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Multi-Resistant *E. coli* In Long-Distance Migratory Geese: How Greylag Geese (*Anser anser*) And Pink-Footed Geese (*Anser brachyrhynchus*) Can Act As Vectors For Antimicrobial Resistance

Multiresistent *E. coli* i langdistansetrekkende gås: Hvordan grågås (*Anser anser*) og kortnebbgås (*Anser brachyrhynchus*) kan fungere som vektorer for antimikrobiell resistens

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Migratory birds can carry antimicrobial resistant *E. coli* over large distances. Recent studies show antimicrobial resistance in bacteria from wildlife, carrying resistance genes against drugs listed on the "WHO's List of Essential Medicines". During autumn 2015 and 2019, a total of 201 cloaca samples were collected from Greylag and Pink-footed geese landing close to farm areas in the middle of Norway during their flight towards their wintering grounds. A total of forty-three samples screened in 2015 (39%) contained *E. coli* expressing a resistance to one or more drugs, whereas 26 of the samples from 2019 (29%) contained resistant strains with close to equally the same resistance patterns. We found isolates resistant to quinolones, tetracyclines and other types of antibiotics. Several isolates had multi-resistant patterns. Isolates resistant against colistin belonged to ST720, which is identified as a possible pathogenic sequence type through phylogenetic identification (phylotype D). Around one third of the birds sampled contained *E. coli* resistant against one or more antimicrobial agents, indicating that migrating birds can act as vectors for resistant bacteria.

Definitions and abbreviations

AMP	Ampicillin
AMR	Antimicrobial resistance
ARG	Antimicrobial resistance gene
AST	Antimicrobial susceptibility testing
AZI	Azithromycin
CHL	Chloramphenicol
CIP	Ciprofloxacin
COL	Colistin
CRDR	Colistin resistance determining region
CTX	Cefotaxim
GEN	Gentamicin
FOT	Cefotaxim
MERO	Meropenem
MIC	Minimum inhibitory concentration
PMQR	Plasmid mediated quinolone resistance
RG	Resistance gene
SMX	Sulfamethoxazole
SXT	Sulfamethoxazole-Trimethoprim
TAZ	Ceftazidime
TET	Tetracycline
TGC	Tigecycline
TMP	Trimpethoprim
QRDR	Quinolone resistance determining region
VG	Virulence gene
WGS	Whole genome sequencing

Introduction

Antibiotics and the global health situation

The World Health Organization (WHO) describes antibiotic resistance as *"one of the biggest threats to global health"* to this day, and has implemented a global action plan to reduce the misuse of antibiotics (1). There is a widespread use of antibiotics throughout both the medical and the veterinary sector, resulting in increased antimicrobial resistance in life-threatening pathogens. In 2016, WHO updated the list for critically important antimicrobials for human medicine, where they rank quinolones, cephalosporins (3rd and higher generation), macrolides and ketolides, glycopeptides and polymyxins as the *"Highest Priority Critically Important Antimicrobials"* (2). In this study, we will primarily focus on the polymyxins, quinolones and the extended-spectrum cephalosporins, in addition to some highly important antimicrobials.

Mechanisms of resistance

Intrinsic resistance

Antimicrobial resistance can be intrinsic, adaptive or acquired.

Intrinsic resistance refers to inherent properties within a microorganism that limits the effect of antimicrobials through various mechanisms. It differs from acquired resistance in that it is independent of previous antibiotic exposure and is not due to horizontal gene transfer (3). Among the most relevant causes of intrinsic resistance are lack of target for the antibiotic, activation of chromosomally encoded antibiotic-inactivity enzymes and reduced uptake of the antibiotic through reduced permeability of the outer membrane (OM) and expression of drug efflux pumps (4). Gram-negative (GN) bacteria, like *E. coli*, are intrinsically more resistant than Gram-positive bacteria (5). This is due to the OM, composed of an outer leaflet of lipopolysaccharides and an inner leaflet of phospholipids, acting as a permeability barrier that

prevents antibiotics from reaching their targets (6). *Pseudomonas Aeruginosa* is a prime example of a GN bacteria with extended intrinsic resistance and a large repertoire of efflux proteins, some of which can transport a wide range of structurally dissimilar substrates and are classified as multidrug resistance (MDR) efflux pumps. Clinically, *E. coli* is intrinsically susceptible to almost all relevant antimicrobial agents, but it has a high capacity to accumulate resistance genes though horizontal gene transfer, rendering some of the most critically important antibiotics useless (7).

Adaptive and acquired resistance

Bacteria can also adapt, acquire or develop resistance to antibiotics. Adaptive resistance is when an organism is able to alter the activity of transcription activators, in response to external stress factors like nutrient limitations or antibiotics, in order to increase its chance of survival. An example of this is through the adjustment of membrane permeability, made possible through down- or up-regulation of membrane porin proteins (4, 8). Acquired resistance is the acquisition of genes or the result of mutations making a previously susceptible organism resistant to a particular antibiotic. Plasmids encoding beta-lactamase genes and mutations in transcription genes of efflux pumps in *E. coli* are examples of acquired resistance (9).

Mobile genetic elements

The pan-genome of *E. coli* consists of a conserved core genome containing genetic information that is essential for normal functioning of the bacteria and a flexible gene pool of useful, but non-essential genetic elements, giving the bacteria specific properties like antibiotic resistance and toxin production. It is the parallel gain and loss of these mobile genetic elements which enabled the evolution of separate clones of *E. coli (10, 11)*. Part of

this flexible gene pool includes mobile genetic elements like plasmids, transposons, insertion elements and gene cassettes. Although not necessary under most growth conditions plasmids carry useful genes for the bacteria and play a major role in the dissemination of resistance genes. *E. coli* represents a large reservoir of genes conferring resistance. Through horizontal gene transfer from one bacterium to another *E. coli* can act as a donor and as a recipient of these genes, leading to treatment failure in human and veterinary medicine. Since plasmids represent an added genetic load they must be maintained by positive selection and would be lost in the absence of antibiotics. This leads to the coselection and persistence of resistance to critically important drugs, through the use of less critical drugs in veterinary medicine (tetracycline, sulfonamides), when the genes are located on the same genetic element (7, 12)

Polymyxin E (Colistin) and polymyxin B

Antimicrobial resistance can occur through plasmid-mediated mechanisms or through single nucleotide polymorphism (SNP) in genes in the chromosome. The discovery of the plasmid-mediated gene, *mcr-1*, in 2015, has proven for the world that the last-resort antibiotic polymyxin E, also known as colistin, is in critical danger of becoming ineffective against diseases caused by bacterial pathogens. Selection pressure by the use of colistin to livestock has contributed to the evolution and spread of *mcr-1*, including spread to bacteria infecting humans (13). Because of the discovery of *mcr-1*, and the implementation of whole genome sequencing, researchers all over the world started looking for the *mcr-1* gene in human, livestock and wildlife samples. By analysing isolates from food animals, the environment, food, and humans, researchers have found *mcr-1* positive isolates in several other countries, in addition to China (14). Acquired resistance mechanisms against colistin are also described as chromosomal mutations in genes that are a part of different two-component regulatory systems like the PmrAB and PhoPQ. These signal transduction systems are the major means

by which bacteria are able to mount an appropriate response to a variety of environmental stimuli. They consist of a membrane bound sensor protein and a corresponding response regulator (15). The chromosomal mutations are activated by mutagens and colistin residues in the environment, leading to modification of the lipopolysaccharides in the cell membrane of the bacteria, which may result in increased resistance against colistin (16). Both the plasmid-mediated resistance mechanism of *mcr-1* (through horizontal transmission) and the acquired chromosome-mediated resistance mechanism (through vertical transmission) of the two-component system can result in ineffective colistin therapy.

