of novel clones has been considered unpredictable (Denamur et al., 2021). The selective pressure in the poultry breeding pyramid is important to consider, and the bacterium's passage through the numerous individuals and generations may stimulate the genetic adaptation of APEC. The result is, not only larger SNP distances than what might be expected in an outbreak setting, but also the potential to induce a shift towards more virulent strains adapted to the chickens environment. Another concern has been the horizontal acquisition of genetic elements facilitating host-switching. Host-switching implies the pathogenic adaptability to another host, potentially a human host. At present, the genetic relatedness between human UPEC and APEC has illustrated the zoonotic potential of APEC, but higher resolution studies are still required to confirm chicken meat as the source of foodborne urinary tract infections in humans (Mehat et al., 2021; Jørgensen et al., 2019; Liu et al., 2018; Logue et al., 2017).

The phylogenetic analysis presented in our studies show a very high genome coverage. In all the core genome phylogenetic analysis presented in these studies, the genome coverage was ≥ 94%. This shows the high resolution of today's molecular techniques, especially when comparing APEC isolates of the same ST.

The high resolution is necessary to report when describing isolates as part of the same outbreak. Less than 10 SNPs have been suggested as a threshold for relatedness of clinically relevant bacteria (Schürch et al., 2018). However, the resolution of the analysis should be included in the assessment of the results, as only describing a SNP distance > or < 10 does not illustrate the entire situation. In the present study, the phylogenetic analysis of isolates identified as ST23 (O78:H4) showed a mean and median SNP-distance of 15. Though the analysis shows results > 10 SNPs, these isolates were considered part of the same outbreak, potentially from the same source. The metadata of the outbreak supports the epidemiological link. Results from analyses where the genome coverage is high, as in this study ≥ 94 %, should only with caution be compared to studies where the genome coverage is lower. In most cases, a lower genome coverage would produce fewer SNPs, than when a larger part of the genome is aligned.

For this reason, future phylogenetic studies should provide the genome coverage of the analysis, including accompanying metadata, so that the results may be properly understood and compared.

High FWM in the previous flock and increased flock size as risk factors for local outbreaks of NC

The frequency in which certain types of APEC cause NC, made us consider these STs (APEC-profiles) as potentially more virulent and at higher risk of causing outbreaks of NC. However, unpublished data from our studies identified the same APEC-types in flocks where FWM remained low (<0.8%). This led us to believe that other factors than the presence of the pathogen may influence the high FWM caused by outbreaks of NC.

To identify non-pathogen risk factors for high FWM associated with NC, a matched case-control study was conducted where the binomial outcome was if a flock had FWM > or < 0.8%. The variables to be included in the study were from the broiler chickens' environment. The results showed that the odds for high FWM caused by NC

increased with 233 % in flocks that had experienced high FWM in the previous flock. Further, by increasing the flock with a 1000 birds, the odds of high FWM would increase with 7%. Though yet to be proven, the risk factor study indicated the potential survival of APEC in the broiler chicken house between flocks, and that there was an increased risk for horizontal spread in larger flocks.

Factors regarding the construction of the broiler chicken housing facilities is interesting in connection with the survival of APEC between flocks, as several studies have identified floor quality, type of ventilation and age of the buildings as risk factors for high FWM (Chou et al., 2004; Heier et al., 2002; Van Limbergen et al., 2020). A report on a single strain of APEC surviving and causing disease in consecutive flocks in Denmark was recently published (Bojesen et al., 2022). Interestingly, the consecutive infections stopped after renovating the drinking system and repairing crevices in the cement floor.

The inclusion of a variable covering routines for disinfection would have been interesting to include in our study and strengthened the discussion concerning reasons for survival of APEC within the broiler chicken house as well. However, as discussed in the chapter "Methodological considerations - data collection"(chapter 5.4.1), the questions or observations regarding routines for disinfection need to be clearly specified, potentially including concentration and method of use.

Since the statistical analysis in this study did not indicate *density* as a variable affecting the odds for high FWM, other factors associated with *flock size* need to be considered. The potential decrease in management capacity is one such factor. The capacity to remove dead or morbid chicks in large flocks requires more time than in smaller flocks, resulting in a potential increased risk for horizontal spread in large flocks (Christensen et al., 2021). Increased pathogen pressure may further influence the speed of horizontal spread of NC within a flock. The survival and build-up of APEC between flocks, or vertical transmission of APEC from the parent layer flock to the broiler chickens, may substantially increase the pathogen pressure.

The hatchery is another unit which has proven to be a source of horizontal spread of APEC, disseminating to the broiler chicks (Poulsen et al., 2017). The critical control points for spread within the hatchery need to be identified. Further, true vertical transmission of APEC from parent or grandparent generations needs further investigation.

Interestingly, two studies (Heier et al., 2002; Yerpes et al., 2020) identified *study year* as a risk factor for high FWM, which supports the findings from our molecular studies of larger, cross-farm outbreaks of NC occurring in intervals with months or even years apart.

Altogether, more than 90 variables were collected for the study, but only 37 variables were validated for use in the forward selection of variables. The results were, therefore, based on just a few of many variables potentially important in the prevention of NC. This means there are several factors surrounding the broiler chicken flock environment which needs further investigation.

On the other hand, the risk factor study did include important variables, such as: moisture, temperature, age of the parent flock and number of parent flocks, which resulted in two factors which indicated increased odds of high FWM. However, we cannot exclude that other factors included in the study may still be important. Nonetheless, the results from this study gave valuable information where to focus future research on APEC, and how to improve management in future outbreaks of NC.

5.5 Concluding remarks

During the PhD study, we have systematically sampled broiler chicken flocks with high FWM for APEC. Samples have been collected from multiple organs (3-5) within a bird, and three-five birds per flock. With this method, we showed that there is almost no diversity of APEC within a bird, but within a flock one-five distinct APECs may be identified. The studies showed the importance of sampling multiple birds from the same flock, to identify the disease causing APEC within a flock. Certain APECs are more often identified to cause disease alone in a flock, and we have shown how outbreaks of NC across farms may be caused by a single strain of APEC.

Ranking APEC according to the prevalence of a ST is challenging, as one ST may be identified with several serotype profiles. The studies illustrate that more than one typing method is needed to accurately present the APEC-profile responsible for disease in a flock. Further, we showed that two strains (ST429, O2/O50:H1 and ST23, O78:H4) were responsible for two individual outbreaks across farms, and were, therefore, considered to be more virulent APECs.

With the use of an extensive database for VAGs we showed what VAGs were present in APEC isolates from flocks with confirmed NC. The results confirmed previous studies, that the VAGs *hlyF and ompT* are present in almost all APECs. However, without a true control group, further ranking of APEC-virulence is limited.

The in-depth phylogenetic analysis of same STs showed what to expect in SNP distances within a flock and between flocks in outbreaks of NC. Further, the analyses implied the potential rapid genomic drift of APEC when submitted to external selective pressure. The phylogenetic analysis showed high resolution which resulted in SNP distances larger than what might be expected in an outbreak setting. Therefore, we addressed the importance of presenting both the genome coverage and SNP distance, together with flock-related metadata when evaluating the relatedness of APEC-isolates.

A study on management risk factors for high FWM caused by NC showed that the odds for high FWM caused by NC more than doubled if the previous flock had experienced high FWM. The results indicate the need for thorough cleaning and disinfection procedures in the poultry-house after flocks have suffered an outbreak of NC to

reduce the pathogenic pressure, and thereby, the odds of NC in the next flock. The results further illustrated the need for more studies on the potential survival of APEC in the poultry house between flocks. Further, the odds for high FWM caused by NC increased with 7% for every 1000 bird increase in flock size. If suspicion of NC, farmers with large flocks should ensure enough capacity to efficiently, remove and cull dead and morbid birds to avoid further horizontal spread and mortality in the flock.

5.6 Future perspectives

With every study more questions arise. There are still many questions regarding APEC that remain unanswered, such as the clear definition of an APEC, and how it differs from the commensal gut *E. coli*. However, I would like to focus future perspectives towards how we can implement procedures to better help the industry prevent larger outbreaks of NC resulting in high FWM in the broiler chicken population.

First of all, does APEC disseminate through the broiler chicken breeding pyramid to cause outbreaks of NC? Several studies indicate that this is the case, but without systematically collected samples from all levels of the broiler breeding pyramid over time, the results will remain vague. I would like to implement sampling and analysis of critical control points (HACCP) through the breeding pyramid, from the grandparent-generation, through the parent generation and hatcheries down to the fattening broiler chicks, to better establish the pathogens route of entrance to the flock. I believe this is important for early detection of outbreaks of NC, and for the implementation of preventive measures.

Early detection requires fast and cheap methods for screening of potentially highly virulent APEC. Methods for screening have been published, but there may be a need to adapt the screening methods according to geographical regions, and even hybrids. For this, the development of publicly available databases to report APEC-sequences including metadata would be beneficial.

Vaccines have proven to be effective against homologous strains of APEC. Early detection of potentially virulent strains of APEC within the broiler chicken breeding pyramid is advantageous for the production of autogenous vaccines against these strains. In the future, the production of autogenous vaccines will need to implement the increased knowledge on genomic traits of APEC, to target highly virulent APEC more specifically. Further, increased knowledge on the degree of immunity passed on through the broiler chicken breeding pyramid would be of interest. Research on other preventive measures, such as: genetic resilience of certain hybrids against NC and immune modulatory strategies against APEC are ongoing and should continue

In addition, I would like to identify why the odds for high FWM in two consecutive flocks is more than 200%. Collecting samples from consecutive flocks and barn-house environment between flocks, could potentially establish if the same strain resides within the house between flocks, and the biofilm-forming potential of these strains. Resistance towards disinfection and mechanisms for survival of APEC in the environment should be included in these studies. Furthermore, in houses with re-occurring outbreaks of NC, routines for washing and disinfection should be thoroughly examined, as should cracks and crevices in the poultry house.

Practical ways to decrease mortality for the broiler chicken producer is of interest. Since *flock size*, but not *density*, showed an increased odds for high FWM, it would be interesting to see if the speed of horizontal spread would decrease in houses where the flock was separated in smaller cells, rather than together in one large room. This could be identified through a cohort study comparing large flocks divided in cells to flocks kept in one large room.

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7 Papers I-III

Paper I

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High sequence similarity between avian pathogenic *E. coli* isolates from individual birds and within broiler chicken flocks during colibacillosis outbreaks

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ABSTRACT

Avian pathogenic *E. coli* (APEC) cause high first week mortality (FWM) in broiler chickens worldwide. In order to investigate the epidemiologic aspects of colibacillosis in broiler flocks it is important to develop reliable and cost-effective sampling guidelines. In this context, it is particularly important to define the minimum number of samples required to reliably identify the causative APEC clone during outbreaks of colibacillosis.

This study describes the diversity of *E. coli* isolates between and within three flocks with high FWM due to colibacillosis. Each flock was represented by five animals, showing typical lesions of colibacillosis, and spleen, liver and one other organ from each animal was sampled for APEC. A total of 47 *E. coli* isolates, one per organ, and approximately 15 isolates per flock were whole genome sequenced and compared by multilocus sequence typing (MLST), serotyping and phylogenetic analysis to deduce their relationship. The results revealed that within individual birds there was little or no sequence type (ST) or serotype diversity between APEC isolates from different organs. Based on phylogenetic analysis, isolates belonging to the same ST and serotype showed a low number of single nucleotide polymorphisms (SNPs) across more than 95 % of the genome. Isolates from the liver always represented the major disease-causing APEC in individual birds, even when more than one ST was detected within an individual bird and flock. This study guides us towards an economically efficient way of sampling for future epidemiological studies on colibacillosis, by determining the causative APEC-clone at flock level.

1. Introduction

Colibacillosis is considered the most common infectious bacterial disease and among the most frequently reported disease in poultry (Nolan et al., 2020). Colibacillosis is a localized or systemic infection of fowl caused entirely or partly by avian pathogenic *E. coli* (APEC). In young broiler chickens, the infection commonly manifests as a polyserositis causing high first week mortality (FWM), poor animal welfare and economical loss to the farmer.

Pathogenic *E. coli* are often grouped according to the anatomical site where the bacteria causes disease. APEC belongs to the group of extra-intestinal *E. coli* (ExPEC), together with neonatal meningitis-causing *E. coli* (NMEC) and uropathogenic *E. coli* (UPEC). As APEC shares certain traits with mammalian ExPEC it can be regarded as a possible zoonotic pathogen (Jørgensen et al., 2019; Nolan et al., 2020). ExPEC, including APEC strains, are characterized by the presence of a diverse set of virulence-associated genes involved in colonization, bacterial invasion, toxin production, serum survival and iron acquisition. Although no specific single gene or gene combination has been identified as essential for virulence, several genetic traits have been shown to contribute to APEC pathogenesis (David et al., 2008; Dziva and Stevens, 2008; Ewers et al., 2007; Johnson, 2008; Nolan et al., 2020; Rodriguez-Siek et al., 2005). Several attempts have been made to define the APEC pathotype in order to separate them from commensal avian fecal *E. coli* (AFEC). However, this has proven difficult and the definition of APEC remains elusive (Johnson, 2008; Kemmett et al., 2013; Rodriguez-Siek et al.,

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2005). The general agreement is that APEC represents a genetically diverse group of *E. coli,* which may exclusively or partly be the cause of colibacillosis in poultry (Collingwood et al., 2014; Delannoy et al., 2020; Guabiraba and Schouler, 2015).

The most common serogroups of APEC found in poultry are O1, O2, O18, O35, O36, O78 and O11 (Nolan et al., 2020). Serotyping may be performed *in silico*, often in combination with other modern molecular methods, such as phylogrouping and multilocus sequence typing (MLST) (Kauffman, F 1947, Joensen et al., 2015). Whereas phylogrouping uses a Triplex-PCR method to group *E. coli* into seven phylogroups (A, B1, B2, C, D, E and F) based on the presence or absence of three genes (*chuA*, *yjaA* and *TspE4.C2*) (Clermont et al., 2000, 2013; Gordon et al., 2008; Logue et al., 2017), MLST analysis of *E. coli* is based on the combination of seven stable, housekeeping genes encountered in the *E. coli* genome: *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *recA*, and *purA* (Wirth et al., 2006). A wide range of STs have been detected in diseased poultry; including ST117, ST23, ST429, ST354, ST350, ST973, ST10, ST57, and ST95 (Cordoni et al., 2016; Cummins et al., 2019; Papouskova et al., 2020). Newer whole genome-based analysis, such as core genome/whole genome MLST or phylogenetic analysis based on Single Nucleotide Polymorphisms (SNPs), can provide detailed information on the relatedness between isolates (Schürch et al., 2018).

Several studies have characterized and compared APEC isolates from various parts of the world (Delannoy et al., 2020; Papouskova et al., 2020) including the Nordic countries (Ronco et al., 2017). Most studies have shown a high diversity of APEC isolates from poultry, possibly reflecting the method of sampling. Detailed genome-based epidemiological studies, involving systematically collected isolates from diseased flocks and healthy control flocks, may facilitate the understanding of the genetic basis for the APEC pathotype (Rasko et al., 2008).

Little is known about the diversity of APEC strains involved in outbreaks of colibacillosis in Norway. This is due to the lack of systematic studies, the high costs of typing *E. coli* isolates, and that typing is not performed routinely when identifying *E.coli* from cases of colibacillosis. To address this shortcoming, we have an ongoing study implementing WGS of APEC isolates, together with registrations related to environment and management in diseased flocks, with the goal of revealing epidemiologic aspects of APEC in the Norwegian broiler population. The study will also provide more information on virulence factors related to the APEC pathotype.

The aim of the present pilot study was to examine the genetic diversity of *E. coli* isolates within individual birds and flocks in local outbreaks of colibacillosis in Norway. The results will be used to define the minimum number of samples required to identify the disease-causing APEC on flock level. This information will provide guidelines for an economically efficient selection of isolates for genomic and epidemiologic studies of colibacillosis without affecting the reliability of the results.

2. Materials and methods

All sampled animals were either culled for animal welfare reasons or recently found dead in the poultry house.

2.1. Study design and sampling

2.1.1. Study design

Three flocks with predicted first week mortality above 2 %, and with lesions indicative of colibacillosis were selected for this study. For comparative reasons, the flocks were all of the same hybrid (Ross 308), but from different regions of Norway (Table 1). The spleen, liver and one other random organ with lesions consistent with colibacillosis were sampled from five representative necropsied birds. Bacteriological examination was performed for the 45 samples and at least one *E. coli* isolate from each sample was selected for WGS. The samples were named according to flock (F1-3), bird (B1-5) and organ: spleen (S), liver (L) and organ with lesion (O). Morphologically different colonies of *E. coli* were selected from each sample/bird to reflect the diversity of APEC in a flock.

2.1.2. Sampling

Reports of high first week mortality (FWM), estimated to reach >2 %, elicited sampling by collaborating veterinarians. From January to May 2019, three random broiler flocks (F1-F3) were sampled; F1 and F2 were sampled in winter season and F3 in the spring. F1 and F3 originated from the same area and hatchery, while F2 was from a geographically distinct area and from a different hatchery (Table 1). Necropsy was performed on ten birds in each flock according to a defined standardized procedure. Five birds were selected for bacteriological sampling, and samples were obtained from the spleen, liver and one other organ presenting typical lesions of colibacillosis. Bacterial sampling was performed by incision of the organ with a sterile scalpel and touching the cut surface with a transport swab (Copan© gel amies with charcoal). The swabs were kept cool and sent with overnight delivery to the Norwegian Veterinary Institute's (NVI's) laboratories.

2.2. Laboratory analysis

2.2.1. Isolation of E. coli

Samples were received and analyzed at the NVI bacteriology lab according to standard procedures for diagnostics: each sample was plated onto two 5 % bovine blood agar (BA) plates (Oxoid, Basingstoke, UK) and one Heart Infusion Agar (HIA) plate with 6 % lactose saccharose solution. The samples were then incubated at 37 °C ± 1 °C for 16–24 hours; one BA plate under anaerobic conditions, the other in a 5 % CO_2 atmosphere and the HIA agar was incubated under normal atmospheric conditions. Following incubation, each sample was registered, and the colony phenotypes were described. A minimum of one colony with typical *E. coli* colony morphology was picked from each sample and re-plated onto BA and incubated in a 5 % CO_2 atmosphere at 37 °C ± 1 °C for 16–24 hours. *E. coli* cultures were confirmed using the MALDI–TOF MS (Bruker Daltonics, Germany). Confirmed *E. coli* cultures were frozen at −80 °C in a media containing 85 % glycerol and Heart Infusion Broth.

2.2.2. DNA extraction and high throughput sequencing

Isolates stored at −80 °C were plated onto BA and incubated in a 5 % CO_2 atmosphere at 37 °C ± 1 °C for 16–24 hours. One colony was inoculated into 5 ml Luria Bertani (LB broth) and incubated under agitation at 37 °C overnight. One tube with only LB broth and no

Table 1
Data related to the three flocks included in the study.

Flock	Sample year	Season	Region[a]	Hatchery	Breed	Age (days)	Major ST[b]	FWM (%)
F1	2019	winter	south west	a	Ross308	6	429	1.8
F2	2019	winter	east	b	Ross308	8	429	4.0
F3	2019	spring	south west	a	Ross308	9	95	2.6

[a] Region = Approximate location of poultry house in Norway.
[b] ST = The dominating sequence type within the flock.

inoculated sample was included in the batch as a negative control.

DNA extraction was performed using the QIAmp® DNA Mini Kit (Qiagen) according to manufacturer's protocol with minor modifications; the optional RNase A step was included and 100 µl 10 mM Tris HCl, pH 8 was used as the elution buffer in the final step. The concentration and purity of the DNA extracts was determined using Qubit dsDNA broad range kit (Thermo Fischer Inc.) and NanoDrop Nucleic Acid Quantification (ThermoFischer). DNA integrity was assessed using gel electrophoresis. Extracted DNA from 43 isolates was sent to The Norwegian Sequencing Centre (https://www.sequencing.uio.no/) for library preparation and sequencing. Four additional isolates (F1-B1-Ls4, F1-B1-O, F1-B2-L, F2-B3-L) were prepared and sequenced in house at NVI. All samples, regardless of where they were sequenced, were prepared with Nextera™ DNA Flex library preparation kit (Illumina), and sequenced on an Illumina MiSeq instrument, resulting in 300 bp paired-end reads.

2.3. In silico analysis

2.3.1. Whole genome sequence assembly and typing

Initial quality control and assembly of samples were done using the Bifrost pipeline (Lagesen, 2020). This pipeline consists of read quality control, quality and adapter trimming, removal of PhiX and assembly. ARIBA (Hunt et al., 2017) was used to determine the sequence types (ST) according to the Achtman scheme (Wirth et al., 2006). Isolates with novel sequence types were uploaded to Enterobase for ST assignment (Zhou et al., 2020). Serotypes were identified using SerotypeFinder (Joensen et al., 2015). Raw data from whole genome sequencing have been uploaded to the ENA database under BioProject accession number PRJEB43441, associated with the single isolates in Table 3.

2.3.2. Phylogenetic analysis

STs found in more than four isolates were subjected to phylogenetic analysis for each ST. First, ParSNP (Treangen et al., 2014) version 1.2 was used to align the pilon-corrected assemblies and to identify the core genome single SNPs. Harvesttools (Treangen et al., 2014) version 1.2 was then used for file conversion, before removing duplicated entries in the alignment with seqkit (Wei Shen and Yan, 2016) version 0.2.0. Then, Gubbins (Croucher et al., 2015) version 2.3.2 was used to identify recombinant areas in the deduplicated alignment, using RaxML, as the treebuilder, and the GTRGAMMA model. Maskrc (https://github.com/kwongj/maskrc-svg) version 0.5 was used to mask the identified recombinant areas from the alignment. The resulting masked alignment was used as an input to IQTree (Nguyen et al., 2015) version 1.6.8 to generate a maximum likelihood tree. Ultrafast Bootstrap approximation (Hoang et al., 2017), integrated in IQTree, was used to determine branch supports, using 1000 bootstrap replicates. The optimal evolutionary model was identified by using model finder plus (Kalyaanamoorthy et al., 2017). The resulting trees were visualized in R (https://www.R-project.org/) version 4.0.2, using the ggtree (Yu et al., 2017) package. Lastly, snp-dists (https://github.com/tseemann/snp-dists) version 0.6.3 was used to determine the number of SNP differences between isolates within each tree.

3. Results

3.1. Pathological findings

Flocks F1, F2 and F3 were sampled on day 6, 8 and 9 after hatching, respectively, and pathological lesions were registered by the field veterinarian. Moist body cavities and edema in the umbilical region was reported from all flocks (F1-F3). Additional pathological findings such as fibrin within the body cavity and an enlarged spleen were found in animals from F2 and F3. A summary of the pathological findings during necropsy are found in Table 2.

Table 3
Overview of the morphotype on HIA, ST and serotype of each isolate.

ID[a]	Morphology on HIA[b]	ST	Serotype	Sample accession
F1-B1-S	nn	40	O109:H21	ERS5886196
F1-B1-L	nn	429	O2/O50:H1	ERS5886197
F1-B1-Ls4	nn	429	O2/O50:H1	ERS5886207
F1-B1-Os1	nn	429	O2/O50:H1	ERS5886208
F1-B1-Os4	nn	6665	O8:H30	ERS5886198
F1-B2-S	nn	429	O2/O50:H1	ERS5886199
F1-B2-L	nn	429	O2/O50:H1	ERS5886210
F1-B2-O	nn	429	O2/O50:H1	ERS5886201
F1-B3-S	nn	429	O2/O50:H1	ERS5886202
F1-B3-L	nn	429	O2/O50:H1	ERS5886203
F1-B3-O	nn	429	O2/O50:H1	ERS5886205
F1-B4-S	nn	429	O2/O50:H1	ERS5886206
F1-B4-L	nn	429	O2/O50:H1	ERS5886209
F1-B4-O	nn	429	O2/O50:H1	ERS5886211
F1-B5-S	nn	429	O2/O50:H1	ERS5886212
F1-B5-L	nn	429	O2/O50:H1	ERS5886214
F1-B5-O	nn	429	O2/O50:H1	ERS5886215
F2-B1-S	o	429	O2/O50:H1	ERS5886193
F2-B1-L	o	429	O2/O50:H1	ERS5886204
F2-B1-O	y	429	O2/O50:H1	ERS5886219
F2-B2-S	y	429	O2/O50:H1	ERS5886230
F2-B2-L	o	429	O2/O50:H1	ERS5886233
F2-B2-O	o	429	O2/O50:H1	ERS5886234
F2-B3-S	o	429	O2/O50:H1	ERS5886191
F2-B3-L	y	429	O2/O50:H1	ERS5886213
F2-B3-O	y	429	O2/O50:H1	ERS5886235
F2-B4-S	y	2485	O15:H45	ERS5886236
F2-B4-L	y	429	O2/O50:H1	ERS5886237
F2-B4-O	o	429	O2/O50:H1	ERS5886192
F2-B5-S	o	429	O2/O50:H1	ERS5886200
F2-B5-L	y	429	O2/O50:H1	ERS5886194
F2-B5-O	y	429	O2/O50:H1	ERS5886195
F3-B1-S	y	95	O2/O50:H5	ERS5886216
F3-B1-L	y	95	O2/O50:H5	ERS5886217
F3-B1-O	yo	95	O2/O50:H5	ERS5886218
F3-B2-S	y	95	O2/O50:H5	ERS5886220
F3-B2-L	yo	95	O2/O50:H5	ERS5886221
F3-B2-O	y	95	O2/O50:H5	ERS5886222
F3-B3-S	y	10836	O41:H45	ERS5886223
F3-B3-Ss2	NLF	10836	O41:H45	ERS5886232
F3-B3-L	y	10836	O41:H45	ERS5886224
F3-B3-O	y	10836	O41:H45	ERS5886225
F3-B4-S	y	457	O11:H25	ERS5886226
F3-B4-L	y	457	O11:H25	ERS5886227
F3-B5-S	yo	95	O2/O50:H5	ERS5886228
F3-B5-L	yo	95	O2/O50:H5	ERS5886229
F3-B5-0	yo	457	O11:H25	ERS5886231

[a] Describing the ID given to each *E. coli* isolates; F1-3 is flock 1–3, B1-5 is bird 1–5, the last is describing which organ the isolate originates from; L = liver, S = spleen, O = other organ. Where more than one *E. coli* has been sequenced from the same organ, the isolates are differentiated by adding s1-4.

[b] Morphotype on HIA: y = yellow, o = orange, yo = mixed yellow and orange, NLF = non lactose fermenting, nn = not noted.

3.2. Colony morphology

Typical *E. coli* colonies appeared on BA plates as medium-large, smooth and greyish white colonies with an underlying, weak greenish discoloration. On HIA, *E. coli* colonies were typically medium-large, smooth and light yellow. Some colonies were described as mucoid while others, as in the case of F2, had an orange discoloration on HIA. Cultures from flock F2 revealed two morphologically distinct colonies on HIA: one morphotype appeared medium-sized, smooth and light yellow, the other was smaller with rough edges and had a deeper orange colour. One isolate (F3-B3-S) from F3 was defined as non-lactose fermenting as it showed a deep blue colour, with no yellow colorization, during growth on HIA. All morphotypes were selected for WGS, and no consistency between colony morphology and ST was observed.

Table 2
Pathological findings and selected data from each individual bird included in the study.

Animal ID[a]	Body Cavity[b]	Heart sack	Gizzard	Liver	Spleen	Umbilicus	Femur Head	Culture[c]	Samples/Bird[d]	ST / Bird[e]
F1B1	f, m	f	–	s	a	b,m,o		mixed	5	3
F1B2	f,m	f	–	s	s	b,m.o	n	pure	3	1
F1B3	m	–	–	s	a	b,m,o	–	rel.pure	3	1
F1B4	m	–	–	s	a	b,m,o	n	pure	3	1
F1B5	f,m	f	–	f,s	s	b,m,o	–	pure	3	1
F2B1	f,m	f	–	f,s,e	e,s	b,m,o	n	pure	3	1
F2B2	f,m	f	–	f,s	s	m	–	pure	3	1
F2B3	f,m	f	–	f,s,e	e,s	b,m,o	n	pure	3	1
F2B4	f,m	f	–	f	s	m	n	pure	3	2
F2B5	f,m	f	–	f	s	m,o	–	pure	3	1
F3B1	f,m	f	u	s	e,s	m	–	pure	3	1
F3B2	f,m	–	u	s	e,s	m	–	pure	3	1
F3B3	f,m	f	u	f,s	e,s	m	–	mixed	4	1
F3B4	f,m	–	u	s	e,s	m,o	n	rel.pure	2	1
F3B5	f,m	f	u	f,s	e,s	m	n	rel.pure	3	2

[a] Each flock is marked as F1–3, and each bird is marked with B1-5.
[b] Pathological findings are denoted according to where the lesion is found, and the lesion is denoted with a letter: f = fibrin, m = moist, e = engorged, s = swollen, u = ulcer, o = open, b = bloody, n = necrosis, a = atrophy.
[c] Culture = Samples plated onto two BA and one HIA plate: pure = pure growth of *E. coli* on all three agars, rel.pure = relatively pure describing a dominant growth of *E. coli* on all three agar, mixed = growth of *E. coli* amongst growth of at least two other types of bacteria.
[d] Samples/bird describes the number of sequenced *E. coli* from the individual bird.
[e] ST/bird = number of *E. coli* sequence types identified within the individual bird.