Quinolones

Quinolones are broad-spectrum antibacterial agents and, as bacterial resistance erodes the effectiveness of other agents, their popularity are increasing. Especially in low- and middle – income countries the consumption rate is climbing rapidly as of 2015 (17) and today quinolones still remain one of the most used group of antibiotics worldwide (18). Nalidixic acid (NA), the first quinolone to be developed, was discovered as a by-product of chloroquine synthesis (19). It is a first generation quinolone with narrow-spectrum against enteric bacteria and have been used for treating urinary tract infections in humans since its introduction into clinical use in 1967 (20). Since then, several derivatives have been synthesized, increasing both their antibacterial potency (21) and importance as key therapies to threat infections. This was made possible, in large, by the breakthrough development of fluroquinolones, like ciprofloxacin, which had a much broader spectrum of activity compared to the first-generation quinolones. These second-, third- and fourth-generation quinolones are active against both Gram-negative and Gram-positive pathogens, as well as *Mycobacterium tuberculosis*, the causative agent of tuberculosis (22), making them useful agents for the treatment of systemic and respiratory tract infections in addition to urinary infections (21). Over the past five

decades the drug has gone from being used on a small scale to becoming one of the most commonly prescribed antibacterial classes in the world (20). Ranging from the use in medical practice to the use in veterinary practice, quinolones like NA and fluroquinolones like ciprofloxacin are widely known antibiotics with increasing prevalence of resistance. A study done in China, where *E. coli* strains were isolated from diseased chickens (n = 71) and swine (n = 89), showed that 100% of the isolates were resistant against NA and 79% were resistant against ciprofloxacin, in addition to being multidrug resistant (MDR) towards other classes of antibiotics (23). This study underlines the critical situation of quinolone resistance and the dire need to reduce the use of critically important antimicrobials in veterinary practice. Plasmid-mediated quinolone resistance (PMQR) has been under surveillance for a longer time than colistin resistance (CSTR) and its resistance mechanisms are well known. There are three families of plasmid-encoded resistance mechanism that have been identified: (1) The *qnr*-like proteins (QnrA, B, C, D and S), protecting DNA from quinolone binding (2) the AAC(6`)-lb-cr resistance protein that modifies fluroquinolones, and (3) efflux pumps like OqxAB, QepA1, and QepA2(24). These resistance mechanisms often lead to reduced susceptibility and do not confer a high level of resistance, but can increase the selection pressure on isolates with higher levels of resistance though chromosomally mediated mechanisms (25). Furthermore, mutations in the quinolone resistance-determining region (QRDR) of the *gyrA* and *parC* genes in the chromosome is the main mechanism behind resistance against quinolones. Single mutations will often confer resistance to narrow-spectrum quinolones, while resistance against broad-spectrum fluroquinolones requires multiple mutations within the gyrA and/or parC and can promote high-level resistance through vertical transmission (7, 26).

Cephalosporins

Cephalosporins can be divided into five different generations according to their antibacterial activity and are part of an important group of antibiotics: the beta-lactam antibiotics (9). Antibiotics within this group share a common feature, which is a beta-lactam ring that is highly reactive and causes cell lysis through binding of penicillin-binding proteins (PBS) and thereby inhibiting cell wall synthesis (27). The newer generation cephalosporins, like 3rd, 4th and 5th generation cephalosporins, have been classified as "critically important antimicrobials" in human medicine by the World Health Organization (WHO). Unfortunately, the production of broad-spectrum beta-lactamases is a major problem in many countries and the occurrence of infection has become more frequent in Norway as well. Through surveillance/screening of clinical isolates from humans, 6.5% of *E. coli* and 6.6% of *Klebsiella spp.,* isolated from blood cultures, were identified as ESBL-positive in 2018. The prevalence, compared to previous years, seemed to be stable for *E. coli* (6.6% in 2017) while it was increasing for *Klebsiella spp.* (5.3% in 2017) (28). In many European countries, there has been a dramatic increase in the prevalence of *E. coli* resistant towards newer generation cephalosporins in broilers and broiler meat (29-31). These isolates likely contribute to the increased incidence of infections with these bacteria in humans and pose an additional concern for the public health (31). A study from Denmark investigating the prevalence of extended-spectrum cephalosporinase (ESC) found a high prevalence of ESC *E. coli* in imported broiler meat (36%) (32) compared to other meat types. Even in Norway resistance towards ESC har been widely disseminated in the broiler production with a total of 43% of flocks being positive for ESC-resistant *E. coli* in 2011. The isolates carried the plasmid-borne blaCMY-2 gene, despite no selection pressure from cephalosporin usage in Norway. Research have clearly demonstrated that the use of cephalosporins in hatcheries, to control the early mortality rate associated with *E. coli*, promotes the selection of cephalosporin resistant

E. coli (33). Restricting, or stopping, the usage of cephalosporin in food animals would be a highly effective measure in preventing this self-promotion of resistance (29). With the source of introduction originating from imported breeding animals, the Norwegian poultry industry had to introduce other measures. In 2018 only a single flock of broilers was positive for ESC-resistant *E. coli (28).* This underlines the importance of ongoing surveillance as a fundamental component of the strategy against microbial resistance, and that, in the presence of widespread resistance, introducing measures to limit the occurrence can be highly effective.

Other important antibiotics in human and veterinary medicine

Even though both mentioned classes of antibiotics are of critical importance, our study also includes less critical, but highly important classes of antimicrobials like tetracyclines, aminoglycosides, trimethoprim and sulfamethoxazole. Multidrug resistance occurs in *E. coli* isolates with resistance against colistin and/or quinolones, and further complicates antibiotic therapy. In 2015, multidrug resistant *E. coli* was isolated from cattle in Spain, carrying the *mcr-1* and *mcr-3* genes, in addition to being resistant against NA, ciprofloxacin, gentamicin, tetracyclines, trimethoprim and sulfamethoxazole, as well as other important antimicrobials (34). In this study, we will try to explain how wild birds can act as vectors for multidrug resistant *E. coli*.

Management of long-distance migratory birds

Norway's ideal geographical position makes a great place for a pit stop for long distance migratory birds. Their Nordic route usually involves heading to Svalbard in the mating season and from Svalbard in the non-breeding season. The stops along the way include farm fields, with either seeds or unharvested crops, making almost all migratory birds a verminous parasite for farmers. Due to strict wildlife management of the Greylag goose (*Anser anser*)

and the Pink-footed goose (*Anser brachyrhynchus*), hunting is limited, and these birds have free access to the farm fields between 1st of March and 30th of September, meaning that the species are red-listed in this period. However, there are legal options, in which the local government can issue a permission to hunt the birds if they are causing too much damage to the farm fields, and thereby compromising the farmer's livelihood. Damage can be extensive, and not only do they feed on the crop, but waste products are also left behind on the ground, containing, amongst other microbiota, different *E. coli* strains from other areas of Europe. In this study, we examined cloacal samples from these birds, and analysed *E. coli* isolates to evaluate the severity of antimicrobial resistance in long distance migratory species.