3.3. In silico analysis

3.3.1. MLST and serotyping

At least three organs were sampled from each individual bird (except F3-B4) and at least one bacterial isolate per organ was whole genome sequenced. In 12 out of 15 individual birds, identical STs were identified in all three organs.

ST429 was identified as the disease-causing ST in flock F1 and F2 with 88 and 93 % representation (15 of 17 isolates and 14 of 15 isolates), respectively (Table 3).

Three major STs were identified in flock three: ST95, ST10836 and ST457. ST95 was the dominating sequence type with 53.3 % representation (8 of 15 isolates).

Of the 47 isolates, three were identified as single STs. These diverging ST's were either sampled from the spleen (S) or the "other" (O) organ, and never the liver (L). In all cases, the isolate sampled from the liver was equivalent to the majority of STs identified in individual birds. (Table 3).

Serotypes were in agreement with the identified ST's: all isolates with ST429 belonged to serotype O2/O50:H1, and all isolates with ST95, ST457 and ST10836 belonged to serotype O2/O50:H5, O11:H25 and O41:H45, respectively (Table 3).

3.3.2. Phylogenetic analysis

A total of 29 isolates of ST429 and eight isolates of ST95 were subjected to phylogenetic analysis. For ST429, the phylogenetic analysis revealed two distinct clades that were separated according to flock (Fig. 1). Clade A comprised isolates from flock one and had a mean SNP distance of 6.2, a median SNP distance of 4 and a SNP-range of 0-21. Clade B comprised isolates from flock two and had a mean SNP distance of 4.45, a median SNP distance of 4 and a SNP-range of 0-11. The mean SNP distance across both clades was 35.97 with a median of 61 and a SNP-range of 0-70. The average genome coverage of the ST429 core genome alignment was 97.5 % (supplementary Table 1).

To better understand the phylogenetic relationship and relatedness between *E. coli* isolates of ST429 in future studies, the phylogenetic analysis of ST429 was repeated with an *E. coli* isolate of ST429 from a different study as reference genome (BioSample: SAMN11444807, Sample name: SurreyAPEC012). The isolate originated from a chicken and was sampled in 2017 in a country outside of Scandinavia. The resulting phylogenetic tree is given in Supplementary Fig. S1. In this case, the average genome coverage of all included ST429 was reduced to

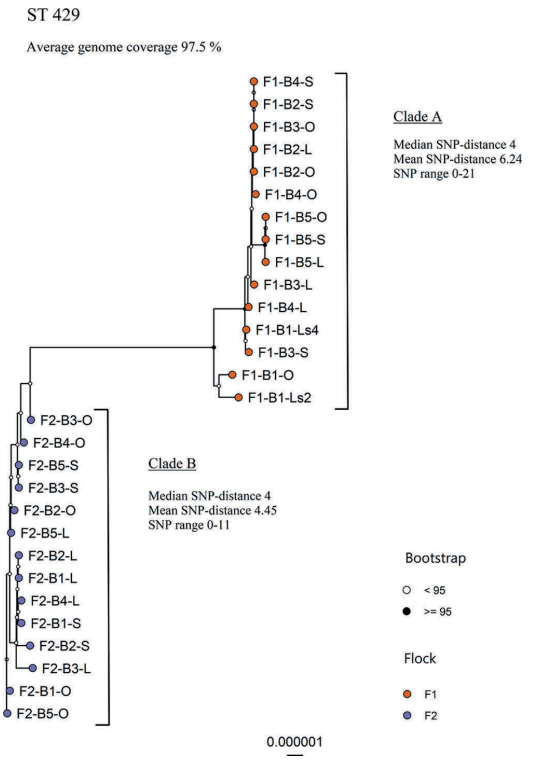

Fig. 1. Maximum likelihood phylogenetic tree of ST429 from flock one and two. Black dots on nodes represent supported bootstrap values. Tips are colored based on the flock of origin. Two clear clades are visible: A and B representing each flock, Clade A = F1 and Clade B = F2. Evolutionary model: HKY + F+I.

85.3 %, the mean SNP distance was 36.48, the median SNP distance was 51 and the SNP-range was 0–147 (Supplementary Table 1).

Eight of 15 isolates in flock three were identified as ST95. The phylogenetic analysis of ST95 revealed a larger diversity within this sequence type, and in this case, smaller clades represented each individual bird (Fig. 2). Here, the core genome alignment had an average genome coverage of 96.7 %. The mean SNP distance was 18.2, the median SNP distance was 20 and the SNP-range was 6-29.

4. Discussion

To the best of our knowledge, this is the first study that systematically samples and performs whole genome sequence-based analyses of several APEC isolates from diseased individual birds and flocks. A few longitudinal studies have recently been published (Delannoy et al., 2020; Kemmett et al., 2013; Oikarainen et al., 2019), but none of them have systematically compared WGS results from a large number of isolates on individual- and flock level.

The identification of STs within an individual bird and flock provides valuable information concerning the disease-causing APEC strains on individual- and flock level.

Three selected chicken flocks, altogether 47 isolates, were analyzed by WGS, and MLST and serotyping were performed *in silico*. The results revealed nearly no sequence diversity between APEC isolates from individual birds. Any diverging isolate was in all cases either sampled from the spleen or the *other* organ such as the pericardium, bone marrow or body cavity, and their presence could be due to contamination during sampling or it could be a secondary, opportunistic *E. coli* gaining systemic access in an already diseased individual. A recent study suggests that colibacillosis could be caused by either a single, highly virulent strain, a combination of less virulent strains acting synergistically, or due to poor environmental conditions (Delannoy et al., 2020). Although based on only three flocks, our results support this hypothesis as two flocks were infected with a single strain while the third was infected with three *E. coli* of different STs. However, larger systematic studies are needed to confirm this.

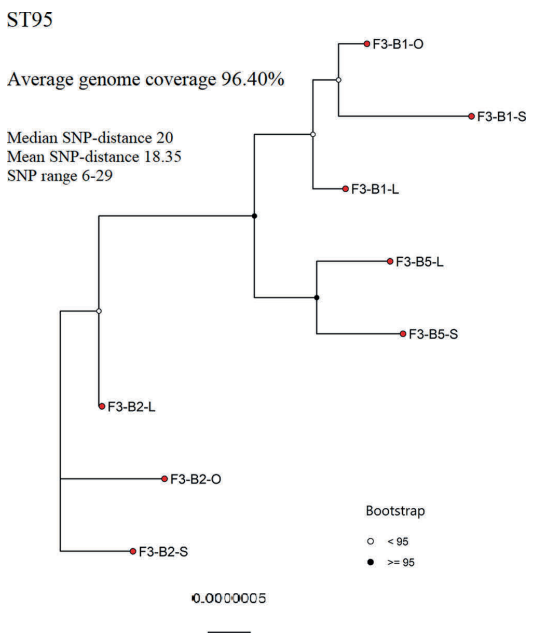

Fig. 2. Maximum likelihood phylogenetic tree of ST95 from flock three. Black dots on nodes represent supported bootstrap values. Evolutionary model: HKY + F+I.

Recent studies have identified *E. coli* ST429 as APEC (Cummins et al., 2019; Oikarainen et al., 2019; Papouskova et al., 2020). ST429 was also the dominant ST in two of the flocks we investigated. Also previously associated with APEC is ST95, which represented more than half of the isolates in the third flock. Notably, ST95 has also been found among human uropathogenic *E. coli* (Cordoni et al., 2016; Cummins et al., 2019; Jørgensen et al., 2019; Ronco et al., 2017).

Both ST429 and ST95 were further analyzed to deduce their phylogenetic relationship. ST429 showed a high sequence similarity among isolates from the same flock while ST95 isolates from a single flock were more diverse. *E. coli* ST95 has also shown high sequence diversity in other studies (Cummins et al., 2019; Jørgensen et al., 2019), however, these studies were focused on ST95 isolates from different sources and time. Whether the higher SNP range in ST95 relates to a more plastic genome compared to ST429, a higher selection pressure as a result of several ST's within the same flock, or a bias due to the low number of isolates needs further investigation. Selection pressure may be the cause of the differences seen in ST429 clade A as one bird had two isolates that diverged from the rest of the clade (Fig. 1). This bird was the only sampled bird in the flock of which *E. coli* of different sequence types were isolated. These two findings might indicate rapid adaptation of *E. coli* under direct selection pressure during co-infection with other STs.

In this study, where several isolates were collected from individual diseased birds and from the same poultry house, one would expect the disease-causing APEC ST to be clonal. Molecular epidemiology based on WGS and phylogenetic analysis, as performed in this study, provides a very high-resolution comparison of isolates. Most criteria for relatedness are based on older, low-resolution methods and it has been suggested that they need to be adjusted to better comply with newer molecular techniques and the genetic stability of individual bacterial species. For *E. coli,* $\leq$10 SNPs has been suggested as a threshold for relatedness (Schürch et al., 2018). In our study, we assume a clonal relationship between isolates within flocks F1 and F2. However, as the SNP distance between the two flocks were about 36 SNPs, no clonal relatedness of isolates between the two flocks could be stated according to above mentioned criteria. Similarly, ST95 isolates from different birds in flock three, could not be considered clonal. The sequence variation seen in ST95 could either indicate that this ST is more ubiquitous in the poultry house, or that the threshold for relatedness within *E. coli* STs varies from the set criteria. This indicates that more studies are needed to evaluate thresholds for stating relationships between isolates, if fixed thresholds are possible at all.

For reasons of interest, comparison and advances in establishing similarity benchmarks, the phylogenetic analysis of ST429 was repeated using an isolate from a different study, but of identical ST, as a reference genome. In this case, the core genome alignment only covered 85.3 % of the genomes on average, while the mean SNP distances were similar (see Supplementary Table 1 and Supplementary Fig. S1). This highlights the importance of careful interpretation of genomic data, and supports the idea that thresholds for relatedness should rather function as guidelines than rules (Schürch et al., 2018). As genomic coverage in phylogenetic analysis varies based on the method and dataset used, the authors encourage the inclusion of genome coverage when describing phylogeny of APEC isolates to allow for better comparison between studies.

Although the low number of isolates included can cause some biased results, the present study provides important guidance for economical sampling for future systematic, epidemiologic and genetic studies on colibacillosis without altering the reliability of the results.

5. Conclusion

Future comparative genomic studies are important to understand the epidemiology and virulence of APEC. Based on this study, where always

one of five birds contained one or more distinct *E. coli* isolates, the authors recommend sampling a minimum three birds from the same flock, to identify the major disease-causing APEC clone at flock level. The results also indicate that *one* sample per bird can represent the diversity of *E. coli* types within a flock, as long as several birds from the same flock are sampled. We recommend sampling from the liver as isolates from liver always represented the major disease-causing APEC in individual birds. When performing necropsy under field conditions, the liver seems to reflect the systemic infection, and the possibility for cross-contamination during sampling is less likely compared to the spleen, due to the organ size. Finally, for successful comparison of isolates, uniform and precise sampling is imperative.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetmic.2022.109378.

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Paper II

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Veterinary Research

RESEARCH ARTICLE

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A molecular epidemiological study on *Escherichia coli* in young chicks with colibacillosis identified two possible outbreaks across farms

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Abstract

Avian pathogenic *Escherichia coli* (APEC) is the cause of colibacillosis outbreaks in young poultry chicks, resulting in acute to peracute death. The high morbidity and mortality caused by colibacillosis results in poor animal welfare, reduced sustainability and economical loss worldwide. To advance the understanding of the molecular epidemiology, genomic relatedness and virulence traits of APEC, we performed systematic sampling from 45 confirmed colibacillosis broiler flocks with high first week mortality (FWM) during 2018–2021. From these flocks, 219 APEC isolates were whole genome sequenced (WGS) and bioinformatic analyses were performed. The bioinformatic analyses included sequence typing (ST), serotyping, detection of virulence-associated genes (VAGs) and phylogenetic analysis. Our results showed a high prevalence of ST23, ST429 and ST95 among APEC isolates from Norwegian broiler flocks, and identified ST23, ST429, ST117 and ST371 to cause disease more often alone, compared to ST95, ST69 and ST10. Phylogenetic analyses, together with associated metadata, identified two distinct outbreaks of colibacillosis across farms caused by ST429 and ST23 and gave insight into expected SNP distances within and between flocks identified with the same ST. Further, our results highlighted the need for combining two typing methods, such as serotyping and sequence typing, to better discriminate strains of APEC. Ultimately, systematic sampling of APEC from multiple birds in a flock, together with WGS as a diagnostic tool is important to identify the disease-causing APEC within a flock and to detect outbreaks of colibacillosis across farms.

Keywords Avian pathogenic *Escherichia coli* (APEC), colibacillosis, poultry, whole genome sequencing, virulence-associated genes (VAGs), outbreak, systematic sampling

Introduction

Colibacillosis is considered a disease of high importance in poultry production as it gives rise to poor animal welfare and high economic losses worldwide. It is caused by avian pathogenic *Escherichia coli* (APEC) which is classified as an extra-intestinal pathogenic *E. coli* (ExPEC), together with neonatal meningitis-causing *E. coli* (NMEC) and uropathogenic *E. coli* (UPEC). Colibacillosis is suspected when the mortality among young chicks in a flock increases rapidly during the first week

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after hatching, usually peaking between two to 5 days after hatching. At necropsy, the chicks show typical macroscopic lesions associated with acute to per acute polyserositis, such as an enlarged spleen, edematous serous membranes and umbilicus. At a later stage of the disease, colisepticemia may lead to fibrinous polyserositis with fibrin exudates covering the serosa of the inner organs like the liver (perihepatitis) and heart (pericarditis). Isolation and identification of *E. coli* confirms the diagnosis [1].

Extra-intestinal pathogenic *E. coli*, including APEC, is considered a diverse group of pathogens, and several studies have attempted to group and identify APEC according to virulence-associated properties [2–4]. Historically, *E. coli* has been serogrouped by detecting somatic O–antigens with antisera. The most common APEC serogroups are O1, O2, and O78 [1, 5]. Later on, phylogrouping, a triplex PCR method to group *E. coli* based on the presence or absence of three genes divides *E. coli* into seven phylogroups: A, B1, B2, C, D, E and F, have been used [6]. MLST is another common method for typing *E. coli,* including APEC, and it is based on a combination of seven housekeeping genes in the *E. coli* genome [7]. Some of the most common APEC STs identified in Europe include ST23, ST69, ST95, ST117, ST131, ST140 and ST428/429 [5].

Whole genome sequencing (WGS), on the other hand, enables an array of in silico characterization methods, such as MLST, serotyping, characterization of VAGs and phylogenetic analysis [5, 8]. Core gene analysis and phylogenetic analysis, detect variations at nucleotide level and may be used to study relatedness, the latter being useful to detect and confirm possible outbreaks and their origin. Phylogenetic methods, however, are continuously developing and the outcomes of such molecular analyses require in-depth genomic understanding, essential bioinformatic knowledge as well as understanding of the pathogens to be studied [9, 10].

Between 2014 and 2016 the Nordic countries, including Norway, experienced a sudden increase in flocks diagnosed with colibacillosis [11]. Whole genome sequencing and genomic investigation of isolates from the affected flocks revealed a predominant lineage of ST117 O78:H4, but also a genetically diverse population. The experience highlighted the need for more knowledge of the molecular epidemiology of APEC in the Norwegian broiler production. In 2018, a systematic sampling of broiler flocks with high first week mortality (FWM) was therefore initiated.

The aim of this study was to systematically collect APEC isolates from Norwegian broiler flocks with high FWM and use WGS and bioinformatic analysis for in depth characterization and comparison of isolates from the same flock and between flocks. Flock-related metadata such as sampling date, hatchery, parent-flocks and hybrid were collected at each sampling occasion, enabling detection of potential association between APEC types identified. Finally, the relationship between the identified STs and their serotype- and VAG- profiles were studied.

Materials and methods

Study design: Necropsy and sampling

Flocks with predicted FWM above 2% elicited sampling from a poultry veterinarian. The flocks were from different regions of Norway and of different hybrids, though the predominant hybrid in Norway is at present Ross 308 (Table 1). Necropsies on ten birds/flock, recently died or euthanized for animal welfare reasons, were performed by a poultry veterinarian. Macroscopic lesions were noted as present/absent in a predefined submission form. Five of the ten examined birds with the most typical macroscopic lesions associated with colibacillosis were selected for bacteriological examination from the spleen, liver and one other organ as previously described in Kravik et al. [12].

Bacteriological examination

Each sample was streaked onto two blood agar (BA) plates and one heart infusion agar (HIA) and incubated at 37 °C anaerobically, in a CO_2 chamber and under normal atmospheric pressure, respectively, according to standard procedures for bacterial diagnostics as described in Kravik et al. [12]. After 18–24 h of incubation all samples were examined for the presence of *E. coli* and the colony morphology on the three agar plates were described. Bacterial growth was divided into sparse, medium, or rich, and the level of purity of *E. coli* was graded from 1 to 4: Pure growth (1), almost pure growth (2), dominating growth of *E. coli* (3) and mixed culture (4). A grade 2 was given if a few colonies of *Enterococcus* spp. or *Proteus* spp. were present on the agar, together with an otherwise pure culture of *E. coli*. Grade 3 was defined by dominating growth of *E. coli*, but in combination with sparse to medium growth of either *Enterococci* spp., *Proteus* spp. or less growth of a different bacterium. A mixed culture, grade 4, was defined based on growth of a minimum of three different bacteria, where *E. coli* was not the dominating bacterium on the three agar plates (Additional file 1). During bacteriological examination, at least one confirmed *E. coli* isolate from each organ was frozen and stored for future analysis.

Flock diagnosis

An individual diagnosis of colibacillosis was given based on the presence of pathological lesions typically

Table 1 Overview of the flocks diagnosed with colibacillosis, with related metadata and sequence types for each flock.

Flock ID	Sampling date	Hybrid	Georgraphical location[a]	Sampled birds/ flock (n)	ST (n)[b]	ST[c]	FWM (%)[d]
1	05.09.2018	Rowan ranger	South-east	3	2	1112, 155	5.06
3	07.09.2018	Ross 308	South-west	5	2	429, 117	2.29
4	29.10.2018	Ross 308	East	5	3	95, 457, 101	4.50
5	09.11.2018	Ross 308	North-east	5	1	135	2.84
6	13.12.2018	Ross 308	East	5	1	429	3.23
7	17.12.2018	Ross 308	East	5	1	429	4.82
9	03.01.2019	Ross 308	South-west	5	1	429	1.82
10	07.01.2019	Ross 308	East	5	1	429	4.07
11	14.01.2019	Ross 308	North-east	4	1	429	3.33
12	17.01.2019	Ross 308	South-west	4	2	95, 457	4.79
16	14.03.2019	Hubbard	Mid	5	2	93, 95	1.64
22	13.05.2019	Hubbard	Mid	5	3	101, 95, 69	13.7
25	31.05.2019	Ross 308	South-west	5	3	95, 10,836, 457	2.60
27	25.06.2019	Hubbard	Mid	5	2	95, 93	2.36
29	12.08.2019	Sasso	East	5	1	429	3.40
30	21.08.2019	Ross 308	South-west	5	1	371	2.06
32	30.08.2019	Hubbard	Mid	5	2	95, 101	3.07
37	17.02.2020	Ross 308	East	5	4	1611, 1170, 2491, 2040	6.01
38	03.03.2020	Hubbard	Mid	5	1	95	2.06
39	06.03.2020	Ross 308	Mid	5	5	2040, 69, 2753, 95, 10	5.43
42	14.07.2020	Ross 308	South-west	5	1	117	2.98
44	20.08.2020	Ross 308	South-west	5	2	371, 1656	2.31
47	18.09.2020	Ross 308	East	5	4	1684, 69, 10, 5340	6.85
48	21.09.2020	Ross 308	East	5	5	2690, 349, 88, 154, 10	2.92
51	02.10.2020	Hubbard	Mid	5	1	117	12.6
52	15.10.2020	Ross 308	South-west	5	4	191, 1640, 1841, 3006	5.22
53	22.10.2020	Ross 308	South-west	5	4	95, 10, 428, 1146	2.57
54	13.11.2020	Ross 308	East	5	3	1146, 1841, 69	2.25
56	17.03.2021	Ross 308	East	5	1	23	4.70
57	17.03.2021	Ross 308	East	5	1	23	7.74
58	23.03.2021	Ross 308	South-west	5	2	23, 69	2.14
59	23.03.2021	Ross 308	South-west	5	1	23	11.51
62	08.04.2021	Ross 308	South-west	5	1	23	2.00
63	08.04.2021	Ross 308	South-west	5	1	23	3.59
65	13.04.2021	Ross 308	South-west	5	1	23	3.34
69	14.04.2021	Ross 308	South-west	5	1	23	2.72
70	14.04.2021	Ross 308	South-west	5	1	23	2.12
73	19.04.2021	Ross 308	North-east	5	1	23	7.73
74	20.04.2021	Ross 308	East	5	1	23	2.04
78	21.04.2021	Ross 308	East	5	1	23	5.09
80	22.04.2021	Ross 308	North-east	3	2	23	3.80
90	30.04.2021	Ross 308	South-west	5	1	23	3.39
101	06.05.2021	Ross 308	East	5	1	23	4.74
103	11.05.2021	Ross 308	East	5	1	23	4.35
104	14.05.2021	Ross 308	South-west	5	1	23	1.53

[a] The geographical location in Norway for broiler production is divided into five regions; South-west, South-east, East, North-east and Mid Norway.

[b] Number of distinct sequence types (STs) identified in the samples from the representative flock.

[c] Sequence types (STs) present within the flock.

[d] Percent first week mortality (FWM) includes all birds euthanized and succumbed within a flock 7 days post hatching.

associated with colibacillosis septicemia in combination with a bacteriological examination graded 1–3. We defined a flock diagnosis if FWM was higher than 1.5% and at least three out of the five sampled birds from the flock were diagnosed with colibacillosis individually.

Whole genome sequencing

From each flock with a confirmed colibacillosis diagnosis, 3–5 birds per flock were sampled and one isolate from each sampled bird was selected for WGS. Each isolate was preferably isolated from the liver and DNA was extracted as described previously [12]. Genomic DNA samples from 204 isolates were prepared and sequenced at the Norwegian Veterinary Institute (NVI). An additional 15 isolates were included, previously described in Kravik et al. [12]. All 219 isolates were subjected to library preparation: Nextera™ DNA Flex library preparation (Illumina), and sequenced on an Illumina MiSeq instrument, resulting in 300 bp paired-end reads. The sequence data analyzed in this study are found publically available in the ENA database with bioprojects PRJEB43441 and PRJEB55163. See Additional file 1 for individual accession numbers.

In silico analysis

Whole genome sequence assembly and typing

The Bifrost pipeline [13] was used for initial quality control and assembly. This pipeline consists of read quality control, trimming, removal of PhiX and assembly. ARIBA [14] version 2.14.6 was used to determine the sequence types (ST) according to the Achtman scheme [7]. Isolates with novel sequence types were uploaded in Enterobase for ST assignment [15]. Serotypes were identified using SerotypeFinder [16] version 2.0.2.

Virulence-associated genes

Analysis for detection of VAGs was performed using VirulenceFinder version 2.0.4. The VirulenceFinder database was extended by adding known APEC-associated genes found in the Virulence Factors of Pathogenic Bacteria database (Additional file 2), [2, 17–19]. The complete database consisted of 629 entries of virulence-associated genes and their variants.

Core gene analysis and phylogenetic analysis

All isolates that passed QC parameters were included in a phylogenetic analysis based on the core genes. The ALPPACA pipeline [20] version 1.0.0 was used to run genome annotation with Prokka [21] version 1.14.6, followed by pangenome analysis with Panaroo [22] version 1.2.9 to detect and align the core genes among the 219 genomes. Constant sites were removed from the alignment by using Snp-sites [23] version 2.5.1. Snp-dists [24] and version 0.8.2 was used to calculate the pairwise SNP distances from the alignment. Lastly, IQTree [25] version 2.1.4 was used to generate the phylogenetic tree, using Ultrafast bootstrapping [26] with 1000 replicates, and model finder plus [27] for model selection.

Within two of the most frequent STs (ST23 and ST429) separate phylogenetic analyses with ALPPACA were performed. ParSNP [28] version 1.6.1 was used to generate a core genome alignment, followed by detection of recombinant regions with Gubbins version 3.2.0 using RaxML as the treebuilder and the GTRGAMMA model. Maskrc-svg [29] version 0.5 was subsequently used to mask recombinant areas from the alignment. Constant sites were removed by using Snp-sites, followed by pairwise SNP distance calculation with snp-dists and phylogenetic inference with IQTree, similar to above. All phylogenetic trees were visualized in R [30] version 4.0.2, using the ggtree package version 3.0.4 [31].

Results

Sampling and colibacillosis confirmation

From September 2018 to June 2021, 45 broiler and four broiler breeder rearing flocks were sampled, resulting in a total of 49 flocks with FWM ranging from 1.53% to 12.6%. The flocks were of different hybrids, all less than 14 days of age, from various regions in Norway and originated from three different hatcheries. Four broiler flocks were not diagnosed with colibacillosis, and thereby excluded from further analysis. Altogether, 45 flocks with high FWM were given a colibacillosis diagnosis and further included in the analysis (Table 1).

Whole genome sequencing and in silico analysis

Quality control

Altogether 219 confirmed *E. coli* isolates were sequenced. One *E. coli* was selected from each bird, and 3–5 birds were sampled from 45 flocks (Additional file 1). Initial quality control of the genome sequences, based on multiQC and Quast report, showed that the GC content of the isolates were between 50.51 and 50.57%, the number of contigs were 43–74 and the total length of the complete genome after assembly was 4.86–4.96 Mbp (See individual quality scores for all isolates in Additional file 1).

MLST and serotyping

The 219 APEC isolates from 45 flocks were sequenced and characterized by ST and in silico serotype. Twenty-six of these flocks exhibited the same ST in all isolates from within a flock, while 35 flocks were identified with up to two STs and therefore identical ST in a minimum of three birds from within the same flock (Table 1 and

Additional file 1). Ten of the 45 flocks were identified having three or more STs within the flock (Table 1).

Altogether 32 different STs were identified, of these, 15 were only identified once. The most common STs identified in this study were ST23, ST429, ST95, ST117, ST371, ST69 and ST101 in descending order (Table 2).

In silico serotyping revealed a total of 38 distinct serotypes. Some serotype profiles were detected within a single ST, whereas other serotype profiles were detected across several STs. Of the ten most common STs, five STs were identified with several serotype profiles. The most common serotypes were O1:H7, O2/O50:H1, O2/O50:H5, O45:H19 and O78:H4 (Table 2).

Phylogenetic analysis

To investigate the relationship across STs and between STs and serotypes, a core gene analysis of all 219 isolates was performed (Figure 1). Among the 219 isolates, the pangenome analysis detected 14,332 unique genes. Out of these, 3303 were defined as core genes as they were present in at least 95% of the genomes. Model finder plus identified GTR + F + I + G4 as the best-fitting model. The phylogenetic tree revealed that the isolates clustered according to their STs and it showed that isolates with the same serotype profile might be genetically distant. Isolates of serotype O78:H4 were identified as both ST23 and ST117, and serotype O2/O50:H1 was identified as both ST429 and ST135. Further, ST95 and ST117 also contained several serotype profiles: O1:H7 and O2/O50:H5 and O24:H4, O78:H4 and O161:H4, respectively.

The two largest clusters in the core gene tree were represented by ST23 and ST429, respectively. Both STs had one distinct serotype profile and all flocks, except one flock with ST429, clustered according to hybrid, sampling dates and ST (Figure 1, Table 1). These two STs were therefore suspected to represent two outbreaks of colibacilllosis and separate phylogenetic analyses were therefore carried out for each of them (Figures. 2 and 3).

A total of 81 isolates from 17 flocks were confirmed to be of ST23. All ST23 isolates were collected from broiler flocks of the hybrid Ross 308 and sampled from March 2021 until the end of May 2021 (Table 1). The phylogenetic analysis of ST23 had an average genome coverage of 94.0%, a SNP range of 0–33 and a mean and median SNP distance of 15. Individual isolates of ST23 clustered, for the most part, according to individual flocks, but there were also isolates which clustered with isolates from different flocks (Figure 2 and Table 3). Within a flock, all but two flocks had mean SNP distances below 10. The SNP range varied from 0 to 1 (smallest range) to 0–33 (highest range) within a flock (Table 3).