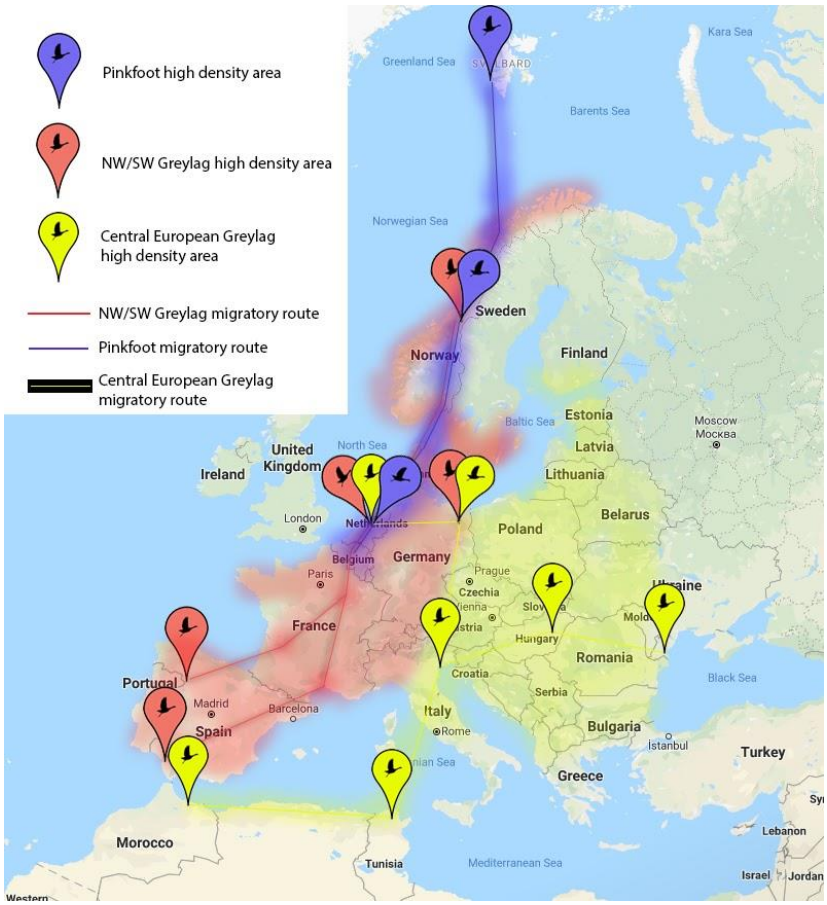


Figure 1: Migratory patterns and habitats of greylag geese and pink-footed geese.

Greylag goose (*Anser anser*)

Habitat and population management

The Greylag goose (*Anser anser*) is considered a highly adaptable species, thriving in a range of different habitats like swamps, farmlands and areas closely connected to sewage spill-offs. However, due to loss of habitat and an overly liberal hunting management plan, the Greylag goose in Western Europe became near extinct in the middle of the 20th century (35). In the 1980s, there was an estimate of 120,000 geese in total in Europe. To counter this problem, the *United Nations Environment Programme* (UNEP) introduced an international management plan under the *Agreement on the Conservation of African-Eurasian Migratory Waterbirds* (AEWA), called *European Goose Management Platform* (EGMP), which focus on four bio-geographic populations. These populations include the Icelandic population, the British/Irish population, the Northwest/Southwest (NW/SW) population, and the Central Europe population. The goal of the management plan was to create a strategic plan to increase the number of individuals within each of these populations, by constructing conservation guidelines (36). As a result, the West- and Central European population increased by a seven-fold, totalling to around 900,000 individuals by the end of 2009, with estimates of a continuous increase in numbers in the 2010s (37). The success of the management plan lead to a socio-ecological disaster, as the sheer number of Greylag geese can be linked to damage on farmland, destructive changes in local ecosystems, and challenges in public health and air safety. An increase in total population also lead to birds nesting closer to human settlements, increasing cross-species interactions through landfills and waste spill-off. Again, the AEWA was forced to act, introducing the *International Single Species Management Plan for the Greylag Goose* (38), in which the populations in NW/SW Europe is to be controlled by defining favourable reference values for the population, provide protocols for adaptive

management, and other guidelines for making sure that the population does not supersede a sustainable amount.

Migratory patterns

The migratory patterns of the Western Greylag goose in the NW/SW flight route can be explained through its northbound flight during the spring and summer, and its southbound flight during the autumn and winter. It is very difficult to determine the exact country of origin for each individual, as the Greylag goose can be found as far south as the African continent. However, tagging of individuals and the use of surveillance programs can help us locate geese and their respective habitats during overwintering and mating season. Even though seasonal changes dictate the time in which the geese should start migrating, studies have shown that there is a shift in the migratory patterns of the Greylag goose, where populations seem to overstay their welcome during mating season. In this study, Greylag goose samples were collected as late as October, during their southbound flight.

Mating season

Mating season in Norway is estimated to occur between the start of April to the start of September. Captures and tagging of individuals helps us understand the usual areas of breeding, where most captures occur along the coastline, ranging from within the Oslo fjord in the south-eastern part of Norway, to the Varanger fjord in Troms og Finnmark, the northernmost county of Norway. The highest density of birds can be found somewhere in the middle of Norway, in the southern part of Nordland county. The density of birds are based on resighting probabilities during the whole year (39). Within this period, hunting is prohibited

and in 2017, the number of breeding Greylag geese was estimated to be around 20 000 – 25 500 pairs in the highest density area.

Overwintering

Overwintering for the NW/SW migrating Greylag goose usually starts in September and lasts until the end of March. Overwintering habitats are distributed all over Europe, and sightings of the Greylag goose have been reported as far south as North Africa. The birds that overwinter in North African countries are mostly connected to the Central Europe/North African flyway, connecting Italy and the Adriatic countries to the African continent through breeding Greylag geese. However, surveillance studies have shown that overwintering in Tunisia and Algeria have not occurred since 2004, due to global climate changes (40). The southbound flight of the birds that breed in Norway, stretches as far as the southernmost point of Spain, with high density populations in the western part of the region of Andalucía. There is also observed a high-density population in the western part of the region of Castilla y Leon. The last high-density population spotted, using resighting probabilities, is in the northern part of Belgium, spreading through the Netherlands and into the western part of Germany. A low density resighting has also been spotted in Nordland county in Norway in the overwintering season, making some breeding goose non-migratory during the year (40). Hunting season is declared open during this period, and there is an estimated amount of 15 000 – 20 000 Greylag geese shot each year. This amount is less than half of what is recommended according to the Norwegian Institute for Nature Research (NINA) to effectively manage the species.

Anser anser rubrirostris and the Asian connection to Europe

Distinctly known for its paler plumage and pink bill (in comparison with the Western Greylag goose, which has an orange bill and an eye ring), the Eastern Greylag Goose (*Anser anser rubrirostris*) is a known subspecies of the Greylag goose. Its biogeographical area stretches across Asia, from the North Western parts of China, through the Anatolian plateau and southern part of Russia, all the way west to Eastern Europe. In addition to this route, populations have also been spotted throughout the southern part of Russia, passing through the Ural Mountains. This latitudinal migration is of interest, as the subspecies integrate with the Central European Greylag goose on the European continent. The species is also able to hybridize with many other Anseriformes species, like the Barnacle goose (*Branta leucopsis*) and the Canada goose (*Branta Canadensis*) (41). There are several different migratory routes, depending on the different subpopulations of the eastern species. There is also a report of a longitudinal migratory route between wintering sites close to the Yangtze River (of the Eastern part of China), and breeding sites in eastern Mongolia (42). By both having long-distance longitudinal and latitudinal migratory routes, the Eastern Greylag goose covers a lot of ground on the Asian continent, as well as the European continent.