ST429 was identified in 33 isolates from seven flocks; six flocks of the hybrid Ross 308 sampled between September 2018 to January 2019, and one flock of the hybrid Sasso sampled in August 2019. The phylogenetic analysis revealed an average genome coverage of 95.8% and the SNP range was 0–172 with a median SNP distance of 62. The tree diagram shows that ST429 isolates separate into three dominating clades: ST429-A, ST429-B and ST429-C (Figure 3). Isolates from the only Sasso flock clustered together in clade ST429-B. Isolates in clade ST429-A and ST429-C originated from Ross 308 flocks from different geographical locations, hatcheries and parent flocks, however, all Ross 308 broiler rearing flocks are distributed from one main hatchery (Figure 3). Within the individual flocks identified with ST429, the mean SNP distances were in all cases < 10 SNPs, except from the Sasso flock, and the SNP range varied from 0 to 1 to 4–21 (Table 3).

The phylogenetic analysis of ST429 was re-run, including only flocks of hybrid Ross 308. The results from this analysis showed an average genome coverage of 95.7%

Table 2 Description of the ten most common sequence types identified.

ST	Isolates (n)	Flocks (n) where ST is identified	Flocks (n) where ST is identified in > 3 isolates	Serotypes
23	81	17	17	O78:H4
429	33	7	7	O2/O50:H1
95	24	10	3	O1:H7, O2/O50:H5
117	11	3	2	O161:H4, O24:H4, O78:H4
371	9	2	2	O45:H19
69	8	5	0	O15:H6, O17/O44/O7:H18, O23:H6
101	7	3	1	O103:H21, O88:H8
135	5	1	1	O2/O50:H1
10	4	4	0	O49:H12, O71:H40, O99:H33
457	4	3	0	O11:H25
93	4	2	1	O5:H10

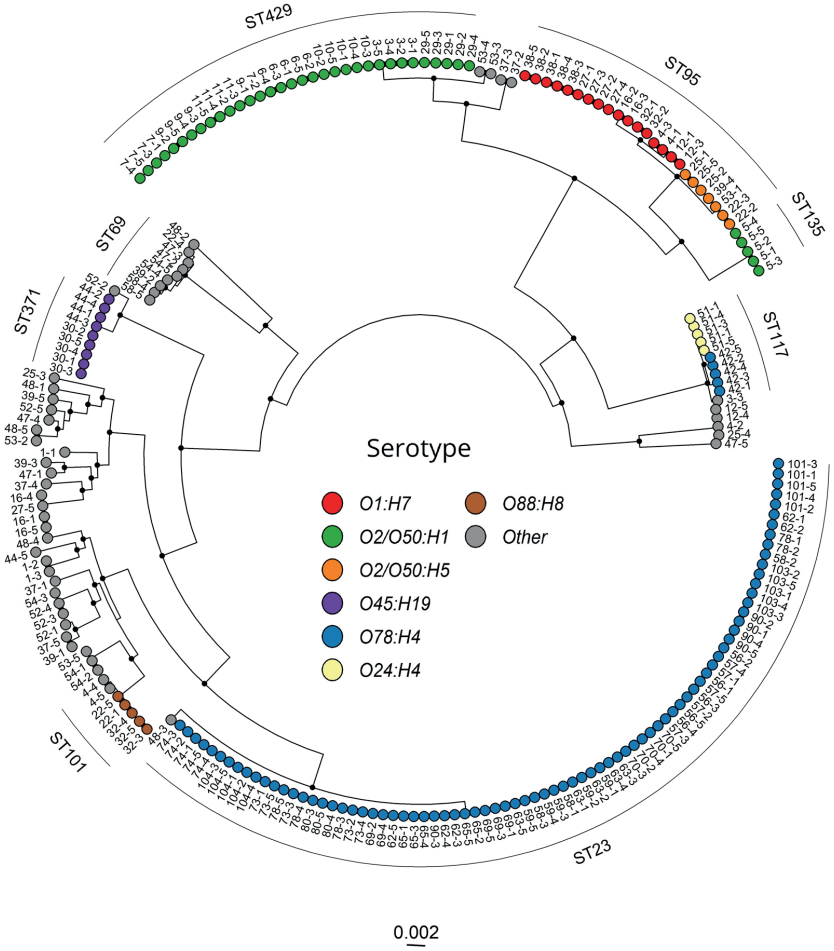

Figure 1 Maximum likelihood core-gene SNP tree of all isolates included in the study. Bootstrap values above or equal to 95 are denoted as black nodes. The core gene tree visualizes the genetic relations between the most common sequence types (STs) identified and what serotype profiles are linked to these. Colors on the tips and clade labels represent serotypes and STs, respectively, represented by more than five isolates. Tip labels represent flock and bird.

and the SNP range was 0–76 with a median SNP distance of 59 (Additional file 3).

Virulence-associated genes

From a database containing 629 entries of VAGs, 112 VAG-variants were identified in at least one of the APEC isolates from the present study (Additional file 1). The frequency of VAGs was described separately for isolates of ST23 and ST429 and a third group that included all other isolates (Figure 4).

A total of 25 VAGs were identified in all isolates of ST23, while five VAGs were identified in only some of the ST23 isolates. These include *tra*T, *cba*, *cia* and *cma* that were identified in only 28%, 17%, 12% and 17% of these isolates, respectively. The *gad* gene was identified in 94% of all the ST23 isolates (Figure 4). Thirty three of the VAGs were present in all isolates of ST429, while four genes were found in some isolates. The *tsh*, *sitC*, *vat* and *gad* genes were identified in 61%, 94%, 12% and 30% of the ST429 isolates, respectively (Figure 4).

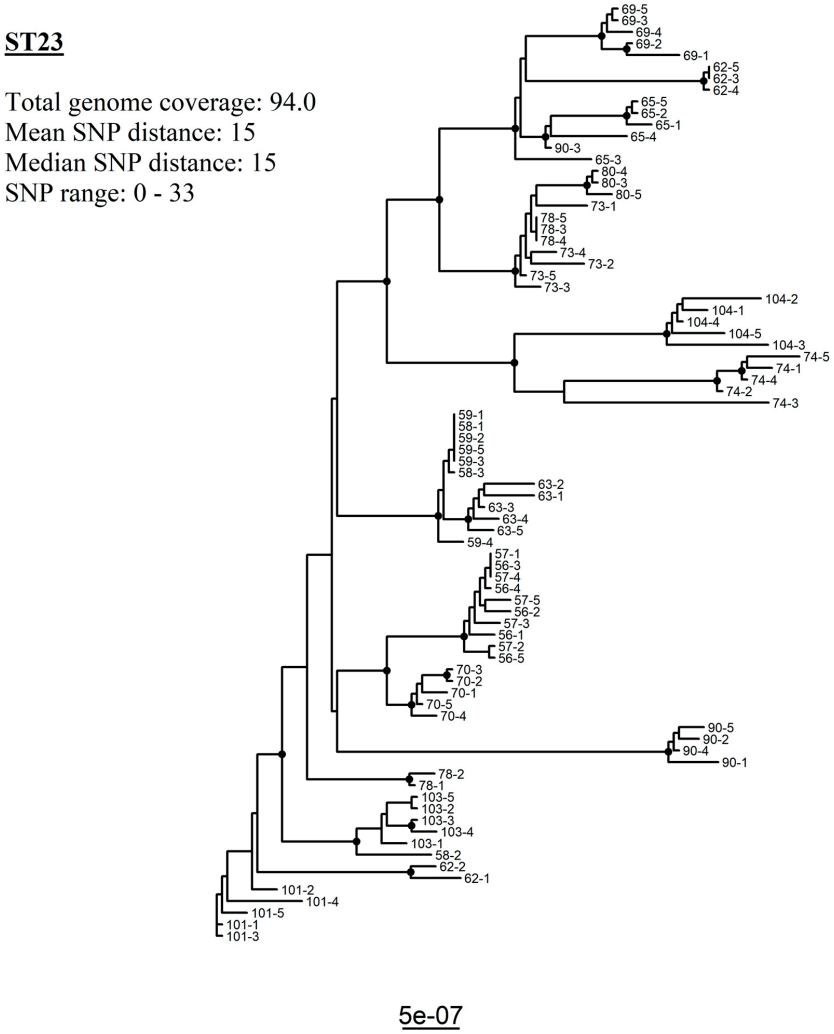

Figure 2 Maximum likelihood core genome tree visualizing the genetic relations of all isolates identified as ST23 from 17 flocks (n = 81 isolates). Bootstrap values above or equal to 95 are denoted as black nodes. Tip labels represent flock and bird.

In the third group containing all other STs, there was higher diversity in the presence of VAGs. Similar to ST23 and ST429, all of the isolates in the third group carried the genes *csg*A-F, *ecp*A, *ibe*B, *ibe*C, *iuc*C, *sit*D and *ter*C. Several other VAGs were present in more than 90% of all the isolates in the third group, but there were also VAGs that were absent or only present in a few isolates (Additional file 1 and Figure 4). The siderophore receptor-genes *fyu*A and *irp*2 were present in all ST23 and ST429 isolates, but only present in 51% and 52% of the other isolates, respectively.

Discussion

There is limited knowledge about APEC in the Norwegian broiler production, and after the peak of outbreaks of colibacillosis on farms in the Nordic countries in 2014, we saw the need for a systematic study of the epidemiology of APEC in the Norwegian broiler production. To

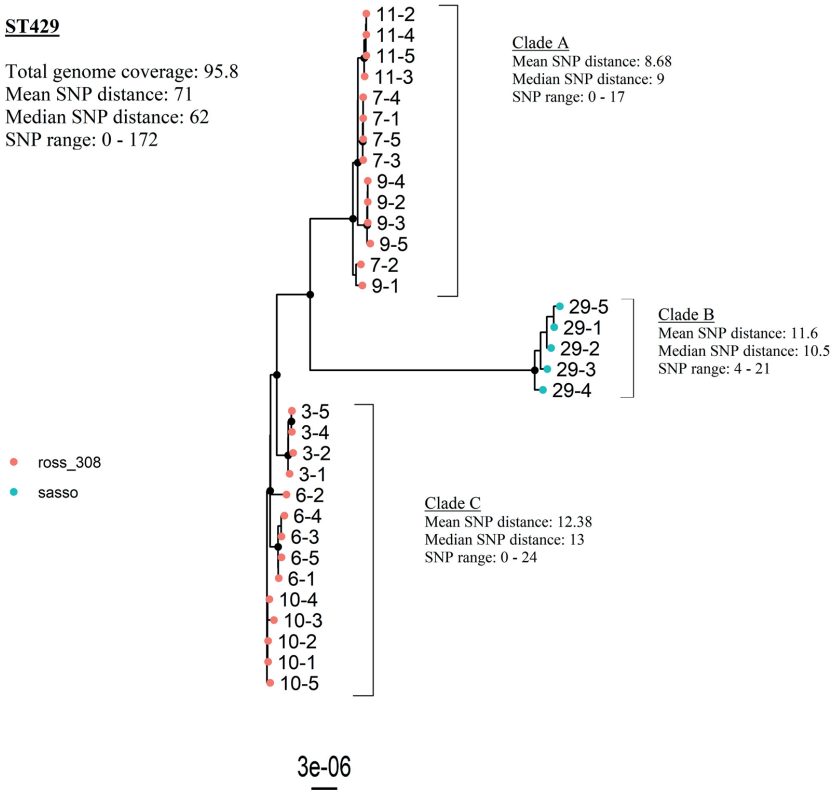

Figure 3 Maximum likelihood core genome tree of all isolates identified as ST429 from seven flocks (n = 33 isolates). Bootstrap values above or equal to 95 are denoted as black nodes. Tip labels represent flock and bird. Colors on the tips represent hybrid. Clade A and C consists of isolates from three flocks each, all of hybrid Ross 308. Clade B represents a single Sasso flock.

identify APEC types within the Norwegian broiler production, we performed systematic sampling of flocks with high FWM and sequenced the genomes of individual isolates from these flocks to study their STs, serotype profile, content of VAGs and their evolutionary relationship.

The selection of isolates was carried out according to the results from a pilot project in 2019. The study concluded (1) low diversity of APEC within individual diseased birds, and (2) the need of sampling a minimum of three birds per flock to identify the diversity of APEC within a flock and determine the main disease-causing APEC at flock level [12].

Out of the 32 distinct STs identified in the present study, almost 50% were only identified once. A similar trend has been observed in previous studies [32–34]. The high number of STs only represented by one or two isolates influences the high diversity of STs reported. Whether these single ST strains are true avian pathogens and important for the colibacillosis etiology, or sporadic findings without importance for pathogenicity, is currently not known. These reports, however, highlight the need for sampling multiple animals and WGS of several isolates from a flock to identify the main disease-causing APEC within a flock.

Altogether, 35 of 45 diseased flocks were identified with a dominating APEC type, and ST23, ST117, ST371 and ST429 were more often found to cause disease in a flock alone than other STs. The latter indicates that these STs are possibly more pathogenic in poultry, compared to the STs more commonly identified in mixed infections with more than one ST within a flock [35]. Altogether, ten flocks exhibited a combination of several STs and the APEC types most commonly identified in combination with other STs were ST10, ST69, ST95 and ST101.

Notably, both ST69 and ST95 are frequently isolated in human infections [5, 36]. However, due to their presence

Table 3 Overview of the calculated SNP distances between isolates of same sequence type (ST23 and ST429) within a flock.

Flock ID	ST	Mean SNP distance	Median SNP distance	SNP range	Isolates/ flock (n)
56	23	1.2	1	0–2	5
57	23	1.2	1	0–2	5
58	23	8.7	13	0–13	3
59	23	0.4	0	0–1	5
62	23	19.8	32	0–33	5
63	23	2.4	2.5	1–4	5
65	23	7.4	7	1–13	5
69	23	6.6	7	1–11	5
70	23	1.4	1.5	0–2	5
73	23	2.4	2.5	1–4	5
74	23	7.4	3	1–17	5
78	23	7.6	12	0–13	5
80	23	0.6	1	0–1	3
90	23	13.1	3	1–31	5
101	23	2.2	2.5	0–4	5
103	23	2	2	0–3	5
104	23	3	4	1–7	5
3	429	3.3	4	0–5	4
6	429	8.8	5	2–19	5
7	429	4.4	0	0–11	5
9	429	6.8	2	0–17	5
10	429	2.8	2.5	0–6	5
11	429	0.7	1	0–1	4
29	429	11.6	10.5	4–21	5

in a majority of mixed infections, it is likely to assume that they are rather opportunistic than highly pathogenic in poultry. This is supported by Kromann et al. where ST95 was identified with the highest prevalence from healthy poultry flocks sampled in a non-outbreak situation [37].