Pink-footed goose (*Anser brachyrhynchus*)

Habitat and population management

Also protected by the AEWA EGMP action plan, the Pink-footed goose (also called "*pink feet*") was close to becoming extinct in the middle of the 20th century, much like the Greylag goose. With estimates as low as 15 000 in the mid-1960s, the pink feet was saved by a management plan worked out by the UNEP. Today, the total population of the Pink-footed goose is estimated to be around 80 000, based on marked birds (43). What differs largely from

the Greylag goose in choice of habitat is their migratory flight from Flanders in Belgium, all the way to western Svalbard during mating season. During their flight, they stop in the far end of Trondheimsfjorden, in the middle of Norway. Here they feed and rest for two to four weeks before continuing their northern or southern flight. This small piece of land is predominantly farming ground and the sheer number of birds landing there during the migratory seasons, poses a problem considering harvest and bird droppings. The last stop before leaving the Norwegian mainland is the Lofoten islands, more specifically in Vesterålen, in north Norway. Most of the geese leave Vesterålen in the third week of May during their northbound flight and throughout September during their southbound flight (44). The barren Arctic Archipelago of Svalbard remains untouched by some of the emerging diseases spreading throughout the mainland of Norway and the rest of Europe. Recent increases in total gees ? population leads to socio-ecological problems regarding, not only agricultural damage, but also human and animal health, and ecosystem effects (35). Reports show that migratory connectivity affects eutrophication in different ecosystems, and can further promote infectious diseases spreading potentially from mainland Europe to the islands of Svalbard (45).

Migratory patterns

Whereas the western Greylag goose can be divided into four subpopulations, the pink feet are divided into two biogeographical populations: the Greenland/Iceland population and the Svalbard population. In this article, we will focus on the latter of the subpopulation. By looking at the migratory patterns of the Pink-footed goose, we might be able to get an understanding of how the species can act as a possible vector for antimicrobial resistance between different types of habitats. It is important to have in mind that the AEWA committee have reported changes in the migratory patterns of several long-distance migratory species during the past few decades. Climate change, economic possibilities, agricultural

intensification, changing traditional wet grasslands into fields by draining and ploughing (Belgium), and overgrowing of grassland habitats (Norway) are amongst some of the root causes for habitat- and land use change (44).

Mating season

According to AEWA, the start of the northbound migration for the Svalbard population was in 2011 said to have advanced by more than a month. Now, mating season starts with sightings of pink feet leaving Denmark in the beginning of April. Population numbers in areas around Trondheimsfjorden peak from late April to mid-May, confirming their path towards their breeding ground on the western parts of Svalbard. Between Trondheimsfjorden and Svalbard, the pink feet stops in Vesterålen and Lofoten in the northern part of Norway in late May, before continuing their flight. In Svalbard, the nesting pink feet resides on islets on the western coast and on the arctic tundra, feeding on rhizomes and roots. Moulting geese gather along coastlines, usually with great distances from different sources of disturbances, avoiding especially humans on foot by one to two km. Mating season lasts until the end of September, before starting their autumn migration (44).

Overwintering

Between the early days of September and late October, the pink feet migrate the same way south as they did during the northbound flight. Stopovers in Vesterålen and Lofoten, as well as stop in the areas around Trondheimsfjorden, poses the same socio-ecological challenging Norway as during the northbound flight. By this time, hunting season is open, and measures are taken to try to reduce the population each year, without any big changes in the total population. The pink foot fly further south towards Denmark, the Netherlands, and ultimately

Belgium. Popular stopover sites are the Friesland and Vlaardingen areas in southwest Netherlands, before a majority of the pink feet travel to the Oostkustpolders in Flanders in Belgium. Numbers usually peak during December and January at this point. Because of global warming the pink feet have been observed on their migration back north towards breeding grounds as early as January when the winters are warm. There is considerable traffic of the different goose types in the areas along the pink feets migratory pattern. Interactions between the Bean Geese (*Anser fabalis*) and the White-fronted Geese (*Anser Albifrons*) occurs along the German Wadden Sea coast line (46).

The arctic challenge of Svalbard

The islands of Svalbard inhabit a diverse range of species, including several bird species and mammals like the Svalbard reindeer (*Rangifer tarandus platyrhynchus*), the Southern vole (*Microtus levis*), and the Arctic fox (*Vulpes lagopus*). All of the species that are endogenous to the islands are a part of a delicate ecosystem and are prone to suffer from changes in the global climate and political decisions made on the continental mainland. A continuous increase in the migratory pink feet population that arrive during mating season is affecting the arctic tundra ecosystem. The pink feet are overgrazing on the vegetation, where they are grubbing for roots and rhizomes, rendering holes and craters, which reduces the floras ability to regenerate. In addition to overgrazing, wild birds can be carriers of infecting agents, like avian influenza virus and the *Campylobacter* species. Even though human-goose interaction is close to none-existing on Svalbard, waste products from droppings can contaminate drinking sources and spread disease (46).

Antimicrobial resistance in wildlife

In human- and veterinary medicine, antimicrobial susceptibility testing and surveillance of antimicrobial resistance have existed for decades, due to the clinical significance of right treatment protocols of infectious diseases. In wildlife, clinical settings are close to non-existing, and therefore, not many AMR studies have been reported before the "One Health" initiative was put into focus. Several new studies report disseminations of different microbiota in areas where wildlife and anthropological activities intertwine. The theory of a shared intestinal microbiota between species can be proved further through new sequencing technology and mapping of microbial sequence types. In this study, we report a diverse range of *E. coli* sequence types in a relatively small number of isolates, whereas some can be linked to human pathogenic strains.

Wild birds as sentinels for antimicrobial resistance

Avian species protrude the natural barriers of the terrestrial and aquatic ecosystems by flight. This allows birds like long distance migratory waterfowl to hit their habitats with pinpoint accuracy, without passing through natural barriers on the Earth's surface. During their migratory season, waterfowls, like geese, make stopovers along the way to their final destination, often in a range of different countries. Stopovers include farm areas, river deltas and fresh water sources close to human populations. Spillover from anthropogenic sources through irrigation and sewage to these stopover sites is theoretically possible, and human interaction happens passively through waste products. The manure and biological solids applied to agricultural land might also contain residues of antimicrobials as well as resistant bacteria from farm animals (47). The significance of this should not be underestimated considering the high prevalence of antimicrobial resistance and wide use of antimicrobials in veterinary medicine, compared to Norway, in countries like the Netherlands, Belgium and

Germany, where the pink-footed and greylag goose overwinter in large numbers (48). Other wild birds, like seagulls, are reported as reservoirs for multidrug-resistant bacteria in beach areas where people congregate, and present a risk to the public health as transmitters of AMR through more direct human contact (49).