Caution should be taken, however, in the discussion of ST95 as this ST is identified with several serotype profiles, and variants of the same ST but with different virulence properties, exists [5, 36, 37]. Our study shows further examples of STs with several serotype profiles, such as ST101 where O88:H8 was identified from three flocks and O103:H21 from another flock and ST117, which exhibits three serotypes O24:H4, O78:H4 and O161:H4 from three different flocks. Serotype O78:H4 is the same serotype as found in ST23. Generally, typing of APEC should be evaluated with caution, as shown in the core gene analysis where ST117 and ST23 exhibit large genetic distances between these STs, even though they share one of the same serotype profiles. Without WGS data, the two distinct peaks of ST117 in 2014 [11] and ST23 in 2021, with possible distinct origins, would be considered the same APEC if only serotyping was performed, but two distinct APEC types if MLST was performed. Sequence types identified with several serotype profiles, on the other hand, suggests that a combination of two typing methods provides better differentiation between APEC isolates (for quick diagnosis in outbreak situations) [35].

Even though APEC is considered a diverse pathogen, there is less variation amongst the most frequent STs reported [12, 33, 35, 38–40]. The high prevalence of certain STs, however, may be due to small outbreaks of colibacillosis from a single source and within a limited period, possibly from higher up in the production pyramid [41].

Phylogenetic analysis is today considered the gold standard to evaluate relatedness between isolates from a possible single source. Accordingly, this study presents the mean SNP-distance within, and between flocks identified with the same ST. The results are valuable for the evaluation and understanding of future outbreaks of colibacillosis across farms. To our knowledge, there is no consensus for defining the acceptable number of SNPs in APEC outbreaks [9, 10, 12]. Factors such as the mutation rate, the number of individuals the pathogen might encounter, pathogen pressure and the duration of source contamination could influence the number of SNPs emerging during an outbreak [9]. The consideration of SNP distances between isolates from an outbreak, should therefore, include the pyramidal structure of the poultry production and the number of individuals and generations the pathogen will encounter in a potential vertical transmission line, before causing disease in a broiler chick. Further, SNP distances alone should not be assessed without the knowledge of the proportion of the genome that has been used in the analysis. Therefore, pathogen- and population metadata, as well as genome coverage, plays as much a role in the understanding if an isolate belongs to an outbreak as the SNP distance alone [9, 10, 12].

Altogether, 112 VAGs were identified at least once in our set of APEC isolates. This correlates well with recent reports by Apostolakos et al., who identified 113 VAGs in their study [32]. However, within each of the two main STs (ST23 and ST429) identified in the present study, there was, with few exceptions, less diversity. This is expected as the isolates within these STs were considered part of the same outbreaks, and therefore likely to be clonal. The genes varying within ST23 were *traT*, *cba*, *cia* and *cma*, and within ST429 *vat* and *gad.* Most of these genes are known to be plasmid-encoded and might therefore vary more frequently between isolates within an outbreak than chromosomally encoded genes. Further,

category	VAG	gene	ST23 (%)	ST429 (%)	Other STs (%)
Adhesins	csg cluster, curly fibers	csgA-F	100	100	100
	e.coli common pilus	ecpA	100	100	100
	e.coli common pilus	ecpR	100	100	99
	fimbrial adhesin	fimC	100	100	91
	fimbrial adhesin	fimH	100	100	94
	heat resistant agglutinin	hra	100	0	52
	long polar fimbriae	lpfA	100	0	44
	p-fimbriae, major structural subunit	papA_F11	0	0	16
	p-fimbriae, pilus assembly	papC	0	100	27
	p-fimbriae, encodes pilus tip adhesin	papG	0	0	11
	Temperature-sensitive hemagglutinine gene	tsh	100	61	33
	Afambrial adhesin AFA -I, Dr family of adhesins	afaD	0	0	13
	Iron-regulated-gene homologue adhesin	iha	0	0	17
	E. coli attaching and effacing gene that encodes intimin	eae	0	0	1
	fimbrial protein	yfcV	0	100	36
Invasins	Promotes invasion of brain microvascular endothelial cells	ibeA	0	100	13
	Promotes invasion of brain microvascular endothelial cells	ibeB	100	100	100
	Promotes invasion of brain microvascular endothelial cells	ibeC	100	100	100
	Type V secretion system, protease involved in intestinal colonisation	pic	0	0	10
Iron acquisition	hemin receptor	chuA	0	100	65
	heme uptake	chuS-W	0	100	65
	heme uptake	chuX	0	0	14
	heme uptake	chuY	0	100	50
	Encodes ferric yersinibactin uptake receptor, siderophore receptor	fyuA	100	100	51
	Encodes iron repressible gene associated with yersiniabactin synthesis	irp2	100	100	52
	Iron regulated, siderophore receptor, outer membrane protein	ireA	0	0	33
	Aerobactin operon, involved in iron uptake and transport	iucA	100	100	80
	Aerobactin operon, involved in iron uptake and transport	iucB	0	0	4
	Involved in aerobactin synthesis	iucC	100	100	100
	Aerobactin operon, involved in iron uptake and transport	iucD	0	0	1
	Ferric aerobactin outer membrane receptor gene	iutA	100	100	80
	Putative iron transport operon	sitA	100	100	98
	iron transport protein	sitB	100	100	92
	iron transport protein	sitC	0	94	62
	iron transport protein	sitD	100	100	100
	Outer mebrane protein for serum resistance	traT	28	100	99
	outer membrane protein for increased serum survival	iss	100	100	95
Protectins	Polysialic acid capsule biosynthesis protein	neuC	0	100	33
	Encodes a protease able to cleave colicin	ompT	100	100	96
	kps cluster, involved in encoding capsular (K) antigens	kpsE	0	100	49
	kps cluster, involved in encoding capsular (K) antigens	kpsMII_K1	0	100	28
	kps cluster, involved in encoding capsular (K) antigens	kpsM_K15	0	0	1
	kps cluster, involved in encoding capsular (K) antigens	kpsMII	0	0	5
	kps cluster, involved in encoding capsular (K) antigens	kpsMII_K4	0	0	1
	kps cluster, involved in encoding capsular (K) antigens	kpsMII_K5	0	0	10
	kps cluster, involved in encoding capsular (K) antigens	kpsMIII_K10	0	0	1
	kps cluster, involved in encoding capsular (K) antigens	kpsMIII_K96	0	0	2
Toxins	Avian *E. coli* hemolysin	hlyF	100	100	89
	Hemolysin	hlyE	0	0	1
	Uropathogenic-specific protein (bacteriocin)	usp	0	100	30
	Vacoulating autotransporter toxin	vat	0	12	37
	cytolethal distending toxin, blocks mitosis	cdtA	0	0	2
	cytolethal distending toxin, blocks mitosis	cdtC	0	0	2
	heat stabile enterotoxin	astA	0	0	38
Miscellaneous	ets operon, Encodes ABC transporter, efflux pump protein	etsC	100	100	79
	Structural gene of the ColV operon	cvaC	100	100	70
	Structural gene for ColM activity	cma	17	0	34
	ter gene cluster, tellurite resistance gene*	terC	100	100	100
	microcin	mchF	100	100	65
	microcin	mchB	0	0	10
	microcin	mchC	0	0	10
	dispersin transporter protein	upaG	0	0	25
	Enteroaggregative immunoglobulin repeat protein	air	0	0	22
	LEE-encoded TTSS effectors	espF	0	0	1
	cell-cycle-inhibitor factor	cif	0	0	1
	colicin	cba	17	0	8
	translocated intimin receptor	tir	0	0	1
	glutamate decarboxylase gene**	gad	96	30	65
	carcinoembryonic antigen, family of cell adhesion	cea	0	0	14
	endonuclease colicin E2	celb	0	0	10
	colicin	cia	12	0	45
	Hybrid non-ribosomal peptide, polypeptide megasynthase	clbB	0	0	7
	hexosyl transferase homologue	capU	0	0	4
	bacteriocin microcin B17	mcbA	0	0	3
	microcin M, part of colicin H	mcmA	0	0	5
	non - LEE encoded effector A and B	nleA,B	0	0	1
	Salmonella HilA homologue	eilA	0	0	22
	Tir domain-containing protein	tcpC	0	0	1

Figure 4 Frequency of virulence-associated genes (VAGs) present in three groups of isolates: ST23 (n = 81), ST429 (n = 33) and a third group comprising the remaining isolates (n = 105). The colors represent the frequency from which each gene is present: red = 100%, black = 0% and yellow = 1–99%. The VAGs are categorized and described as previously presented by Nolan et al. [1] and Kathayat et al. [18].

fyuA and *irp2* were represented in all outbreak isolates, but only in approximately half of the non-outbreak isolates. Both these VAGs belong to the Yersiniabactin operon which is responsible for iron acquisition and of high importance in the pathogenesis of avian colibacillosis [1].

A few VAGs were, on the other hand, identified in all isolates in this study and could therefore be considered important for the virulence of APEC. However, without a true, non-pathogenic control group for comparison, no valid conclusions may be drawn based on these data as these VAGs might be identified in all avian *E. coli* isolates regardless of pathogenicity. A recent study from Johnson et al. [35] suggested two conserved VAGs associated with APEC plasmid, *hlyF* and *ompT* as potential markers for increased virulence potential in combination with other genetic features. In the present study, *hlyF* and *ompT* were identified in all ST23 and ST429 isolates and in 89 and 96% of isolates of other STs, respectively.

In this study, the virulence of an APEC strain was evaluated based on the prevalence of the identified ST and its' ability to cause high FWM within a flock and across farms alone or in combination. Further, we have given insight into the relatedness of outbreak strains, and presented the most prevalent VAGs associated with the two outbreak strains ST23 and ST429 in our study. However, APEC strains emerge from multiple *E. coli* lineages, and for the future it would be interesting to follow single ST outbreaks to identify if the same ST reappear as more prone to cause outbreaks across farms or if other STs, which in this study appear of lesser importance, could be the cause of future outbreaks. It would be interesting to better identify the transmission routes of the pathogen in outbreak situations between farms. A common database for the control and prevention of APEC outbreaks has been suggested [42]. For the future, the authors supports an initiative for such a database, including well-defined metadata as well as comparable sampling and diagnostic methods. Such a database could aid in the identification of pathogen transmission routes through the broiler poultry pyramid. Further, the comparison of VAGs from systematically sampled outbreak strains to a proper control group would be of interest to further unravel the virulence potential of individual APEC. The importance of defining what an APEC control is, should however, be further discussed as commensal *E. coli* might have the potential to become APEC and the pathophysiology behind is still not well defined [38].

In conclusion, this study shows the presence and distribution of APEC types identified from local outbreaks of colibacillosis-septicemia across Norway during 2018–2021. Further, it identifies how peaks of high FWM due to colibacillosis may be caused by a single, distinct ST. Phylogenetic analysis gives insight into the relatedness between isolates belonging to the same ST, but also across STs and serotypes, identifying the need for combining typing methods to better discriminate between APEC types. This study also highlights the value of using WGS as a diagnostic tool for surveillance as well as in the identification of future outbreaks of colibacillosis.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13567-023-01140-6.

Additional file 1. Metadata linked to each isolate sequenced in this study including flock and sampling data, bacteriological grading, sequencing quality measures, virulence-associated genes identified and accession numbers to ENA. [1]The sample ID explains from what flock (first number) and bird (last number) the isolate was sampled. [2]The bacterial culture was graded according to purity of growth on three agar plates incubated over night in either normal oxygen pressure, CO2 chamber or anaerobically: grade 1 = pure growth, grade 2 = few colonies of *Enterococcus* spp. or *Proteus* spp. were present on the agar, together with an otherwise pure culture of *E. coli*. Grade 3 = dominating growth of *E. coli* with sparse to medium growth of either *Enterococci* spp, *Proteus* spp. or less growth of a different bacteria, grade 4 = mixed culture: minimum of three different bacteria, where *E. coli* was not the dominating bacteria. [3]The presence of pathological lesions associated with colibacillosis (colisepticaemia) within an individual bird during necropsy, 1 if ≥2 lesions present, 0 if <2 lesions present. [4]Each individual bird received a colibacillosis diagnosis (1) if the bird had lesions of colisepticaemia and the bacteriological examination from the individual sample was graded 1-3.

Additional file 2. List of genes included in the extended database of genes uploaded to VirulenceFinder for the identification of virulence-associated genes in this study. Included in the table is the name of gene, short description of gene and from what database the gene was identified (VirulenceFinder or VFDB).

Additional file 3. Maximum likelihood core genome tree visualizing the genetic relations of all isolates identified as ST429 from all Ross 308 flocks (n = 28), excluding isolates from one Sasso flock. Bootstrap values above or equal to 95 are denoted as black nodes. Tip labels represent flock and bird. Clade A and C consists of isolates from three flocks each, all of hybrid Ross 308.

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Authors' contributions

SKS, BD, MA, CS conceptualized and designed the study. IHK, SKS, CS organized the sampling and the laboratory analysis. IHK, HK analyzed the data. HK, KDR assisted in statistical and bioinformatical methods. IHK, HK, SKS, BD, CS

interpreted the data. IHK prepared the first draft of the manuscript. HK, SKS, KDR, BD, MA, CS read, contributed to and approved the final manuscript. All authors read and approved the final manuscript. All authors read and approved the final manuscript.

Declarations

Competing interests
The authors declare that they have no competing interests.

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Publisher's Note

Paper III

Flock Size and High Previous First Week Mortality as risk factors for neonatal colisepticemia in broiler chicken flocks

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ABSTRACT

Good management and high biosecurity ensure that the yearly average first week mortality (FWM) remains low in Norwegian broiler production, the goal being to keep this statistic below 0.8%. However, outbreaks of colisepticemia caused by avian pathogenic *E. coli* (APEC) have been known to occur periodically, resulting in poor animal welfare and high economic loss. Up to five strains of APEC may be isolated in the same outbreak of colisepticemia, and some variants of APEC are identified more often in single strain outbreaks. The same strains of APEC that seem to be instrumental in outbreaks have also been identified in individual birds with colisepticemia from flocks where FWM has remained low (<0.8%).

With this matched case-control study, we aimed to identify management risk factors for high FWM due to colisepticemia within chicken broiler flocks. Of the 37variables used in the analysis, we identified *Flock Size* and *High FWM in the Previous Flock* as variables that increase the risk for high FWM due to colisepticemia, with an odds ratio (OR) of 1.07 and 3.33 respectively. The results suggest potential horizontal transmission of APEC to consecutive flocks and highlight the need for further studies of persistence and transmission pathways of APEC within a poultry house. The resulting knowledge can be applied to control transmission of APEC and aid in the implementation of preventive measures in an APEC outbreak situation.