Aim

The aim of this study was to investigate the prevalence of antimicrobial resistant *E. coli* isolates in cloaca samples collected from long-distance migratory geese in Norway, using phenotypical and genotypical approaches. Population of geese were chosen on the basis of their huge increase in numbers the past 50 years, and their ability to travel large distances by using a diverse range of habitats throughout different countries.

Materials and methods

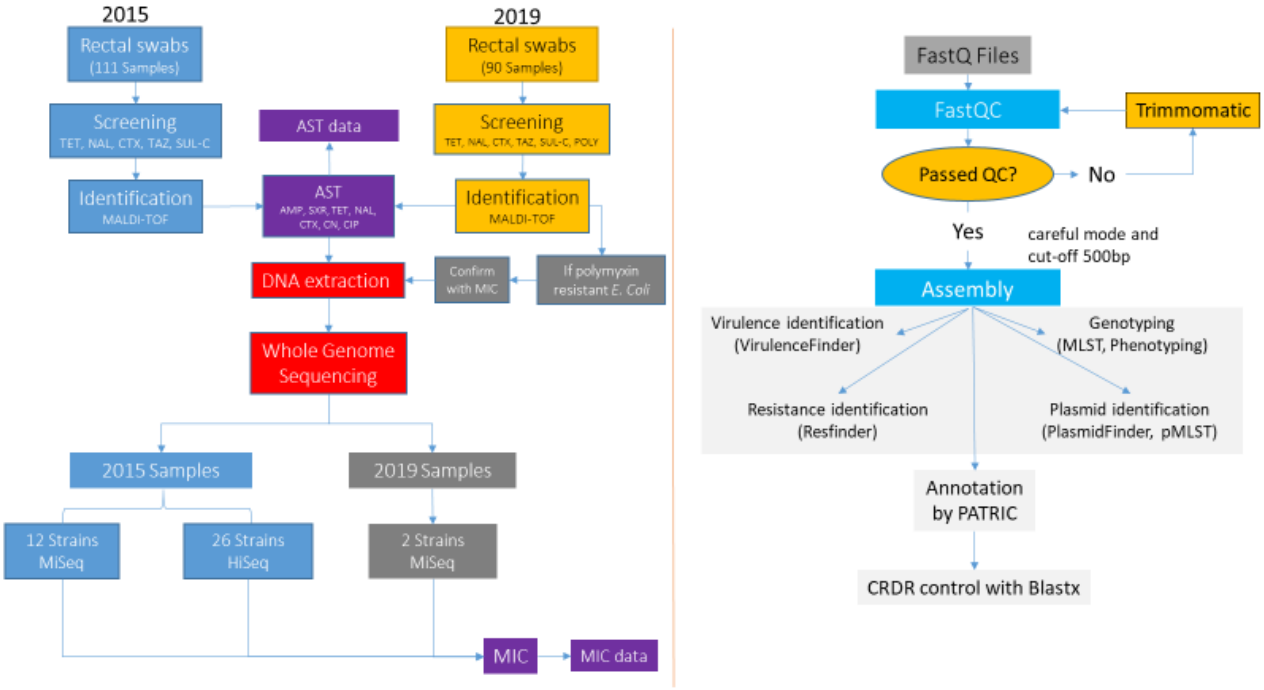


Figure 2: Schematic overview of the protocol used. Left side includes sampling, phenotypic analysis and DNA extraction. Right side includes the genotypic analysis of data from whole genome sequencing.

Sampling

During the autumn period of 2015 and 2019, a total of 201 cloaca samples were collected from Greylag geese and Pink-footed geese in Norway. The distribution of samples between the years collected is 111 and 90 respectively. Sampling took place on a farm field close to Nesset in the county of Trøndelag, where the geese are known to reside for a brief period of time before continuing their migratory flight. This converging point between the two species makes for an ideal place for hunting, due to the massive number of individuals landing there. However, hunting geese requires experienced waterfowl hunters, as the geese tend to react to any sudden sound or movement on the ground. In this study we used a local hunting team lead

by a veterinarian working for the Norwegian Veterinary Institute (NVI). This way, we managed to standardize the sampling procedure and uphold the emphasis on correct sampling and risk of contamination. Charcoal swabs were used during sampling. Transportation of the swabs was performed by using the postal service. Swabs were stored at cooling temperature for a maximum of 2-3 days upon arrival, before being utilized in the lab. It is worth mentioning that most of these samples were mainly gathered for the avian influenza surveillance program, which has been running for several years prior to our first sampling. This way, hunting experience and standardization of sampling from these specific host species can be recognized through other research projects run by the NVI.

Figure 3: Illustration of the location of sampling in Norway.

Phenotypical analysis

To determine the species of the bacteria and evaluate their resistance patterns, a phenotypic analysis in combination with mass-spectrometry was performed. By combining selective screening on MacConkey, antimicrobial susceptibility testing (AST) and minimum inhibition concentration (MIC), we were able to find resistance patterns in the samples collected. Further analysis using MALDI-TOF helped us determine the species using mass-spectrometry.

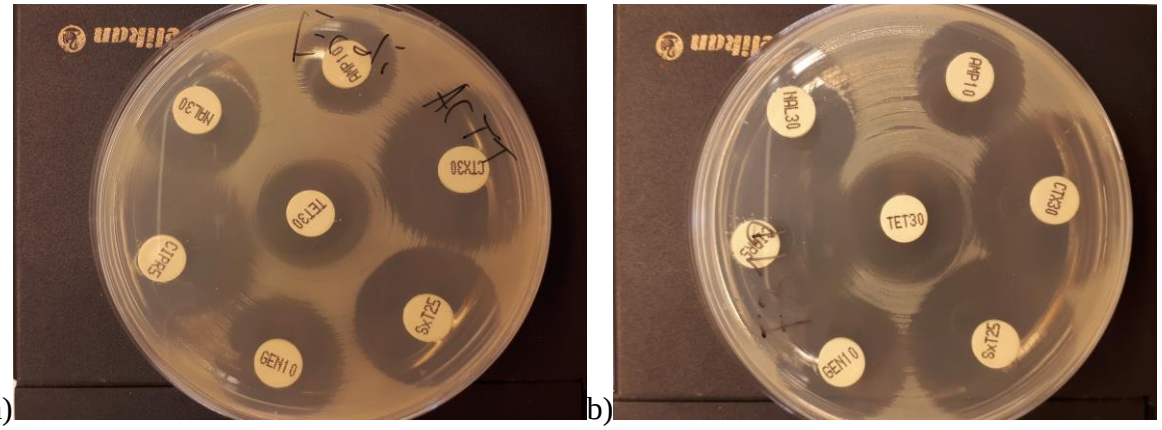
Selective screening

The following selective screening method for detection of resistant *Escherichia coli* was used: MacConkey agar (MC) and MC agars mixed with SUL-C [256 mg/L], TET [8 mg/L], NAL [16 mg/L], TAZ, CTX [1 mg/L] and Superpolymyxin [3,5 mg/L]. Charcoal swabs carrying cloaca samples were mixed in a 5 ml sterile water solution, before being smeared onto each of the plates. Incubation happened at 37° C, over 24 hours. A total of 111 cloaca samples from 2015 were tested, in addition to 90 cloaca samples from 2019. Blood agar was used for subculture and cold room storage.

Picture 1: Tetracycline resistant *E. coli* isolate subcultered on MacConkey.

Antimicrobial susceptibility testing (AST)

Testing using disc diffusion method was performed by following the EUCAST guidelines for area susceptibility testing. Isolates were smeared on to Mueller-Hinton agar, from recently subcultured isolates, and the following antibiotic tablets were used: AMP (10µg), SXT (25µg), TET (30µg), NAL (30µg), CN (10µg), CTX (5µg) and CIP (5µg). Incubation happened at 37° C, over 24 hours. EUCAST clinical breakpoints (50) were used as reference for our findings. 33 samples were tested using AST from the samples collected in 2015, in addition to 58 samples that were gathered in 2019. All in all, a total of 91 samples were tested using AST. ATCC *E. coli* 25922 strain was used a negative control sample.



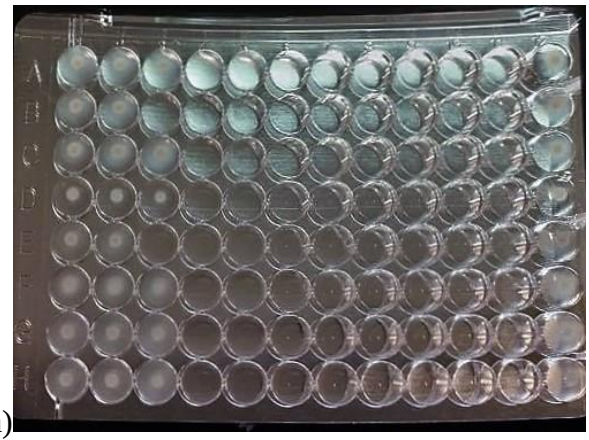
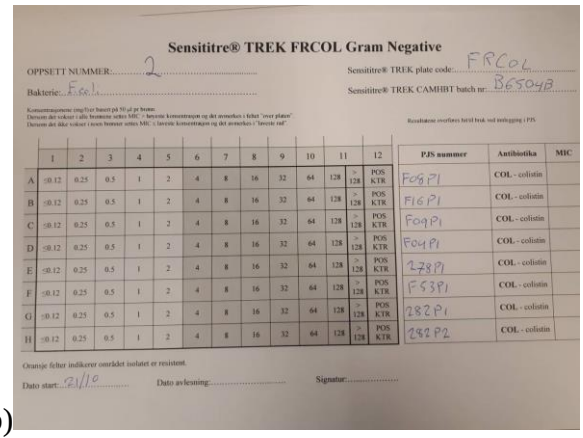
a) b)

Picture 2: a) Negative control ACTT b) Disc-diffusion on random isolate

Minimum Inhibition Concentration (MIC)

To further determine the antimicrobial resistance of our isolates, a micro broth dilution method (Thermofisher, Sensititre™) was performed on all of the isolates from 2015, in addition to the superpolymyxin agar resistant isolates from 2019. Inoculum preparation was performed using 9,5 mcfarland standard and sensititre sterile water. Plate type was *Sensititre EU surveillance Salmonella/E. coli EUVSEC*. The following antimicrobials were tested for: SMX, TMP, CIP, TET, MERO, AZI, NAL, FOT, CHL, TGC, TAZ, COL, AMP and GEN. 34

of the isolates that were collected in 2015 were tested using MIC testing, in addition to 22 colistin positive isolates from selective screening, sampled in 2019. A total of 56 samples were tested using MIC.

a)  b) 

Sensititre® TREK FRCOL Gram Negative

OPPSETT NUMMER: 2 Sensititre® TREK plate code: FRCOL

Bakterie: E.coli Sensititre® TREK CAMHBT batch nr: B6504B

	1	2	3	4	5	6	7	8	9	10	11	12	PJS nummer	Antibiotika	MIC
A	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	> 128	F08P1	COL - colistin	
B	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	> 128	F16P1	COL - colistin	
C	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	> 128	F09P1	COL - colistin	
D	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	> 128	F04P1	COL - colistin	
E	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	> 128	278P1	COL - colistin	
F	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	> 128	F53P1	COL - colistin	
G	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	> 128	282P1	COL - colistin	
H	≤0.12	0.25	0.5	1	2	4	8	16	32	64	128	> 128	282P2	COL - colistin	

Oransje felter indikerer området isolatet er resistent.

Dato start: 21/10 Dato avlesning: Signatur:

Picture 3: a) MIC-values for colistin in 8 different isolates of *E. coli.* The concentration of colistin in the wells increases from left to right. Transition between wells with/without growth of bacteria indicates the MIC-value. Right colon is the positive control sample. b) Form used when reading off the MIC-values.

Mass-spectrometry using Matrix-Assisted Laser Desorption-Ionization Time Of Flight (MALDI-TOF)

To determine the species of our isolates, mass-spectrometry with MALDI-TOF, Bruker MALDI Biotyper (Version 4.1.90 PYTH), was performed on all isolates resistant to either TAZ, CTX, TET, NAL or COL in selective screening. A small fraction of each colony was smeared onto MALDI-TOF plates using a toothpick. They were then added 0,5 µl of reagent and airdried for one minute. The plates were then placed in the MALDI-TOF machine for analysis.

Genotypical analysis

To determine the genetic properties of our resistant *E. coli* isolates, whole-genome sequencing (WGS) was indicated for further analysis. Prior to the sequencing, DNA extraction followed by spectrophotometric and fluorometric analysis was performed to determine the DNA quality of the isolates. The isolates were either stored in broth in freezing temperatures at -80° C or on blood agar in cooling temperatures at around 5° C, depending on the timeframe, prior to extraction.

DNA extraction

Forty isolates were prepared for DNA extraction using a QIAGEN®, QIAamp® DNA extraction kit and by following the QIAGEN kit 10-step user manual.

Spectrophotometric analysis using NanoDrop™

To evaluate the DNA purity of the isolates prior to sequencing, spectrophotometric analysis was performed using NanoDrop One™ (Thermofisher Scientific). 1 µl of sample material was pipetted and placed on the machine's pedestal. Results ranging between 1.8-2.0 OD is considered as sample of good quality. Anything below or above this range is considered lacking or contamination of DNA, respectively.

Fluorometric quantification using Qubit 4™

By performing a fluorometric quantification on our isolates, we were able to achieve an acceptable value of DNA concentration before sending them off for sequencing. Qubit 4™ fluorometer (Thermofisher, Invitrogen) was used to determine the concentration value. 1 µl of sample material was pipetted on to the machine, in combination with a selected assay, for bacterial analysis.

Genotyping using Whole-Genome Sequencing (WGS)

Whole-genome sequencing was performed both in situ and ex situ, depending on the year the sample was collected. 38 samples from 2015, of which 13 strains were analysed using Illumina™ Miseq and 25 strains were analysed using Illumina™ Hiseq, was analysed at the Ullevål University Hospital in Oslo. The two remaining samples from 2019, were analysed at the Norwegian Veterinary Institute, using Illumina™ Miseq. The total of samples analysed using WGS was 40.

In silico analysis

To evaluate the genomic data from our samples, a number of programs were used after receiving the raw data files. By using Conda as a platform, an open-source environment management system, we were able to assemble our DNA strains and ready them for further in silico analysis. A text-based access, by using a command line interface, was used during most of the data processing. Terminal (Apple Inc.) was the preferred command line interface, due to its compatibility to Linux based language. FASTQC and MEGAx were the programs that offered a graphics user interface (GUI), and command line interface could be avoided in cases where these programs were needed. Database query, using a range of internet-based databases was also performed.