INTRODUCTION

Colisepticaemia in young chicks is a continuous problem worldwide. It is an acute, systemic disease caused by avian pathogenic *E. coli* (APEC). Flocks affected by the disease have been known to experience a first week mortality (FWM) of up to 20%, resulting in poor animal welfare, substantial economic loss and poor food sustainability (Nolan et al., 2020)

Avian pathogenic *E. coli* is considered an opportunistic bacterium, a commensal of the gut, which by entering an extra-intestinal site may cause disease. Recent research points to types of APEC with potentially higher virulence to be the cause of single strain outbreaks of colisepticemia (Mehat, et al., 2021; Johnson et al., 2022; Kravik et al., 2023). However, the same APEC types may be identified in birds with colisepticemia from

flocks where FWM remains low (Kromann et al., 2022). APEC is thought to potentially spread both vertically in the breeding pyramid and horizontally at the hatchery and in the broiler house (poulsen et al., 2017; Projahn 2017; Petersen et al., 2005; Bojesen et al., 2022). To our knowledge, little is known about environmental factors that contribute to outbreaks of colisepticemia with high FWM in one house on a farm, while in another house or on another farm, FWM remains low.

Previously, Heier *et al.* studied risk factors for cumulative mortality and FWM of broiler chicken flocks in Norway from 1996-1999. This study was done retrospectively from a regularly recorded database (Heier et al., 2002). A similar study was published from Taiwan in 2004, analyzing factors influencing survival of chickens during the first week of life (Chou et al., 2004). Even though the production systems in Norway and Taiwan are very different, both the above-mentioned studies identified variables such as *Flock Size* and *Ventilation Systems* to be of importance for FWM in broiler chickens. Other important risk factors from the Norwegian study included *Study Year*, *Use of Paper Underlay* for feeding during the first week, *Stocking Density* and use of *Floor Insulation*. The variable *Study Year* was also identified as a risk factor for cumulative first week mortality in broiler chicken flocks in a recent study from Spain. The same study identified *Type of Broiler House*, *Presence or Absence of Drip Cups*, *Egg Storage* and *Season* as possible risk factors for high FWM (Yerpes, et al., 2020). Another study from Belgium identified *Floor Quality*, *Ventilation Type*, *Other Professional Activities of the Farmer* and *Neonatal Septicemia* as risk factors for high FWM (Van Limbergen, et al., 2020). The most common cause of neonatal septicemia in poultry being colisepticemia (Dziva & Stevens 2008)

For years, the Norwegian poultry industry has worked towards the goal of lowering the yearly average broiler chicken FWM. In 2013, an action plan for animal health and welfare in the Norwegian poultry industry was published, in which one of the goals was to keep the FWM in chicken broiler flocks <0.8% (Refsum, 2014). To achieve this goal, focus was placed on improving biosecurity, disinfection routines and early feed access. To improve biosecurity, recommendations were made to reduce human traffic into the poultry house, use disinfection sluices, effective vermin control and the implementation of hygienic routines for import of feedstuffs and equipment into the poultry house. Furthermore, increased focus on down time and proper disinfection between flocks, in addition to continued use of the "all out all in" principle, has helped to improve the biosecurity (Refsum, 2014). Today, even with occasional local outbreaks of neonatal colisepticemia, the industry has largely reached its goal and the average FWM in 2020 registered by the largest company of broiler chickens in Norway was 0.8% (Personal communication, statistics from Nortura SA). In comparison, the average FWM reported by Heier *et al*. on the Norwegian broiler chicken population during 1996-1999 was 1.5%.

Despite improved management of Norwegian broiler chicken production, cross-farm outbreaks in the Nordic countries in 2014 and 2021 have indicated the need for more knowledge on the risk factors contributing to

high FWM caused by colisepticemia in well-managed broiler chicken productions (Kravik et al., 2023 ; Ronco et al., 2017).

In this study, we investigate risk factors contributing to high FWM caused by colisepticemia in the Norwegian broiler chicken production.

MATERIALS AND METHODS

Study Design and Flocks:

This study was designed as a matched case-control study. Case flocks were selected based on high FWM, macropathological lesions typical of colisepticemia and close to pure culture of *E. coli* as described in Kravik *et al.* (Kravik et al.,2023). Defining a control flock proved to be challenging, as sufficient examination for the presence of individual cases of colisepticemia within each control flock was not economically feasible. Control flocks were therefore, assumed to have sporadic cases of colisepticemia, but with FWM remaining <0.8%. Hence, this study compares a population of broiler chickens with generally good management and high level of biosecurity, which underwent outbreaks of neonatal colisepticemia within the first week of life, to flocks where neonatal septicaemia did not spread throughout the flock. The control flocks were matched using the following criteria: geographical location, hybrid and hatching date (within 14 days). For each case flock, two control flocks were selected with the use of a freeware randomization tool. The distribution of flocks included in the study is depicted in Table 1.

Table 1 Number of poultry broiler flocks included in the study and their distribution according to company, hybrid and geographical area.

Company[a]	Hybrid	Geographical area[b]	Case[c]	N[d]
A	Ross308	East	Case	13
A	Ross308	East	Control	26
A	Ross308	Mid	Case	2
A	Ross308	Mid	Control	4
A	Ross308	North-East	Case	4
A	Ross308	North-East	Control	8
A	Ross308	South-East	Control	4
A	Ross308	South-West	Case	1
A	Ross308	South-West	Control	2
A	Rowan Ranger	South-East	Case	2
B	Ross308	South-West	Case	19
B	Ross308	South-West	Control	38
C	Hubbard JA787	Mid	Case	4
C	Hubbard JA787	Mid	Control	8
D	Ross308	Mid	Case	1
D	Ross308	Mid	Control	2

[a]Company describes which of the four companies (A-D) represented data from the flock.

[b]Geographical area describes what part of Norway the flock was situated.

[c]Case: if the flock was a case flock with first week mortality (FWM) > 0.8% and with a colisepticemia diagnosis or, if the flock was a control flock with first week mortality (FWM) ≤ 0.8% but from same geographical area and time period as a control flock.

[d]N represents the number of flocks from which data was collected

Data Selection and Collection

Data was collected from broiler chicken flocks hatched between 2018 and 2021. The selection of questions for the study was compiled after interviewing collaborating poultry veterinarians. From the case flocks, data was collected through prewritten questionnaires filled in by the veterinarians at time of sampling for a recent molecular study on APEC (Kravik et al., 2023). From the control flocks, the same data was collected retrospectively from electronically stored production control databases or, from daily recordings of mortality, feed- and water intake and environmental conditions stored locally at the farm. The raw data are confidential, but the cleaned data may be retrieved by contacting the corresponding author.

Data Validation

Received data was cleaned using R (R Core Team, 2021). A cut-off for lacking data was set to >50% per variable. Further, we excluded variables based on: high possibility of imprecise depiction in the questionnaires (human error), being part of the matching criteria, too many factorial levels in comparison to the number of flocks included in the study or due to multicollinearity with other variables. All variables kept for further analyses are described in Table 2.

Data Analyses

We performed descriptive analysis including mean, median, maximum and minimum values for each variable and category (Table 2). Missing values underwent median imputation, before we performed a univariate regression analysis with High FWM (yes or no) as the outcome (Table 3). To identify the relationship between significant (P <0.05) variables from the univariate analysis, we drew a directed-acyclic-graph (DAG)-diagram and set up two correlation analyses for the variables *Flock Size* and *High FWM Previous Flock* (Fig 1 and Table 2) (Kendall,1938; Kendall, 1945; Becker et al., 1989; R Core Team, 2021)

We set up a multivariate logistic regression model with all the variables that were significant in the univariate analysis. However, to reduce multicollinearity we replaced the variables *House m2* with *Flock Density*, *Flock Size Previous* with *Difference in Flock Size* and *Number of Offspring from the Second Parent flock* with *Max Fraction from a Parent Flock* (Table 4). Finally, a receiver-operating characteristics (ROC)-curve was used to assess the predictive ability of the model and from this we calculated the area under the curve (AUC) using R programming, pROC package version 1.18.0 (Robin et al., 2011; R Core Team, 2021).

Table 2 Variables included in the study, including descriptive statistics: number of missing variables, number of imputed variables and the variables correlation to Flock Size and FWM Previous Flock

[a]Variable	[b]Description	Min[c]	Median[d]	Mean[e]	Max[f]	% Missing case[g]	% Missing control[h]	N imputed[i]	Corr. Flock Size[j]	Corr. FWM Prev Flock[k]
age_parents_f1	numerical: age of parent flock number 1 in weeks	25	38	39.44	64	0	2	2	-0.13	-0.09
age_parents_f2	numerical: age of parent flock number 2 in weeks	27	38	39.58	60	28	26	37	-0.17	-0.07
avg_parent_age_weighted	numerical: average parent age weighted	25	37.56	39.09	64	0	2	2	-0.2	-0.08
avg_progeny_weight_p1	numerical: average weight (grams) of day old chicks from first parent flock	24	44.4	44.61	70	2	49	46	-0.19	0.21
avg_progeny_weight_p2	numerical: average weight (grams) of day old chicks from second parent flock	34	44.8	45.7	66	26	57	64	-0.28	0.15
cat_parent_age	categorical: variable dividing age of parent flocks > 40 weeks or ≤40 weeks					0	2	2		
cat_parent_flock	categorical: variable describing if one or more than one parent flocks contributed to the flock					0	2	2		
difference_parent_ages	numerical: difference in parent age between youngest and oldest contributing parent flock	0	1	3.02	28	0	2	2	0.19	0.04
empty_days	numerical: number of days the poultry house was empty before the new flock arrived	7	13.5	18.28	198	0	0	0	0.14	-0.1
flock_density	numerical: a calculated value based on size of flock and size of farm house	10.77	16.53	16.3	21.37	2	1	2	0.12	-0.07
flock_size_diff_relative	numerical: the relative difference in flock size from previous to present flock	-0.18	0	0	0.26	0	0	0	0.01	-0.11
flock_size_previous_1000	numerical: the total number of chicks in the previous flock	2.6	18.45	19.95	43.92	0	0	0	0.99	0.07
flocks_size_1000	numerical: number of chicks in the flock divided by thousand	2.5	18.85	19.88	43.2	0	0	0	1	0.09
FWM	numerical: first week mortality in percent	0.26	0.68	1.62	11.51	0	0	0	0.18	0.22
FWM_previous_12mnths	categorical: high first week mortality (FWM) the previous 12 months: yes (1), no (0)					0	0	0		
FWM_previous_flock	numerical: first week mortality of previous flock in percent	0.15	0.63	0.76	4.49	2	0	1	0.09	1
house_1000m2	numerical: size of the farm house in square meters divided by thousand	0.15	1.2	1.21	2.68	2	1	2	0.98	0.07
max_parent_age	numerical: maximum parent age of the three contributing flocks	25	39.5	40.72	64	0	2	2	-0.1	-0.09
n_parent_flocks	numerical: number of parent flocks contributing to the broiler flock	1	2	1.97	4	0	2	2	0.33	-0.06
n_parents_f1	numerical: total number of offspring contributing to the flock from parent flock 1	177	11415	11644.69	43200	0	2	2	0.48	0.18
n_parents_f2	numerical: total number of offspring contributing to the flock from parent flock 2	119	8249	8692.94	23600	28	26	37	0.55	-0.03
parents_f_fraction_max	numerical: parent flock contributing with the largest fraction to a flock	0.37	0.81	0.77	1	0	0	0	-0.39	0.09
RHpct_d1_max	numerical: highest measured relative humidity day 1	2	52	51.03	79	24	27	36	0.12	0.03
RHpct_d1_min	numerical: lowest measured relative humidity day 1	1	47	44.34	61	28	35	45	0.06	0.01
RHpct_d2_max	numerical: highest measured relative humidity day 2	3	53	52.4	72	22	29	37	0.07	0.01
RHpct_d2_min	numerical: lowest measured relative humidity day 2	4	48	46.59	62	26	37	46	0.04	0
RHpct_d3_max	numerical: highest measured relative humidity day 3	28	55	55.26	90	22	26	34	0.09	0.05
RHpct_d3_min	numerical: lowest measured relative humidity day 3	12	48	48.03	66	24	35	43	0	-0.01
temp_d1_max	numerical: highest measured temperature on day 1 in degrees Celsius	31	33.45	33.65	36.9	20	27	34	0.17	0.25
temp_d1_min	numerical: lowest measured temperature day 1 in degrees Celsius	17.6	32.3	32.03	34.7	24	35	43	-0.04	0.07
temp_d2_max	numerical: highest measured temperature on day 2 in degrees Celsius	29.8	33	33.07	35.8	17	28	34	0.14	0.19
temp_d2_min	numerical: lowest measured temperature day 2 in degrees Celsius	28.2	32	31.75	34.4	22	38	45	0.13	0.12
temp_d3_max	numerical: highest measured temperature on day 3 in degrees Celsius	28.9	32.5	32.57	35.8	15	27	32	0.13	0.18
temp_d3_min	numerical: lowest measured temperature day 3 in degrees Celsius	21.4	31.2	31	34	20	37	43	0.08	0.11
total_dark_d1	numerical: total number of hours darkness on day one	0	1	1.5	8	30	30	42	-0.18	0.18
total_dark_d2	numerical: total number of hours darkness on day two	0	1	2.32	8	30	32	43	-0.17	0.18
total_dark_d3	numerical: total number of hours darkness on day three	0	3	3.32	8	28	24	35	-0.19	0.17

[a]Name of the variables used in the analysis

[b]Extended description of the variable name used in the analysis

[c]The minimum value recorded for the variable identified in the descriptive analysis of the data

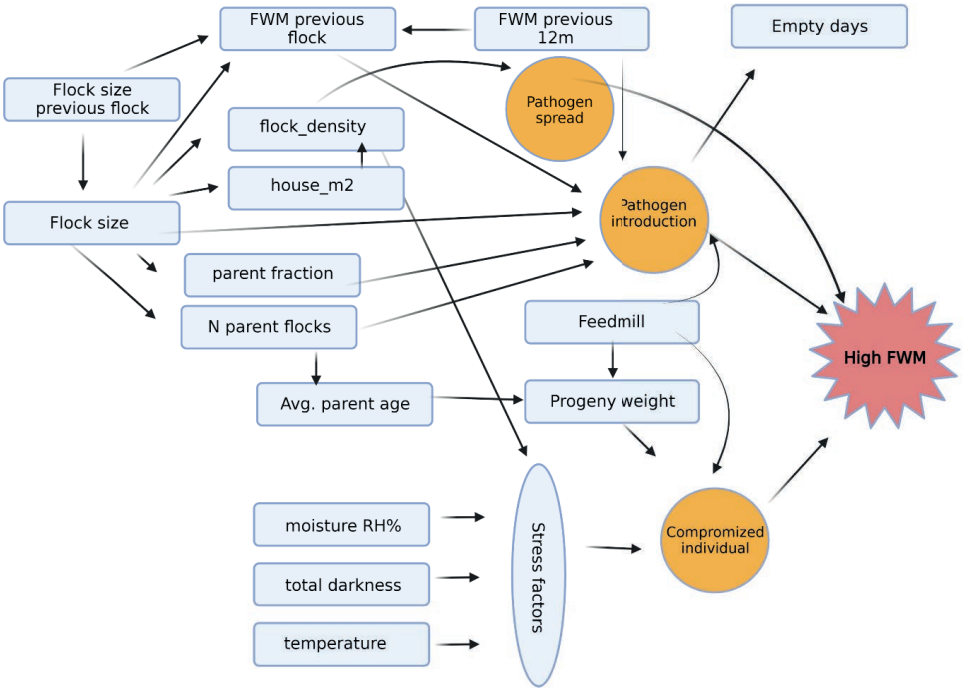
[d]The median value recorded for the variable identified in the descriptive analysis of the data