Quality control and trimming

The first step in the process of analysing the whole-genome data, was to perform a quality control of the raw data file, from now on called the FASTQ file. FASTQC was the software of preference, as it offers a high throughput sequence quality control report, and the program comes as a GUI. During the analysis, the program evaluates the forward- and backwards reads of each isolate. After the read is done, the program determines if the sequences have undergone a bad sequencing or not. In some cases, the reads still have the Illumina primers

and ends sequences attached. To remove these reads, trimming is required, and Trimmomatic(51) was the preferred program. The following command line for single end reads was utilized:

java -jar trimmomatic-0.35.jar SE -phred33 input.fq.gz output.fq.gz ILLUMINACLIP:TruSeq3-SE:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:36

Adapter sequences was found online. Any reads that was considered a "bad read" were discarded and left out of the continuing process. In our case, only one of the samples was discarded.

Assembly

Single-cell sequencing (SCS) and bacterial sequencing requires sufficient software to process the input data and ultimately generate a dataset of the whole genome. The input data, from the FASTQ file, is run through an assembly program in order to get a whole read of the organisms' genome. Assembly of the FASTQ file is required to put the forward- and backward read together into a whole set of genome data. Assembly was performed using the SPAdes software (52). SPAdes' main algorithm is based on constructing a set of k-mers for assembling sets of fragmented DNA parts, using an n-dimensional De Bruijn graph. Several variations of the De Bruijn graph, more specifically called the A-Bruijn graph, is used during assembly, and the operations performed in the program is based on topology, coverage and sequence lengths. The output data comes out as a FASTA file, which is readable in several other applications used later on in the study.

Annotation

To identify the different genes' and coding regions' location, annotation is required. By running the FASTA file through the Pathosystems Resource Integration Center (PATRIC) (53), different genes and regions are identified and systemized into a table. This way, the base

reads can be converted into plain text when it comes to name and function. Furthermore, the base reads are also converted to protein reads, and location of starting and ending sequences are more easily identified. Our data was uploaded to the PATRIC workspace, and identification of specific reads interesting to our case was identified.

Database query using different databases

In order to more easily identify the genes' ability to express certain properties, a database query is the most efficient way of finding these properties. By using the U.S. National Library of Medicine's (NLM) Basic Local Alignment Search Tool (BLAST), we could search for certain nucleotides in our FASTA dataset. In addition to BLAST, we also used a range of databases created by the Center for Genome Epidemiology to detect resistance genes, virulence genes and plasmids. Pubmlst was used to find the multilocus sequence types (MLST) of the isolates.

Detection of acquired AMR resistance genes using Resfinder

By using Resfinder (54), we were able to detect resistance genes and chromosomal mutations in our isolates, using an updated database. By setting a predetermined coverage and likelihood in the search parameters before inducting a search, the search was limited to the variables we considered relevant for our research. Results with a =100% probability were considered positive for carrying a resistance gene or a chromosomal mutation. Any results with a <100% probability was noted and measured up with a possible phenotypical resistance. This was the basis of our genotypical findings regarding AMR.

Detection of virulence genes using Virulencefinder

By using the Virulencefinder(55) database, we were able to detect certain virulence genes in our isolates. The parameters for the search were determined by coverage and identity, and the

result of our search was noted either as =100% probability or a <100% probability for carrying a virulence gene.

Identification of plasmids using Plasmidfinder

By using Plasmidfinder 2.1 (56), we were able to determine the type of plasmid replicons the isolates were carrying. This is important to evaluate the probability for carrying and transferring certain resistance genes. Database were set to Enterobacteriaceae, minimum identity threshold at 80% and minimum coverage at 60%. The (in)compatibility of genes were determined and noted.

Characterization of isolates using Pubmlst for Multilocus Sequence Typing (MLST)

To identify and evaluate sequence types and clonal complexes in our isolates, this publication made use of the PubMLST website (https://pubmlst.org/) developed by Keith Jolley (Jolley *et al. Wellcome Open Res* 2018, 3:124 [version 1; referees: 2 approved]) and sited at the University of Oxford. The development of that website was funded by the Wellcome Trust. The MLST is a way of identifying a specific type of bacterial subspecies, using genotypical approaches to identify specific housekeeping genes within the genome. Serotyping and phylogroups precedes this method of identification.

Phylogenetic analysis

In order to determine the relatability between our isolates, a phylogenetic analysis was performed. This way, we could get a closer look at the relative phylogenetic range between our isolates and evaluate if the diversity and spread were correlating with pathogenicity and resistance. In our case, we had several birds shot down at different periods, meaning the chance of diversity between their origin of flight could correlate with our genotypical findings.

Identification of pan genome using Roary software

By using the Roary software, we were able to identify the pan genome of our isolates. This is required to do extraction of SNPs in the genome and calculating the maximum likelihood of relatability between our isolates later on in the software chain.

Extraction of Single Nucleotide Polymorphism (SNP) sites using SNP-sites software

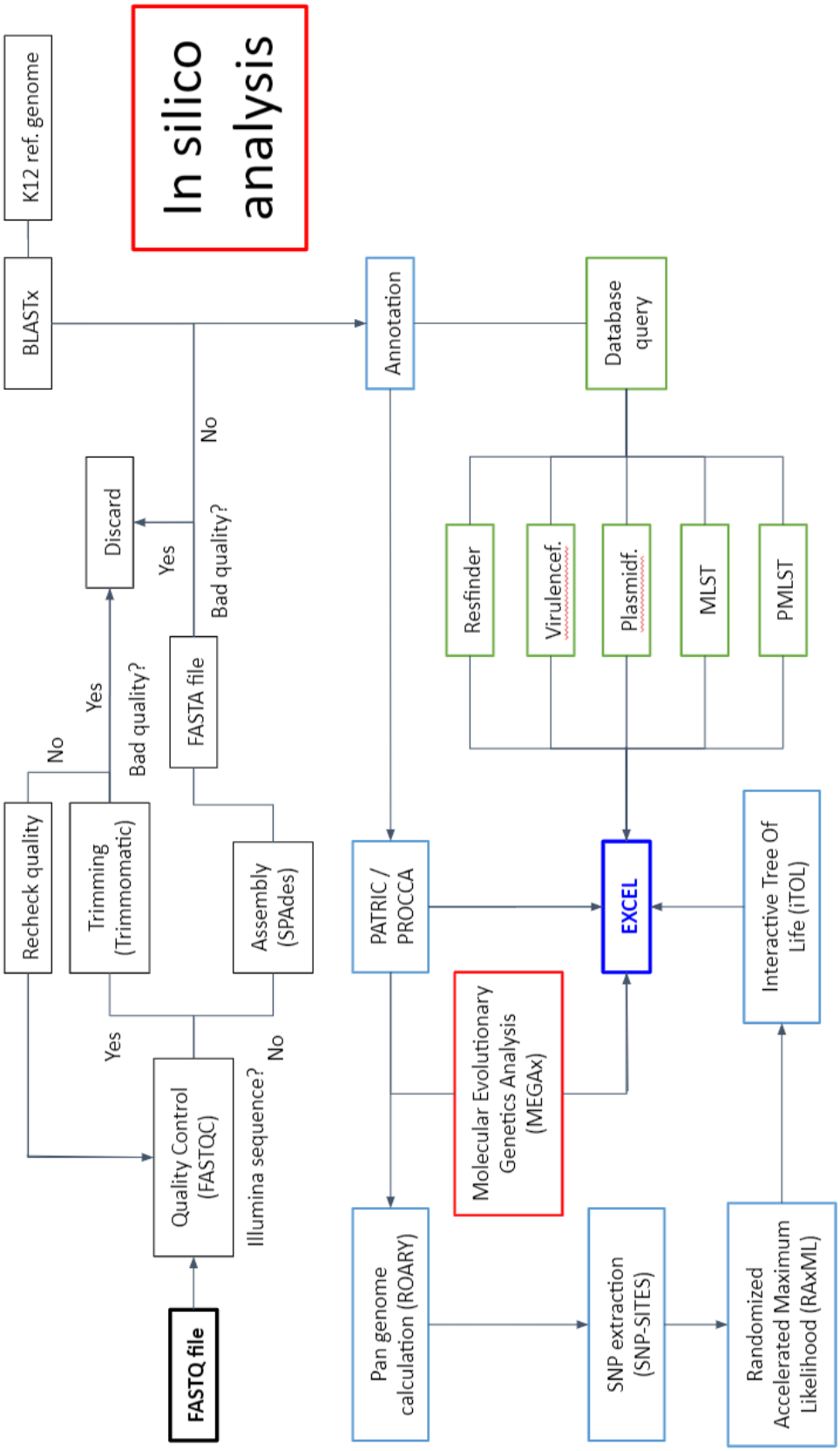
In order to determine the phylogenetic maximum-likelihood, an extraction of SNPs is required. These SNPs are lined up in order to do further statistical analysis later on in the software chain.