[e]The mean value calculated for the variable from the descriptive analysis of the data

[f]*The maximum value recorded for the variable identified in the descriptive analysis of the data*
[g]*The percentage of missing data for the variable in question from all case flocks*
[h]*The percentage of missing data for the variable in question from all control flocks*
[i]*The total number of median imputations performed for the variable in question*
[j]*The correlation factor for Flock Size to the variable in question*
[k]*The correlation factor for First Week Mortality (FWM) in the Previous Flock to the variable in question*

Table 3 Univariate analysis with FWM > 0.8 yes / no as the outcome

Variable	**Estimate**	**P.value**	**Conf.low**	**Conf.high**
house_1000m2	1.21	0.002**	0.47	2.04
flocks_size_1000	0.07	0.003**	0.03	0.12
flock_size_previous_1000	0.07	0.003**	0.02	0.11
n_parents_f2	0	0.005**	0	0
FWM_previous_flock	1.34	0.005**	0.49	2.34
avg_parent_age_weighted	-0.04	0.052*	-0.09	0
age_parents_f2	-0.05	0.053*	-0.1	0
avg_progeny_weight_p2	-0.08	0.083	-0.17	0
total_dark_d2	-0.15	0.111	-0.36	0.02
FWM_previous_12mnths	0.58	0.113	-0.14	1.3
empty_days	0.01	0.121	0	0.03
total_dark_d3	-0.1	0.192	-0.26	0.05
max_parent_age	-0.02	0.208	-0.06	0.01
n_parents_f1	0	0.235	0	0
age_parents_f1	-0.02	0.273	-0.06	0.02
temp_d2_max	0.16	0.408	-0.22	0.56
temp_d2_min	0.15	0.409	-0.2	0.52
avg_progeny_weight_p1	-0.03	0.41	-0.12	0.05
flock_size_diff_relative	2.15	0.448	-3.47	7.87
temp_d1_min	0.1	0.45	-0.13	0.42
flock_density	-0.07	0.515	-0.3	0.15
RHpct_d3_max	0.02	0.52	-0.03	0.07
temp_d3_max	0.1	0.566	-0.25	0.46
temp_d3_min	-0.05	0.715	-0.32	0.22
RHpct_d1_min	0.01	0.737	-0.03	0.05
RHpct_d2_max	0.01	0.768	-0.03	0.05
total_dark_d1	-0.03	0.791	-0.25	0.17
parents_f_fraction_max	-0.19	0.828	-1.93	1.55
RHpct_d3_min	0.01	0.841	-0.05	0.06
temp_d1_max	0.04	0.856	-0.36	0.43
n_parent_flocks	0.02	0.929	-0.48	0.52
difference_parent_ages	0	0.936	-0.09	0.07
RHpct_d1_max	0	0.942	-0.04	0.03
RHpct_d2_min	0	0.945	-0.05	0.05



RESULTS

Flocks and Data Collection

The study included data from 46 case flocks and 92 control flocks, a total of 138 flocks. The 138 flocks were distributed at farms from mid- to south-west and south-east Norway. The flocks were from three different hatcheries and three different hybrids (Table 1). The matching criteria *Geographical Location* and *Hatching Date* were fulfilled for all flocks, but for *Hybrid* there were six exceptions where the hybrid did not match. The four control flocks that did not match on hybrid were of hybrid Ross 308, and the two matching case flocks were of hybrid Rowan Ranger.

From each flock, 91 variables were collected from data compiled by the companies included in the study. Thirteen additional variables were constructed from the original variables, resulting in a total of 104 variables.

Descriptive Results and Data Analysis

Of the 104 variables, 34 variables were removed due to a lack of data from ≥70 flocks. Another 33 variables were removed from the analysis due to; matching criteria (5), data describing the flock after day three of life (17), variables identifying the flock (2), too many factor levels for the number of flocks included in the analysis

(3), unreliable data (human error) (3) and similarity to other variables (redundant) (3). Altogether 37 variables were kept for further analysis and are depicted with the results of the descriptive analysis in (Table 2).

The univariate analysis showed seven variables with *P*-values <0.05: *Size of Poultry House*, *Flock Size*, *Flock Size Previous Flock*, *Number of Offspring from Parent Flock Two*, *High First Week Mortality in Previous Flock*, *Average Parent Age* and *Age of Parent Flock 2* (Table 3).

The average *Size of the Poultry House* was 1210m^2, ranging from 150 to 2680m^2. The *Flock Size* (present) ranged from 2500 to 43200 birds, with an average of 19880 birds. The *Flock Size Previous flock* ranged from 2600 to 43920 birds, with an average of 19950 birds. *Number of Offspring from Parent Flock Two* ranged from 119 to 23600 chicks with an average of 8692.94, and the *Average Parent Age* was 39.09 weeks, ranging from 25 to 64 weeks (Table 2).

In 69 out of 92 (75%) of the control flocks, the previous flocks also had low FWM, while 21 out of 45 (46%) of the case flocks had low FWM in the previous flock. Further, 23 out of 92 (25%) of the control flocks experienced high FWM in the previous flock, while 24 out of 45 (53%) of the case flocks also experienced high FWM in the previous flock (Table 5).

From the correlation analysis three absolute values were above 0.5: *Flock Size-Flock Size Previous Flock* (0.99), *Flock Size-Size of Poultry House* (0.98) and *Flock Size-Number of Offspring from Parent Flock 2* (0.55) (Table 2).

Risk Analysis

A logistic regression model including all variables with a *P*-value <0.05 from the univariate analysis, but replacing correlated (>0.5) variables (*House m2* with *Flock Density*, *Flock Size Previous* with *Difference in Flock Size* and *Number of Offspring from the Second Parent flock* with *Max Fraction from a Parent Flock*) resulted in two variables with *P*-values <0.05. The two variables were *FWM Previous Flock* and *Flock Size* (both $P = 0.011$) with an estimated risk (OR) of 3.33 (CI = 1.45 – 9.16) and 1.07 (CI = 1.02 – 1.13) respectively (Table 4). The AUC was calculated to be 0.747.

This translates to a 233% increase in odds for high FWM if the previous flock also had high FWM. Further, by increasing the flock size by 1000 birds the odds for high FWM increases by 7%.

Table 4: Multivariate regression model

Variable	**OR**	**P.value**	**Conf.low[a]**	**Conf.high[b]**
FWM_previous_flock	3.33	0.011*	1.45	9.16
flocks_size_1000	1.07	0.011*	1.02	1.13
flock_size_diff_relative	366.98	0.103	0.32	525390.67
flock_density	0.82	0.139	0.62	1.06
avg_parent_age_weighted	0.97	0.21	0.92	1.02
parents_f_fraction_max	1.4	0.754	0.17	12.08

The area under the curve (AUC) was for this model calculated to be 0.747

[a]*The lower range of the confidence interval for the odds ratio (OR) for the variable*
[b]*The upper range of the confidence interval for the odds ration (OR) for the variable*

Table 5 Two by two table for High First Week Mortality Previous Flock*, including the frequency in percent for the case and control flocks.*

	(n)Low FWM Prev.[a]	(n) High FWM Prev.[b]	(n)Total[c]	(%) Frequency of High FWM Prev.[d]
Control Flocks	69	23	92	25
Case Flocks	21	24	45	53,33
Total	90	47	137	

[a]*Number of flocks with First Week Mortality (FWM) below 0.8% in the previous flock*
[b]*Number of flocks with First Week Mortality (FWM) above 0.8% in the previous flock*
[c]*The total number of flocks included in the study where First Week Mortality in the previous flock was reported*
[d]*The frequency of high First Week Mortality (FWM) in percent*

DISCUSSION

This study identifies an increase in *Flock Size* and *High FWM in the Previous Flock* as possible risk factors for high FWM due to colisepticemia in broiler chicken flocks where biosecurity is normally considered high.

In Norway, the largest broiler chicken flocks are kept in modern poultry houses built after 2015. At slaughter, all flocks are checked for footpad lesions as an indicator for animal welfare, independent of flock size. If one flock scores high on footpad lesions, the farmer is penalized by restricting the size of the following flock according to the national Animal Welfare Program for broilers as described in the regulations on the keeping of chickens and turkeys (Refsum, 2014; Animal Welfare Regulation, 2017). Animal density in Norwegian broiler chicken flocks is the same for large and small flocks, except for those that have been penalized for high footpad scores.

As *Flock Size* is not an indicator of high density, *Flock Size* might be an indicator of management capacity in a disease or outbreak situation.

In case of disease outbreaks, the capacity to remove dead and morbid chicks within a reasonable time period has been demonstrated to be important in the reduction of the horizontal spread of disease within the flock (Christensen, et al., 2021). Factors affecting this capacity, such as *Flock Size* and *Other Professional Activities* outside the broiler house should be considered.

Van Limbergen *et al.* (Van Limbergen et al., 2020) identified that the FWM was higher if the farmer had professional activities outside of broiler chicken production, compared to if the farmer had no other professional activities. This may reflect the farmer's capacity to spend an increased amount of time tending to the flock in certain situations. One might assume that farmers with smaller flocks would need an extra income

from other professional activities, and that larger *Flock Size* and *Other Professional Activities* would therefore, be negatively correlated. Individually, both larger *Flock Size* and *Other Professional Activities* may point towards the farmer's inability to tend to the flock when an increased workload is required, such as in a disease outbreak situation. Increased work force was suggested as one of the reasons as to why Chou *et al.* identified larger flocks as having a positive effect on FWM (Chou et al., 2010).

Increasing work force in Norway might not be an economically feasible solution. Another approach might be to divide flocks between several houses if available, or to divide large houses into smaller cells, in order to reduce the chance of horizontal transmission within a flock.

High FWM in the Previous Flock was also identified as a variable of importance for increased FWM in consecutive flocks. However, the data collected did not provide any information as to whether this is due to pathogen survival, or an indirect consequence of *Flock Size* in the previous flock.

Incomplete cleaning of drinking water lines has been identified as a risk factor for APEC in the consecutive flock (Vandekerchove et al., 2004; Christensen et al., 2021). Further, studies conducted in Norway during 2014 and 2015, showed that the presence of extended-spectrum beta-lactamase (ESBL) resistant *E. coli* in the previous flock increased the risk for the subsequent flock in the house being ESBL positive (OR of 3.1). It was also shown that disinfection of the floor between production cycles reduced the odds of positive ESBL status (Mo et al., 2016; Mo et al., 2019). This coincides well with the results from the present study, where the OR for high FWM was 3.3 if the previous flock experienced high FWM and highlights the survival of APEC between flocks as an interesting focus area for future research.

Escherichia coli is ubiquitous in the poultry environment and, even with increased focus on disinfection and other hygienic measures, is possible that it survives between each flock. What is not clear is how the pathogen survives in the house (Bojesen et al., 2022).

Survival of APEC in the poultry environment between flocks and after wash and disinfection could be a result of human error (incorrect dilution of disinfectant, insufficient pre-cleaning and inappropriate disinfectant), *E. coli* resistance to disinfectants or biofilm formation in drinking water, cracks or crevices. A study by Newman *et al.* (Newman et al., 2021) identified strong and moderate biofilm-producing properties in more than 50% of the APECs examined in their study, further supported in studies by Projahn *et al.* (Projahn et al.,2017). Our study supports the need to study the presence, prevalence and potential reasons for *E. coli* survival between flocks within the poultry house (Bojesen et al 2022; Christensen et al., 2021).

The possible persistence of APEC within the house indicates not only the need for future studies, but also perhaps a more urgent need to professionalize the cleaning and disinfection routines on the broiler chicken farm. Our data revealed that several farmers employ professional companies for the cleaning and disinfection of the broiler house between flocks. However, due to uncertainties in the answers provided in the questionnaires and a lack of data, we decided to exclude this variable from the present study. We can therefore

not conclude that the professionalization of the disinfection routines will further prevent infection in consecutive flocks.

The size of this study limits the use of multilevel variables describing type of disinfectants used, parent flocks contributing to the broiler chicken flock (vertical transmission) and feed company or feed-mill supplying fodder. A larger study would be able to investigate these variables as potential risk factors.

Other variables were excluded as they were part of the matching criteria, such as: *Season* (hatching date) and *Hybrid*. In this study, both Hubbard JA 787 (slower growing hybrid) and Ross 308 (fast growing hybrid) were included. Hybrid selection may have been an interesting variable, as it has been hypothesized that selection for rapid growth requires a redirection of nutrients towards growth at the expense of bacterial resistance (Nolan et al., 2020). *Season* was also identified as a risk factor for cumulative FWM in 2020 (Yerpes, et al. 2020).

This study was a retrospective matched case-control study. However, care must be taken interpreting statistical significance via *P*-values in the context of retrospective matched case-control studies. The theory of statistical significance and *P*- values is well-suited for randomized controlled trials, but such trials are not suited for exploratory investigation. When we discuss statistical significance ($P < 0.05$), we only claim that results are not random artefacts. We do not claim to have established any causal links through this study alone, but we explore which links are more likely than others. Additional studies are therefore, needed to conclude the effectiveness of our recommendations and to guide the development of recommendations for the prevention of high FWM due to colisepticemia within a farm.

Norway has for the most part reached its goal of low first week mortality rates within broiler chicken production. In comparison, Yerpes *et al.* (Yerpes et al., 2020) reported 1.82% as the average FWM in a Spanish study conducted during 2015- 2018, and Heier *et al.* (Heier et al., 2002) reported a FWM of 1.5% in Norway during 1996-1999. This study has compared outbreaks of neonatal colisepticemia in a population of broiler chickens with presumably good management and a high level of biosecurity, to flocks where neonatal colisepticemia did not spread throughout the flock.

The present study showed an OR of 1.07 for high FWM with every thousand birds increase in a flock. Further, the study showed an OR of 3.33 for high FWM if the previous flock also experienced high FWM. Well-planned studies for the reasons behind these observations are of interest and the results may guide the poultry industry to implement recommendations for the prevention of high FWM due to colisepticemia within a farm.

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