Maximum likelihood based phylogenetic inference using RAxML software

By using a maximum likelihood based software, we could determine the statistical probability of relationship between our isolates. This step was the last of the step in the software chain determined to calculate genotypical relatability.

Phylogenetic identification of isolates using Interactive Tree of Life (iTOL)

To get a visual view of the phenotypical relatability of our samples, we used the iTOL website to create a tree of life. The tree helped us determine the probable relationship between our isolates in either a linear of circular form, both of which was used in interpreting our results.


In silico analysis
K12 ref. genome
BLASTx
Annotation
Database query
Discard
Yes
Bad quality?
No
FASTA file
Resfinder
Virulencef.
Plasmidf.
MLST
PMLST
Recheck quality
Trimming (Trimmomatic)
Assembly (SPAdes)
No
Yes
Bad quality?
PATRIC / PROCCA
EXCEL
Interactive Tree Of Life (iTOL)
Yes
No
Illumina sequence?
Quality Control (FASTQC)
Molecular Evolutionary Genetics Analysis (MEGAx)
FASTQ file
Pan genome calculation (ROARY)
SNP extraction (SNP-SITES)
Randomized Accelerated Maximum Likelihood (RAxML)

Results

Carriage rate of quinolone-, tetracycline-, and extended-spectrum cephalosporin resistant *E. coli*

A total of 201 avian faecal samples were collected from two different species, Greylag goose (n=76) and Pink-footed goose (n=125), of which 111 and 90 were obtained in 2015 and 2019, respectively. From 69 of the faecal samples, a total of 88 *E. coli* isolates were successfully obtained by selective cultivation on MacConkey agar plates infused with either nalidixic acid (NAL), tetracycline (TET) or cefotaxime/ceftazidime (CTX/CAZ). The total prevalence of geese carrying *E. coli* isolates with phenotypic resistance against either quinolone-, tetracycline or ESC, three important groups of antibiotics, were therefor at 34% (69/201). The most common resistance phenotypes observed through screening were to tetracycline (32% of geese) and nalidixic acid (16% of geese). Resistance against ESC was not observed on screening.

Isolation of resistant *E. coli* from geese samples obtained in 2015

Out of 111 faecal samples collected in 2015, approximately a third (39%) carried *E. coli* isolates resistant against either quinolone or tetracycline. One sample (1%) had only NAL resistant isolates, 19 samples (17%) had NAL and TET resistant isolates and 23 samples (21%) had only TET resistant isolates. Growth on CTX and CAZ screening media was observed for one sample, but identification later revealed it not to be *E. coli*. A total of 62 *E. coli* isolates were obtained from 43 samples, of which 42 had growth on media with TET and 20 had growth on media with NAL. There was an uneven distribution of samples obtained from Pink-footed geese (n=100) and Greylag geese (n=11) making comparison of resistance

among the two species difficult. Within the Pink-footed goose, difference in age did not seem to affect the carriage rate of resistant *E. coli* isolates (Table 1).

		Number of geese with resistant *E. coli* / number of geese hunted		
		Pink-footed goose	**Greylag goose**	
Antibiotic	**Age**	***A. brachyrhynchus***	***A. anser***	**Total**
Nalidixic Acid	Young	15/70 (21%)	0/8	15/78 (19%)
	Adult	4/30 (13%)	1/3 (33%)	5/33 (15%)
Tetracycline	Young	28/70 (40%)	2/8 (67%)	30/78 (38%)
	Adult	11/30 (37%)	1/3 (33%)	12/33 (36%)
Cefotaxime	Young	0/70 (0%)	0/8 (0%)	0/78 (0%)
	Adult	0/30 (0%)	0/3 (0%)	0/33 (0%)
Total with AMR	Young	29/70 (41%)	2/8 (67%)	31/78 (40%)
	Adult	11/30 (37%)	1/3 (33%)	12/33 (36%)

Table 1: The carriage rate of antimicrobial resistant strains of *E. coli* according to phenotype, age, and host species. Samples collected in 2015.

Isolation of resistant *E. coli* from geese samples obtained in 2019

Results from screening of faecal samples collected in 2019 (n=90) showed that 29% (26/90) of the geese carried *E. coli* with phenotypic resistance against either quinolones or tetracycline. We were not able to detect any ESC resistance through initial screening on media with CTX and CAZ. Three samples (3%) had only NAL resistant isolates, 10 samples (11%) had NAL and TET resistant isolates and 13 samples (14%) had only TET resistant isolates. From 26 of the faecal samples, a total of 36 *E. coli* isolates were obtained from initial screening for resistant isolates, of which 23 expressed resistance against TET and 13 expressed resistance against NAL. Similarly, to the samples collected in 2015, there was an

uneven distribution of samples between Greylag geese (n=25) and Pink-footed geese (n=65) in 2019. Once again making comparison between the two species difficult. From the available data there seems to be no major differences between the species when it comes to the prevalence of geese carrying resistant *E. coli*. Unfortunately, some of the samples lack information about the age as the hunters were unable to age specify 37 of the geese that were sampled because of bad weather. Within the remaining 53 samples, of which age information is available, there seems to be no significant difference between young and adult individuals (Table 2).

		Number of geese with resistant *E. coli* / number of geese hunted		
		Pink-footed goose	**Greylag goose**	
Antibiotic	**Age**	***A. brachyrhynchus***	***A. anser***	**Total**
Nalidixic Acid	Young	1/10 (10%)	3/16 (19%)	4/26 (15%)
	Adult	3/15 /20%)	2/12 (17%)	5/27 (19%)
	Unknown	0/0	4/37 (11%)	4/37 (11%)
Tetracycline	Young	3/10 (30%)	4/16 (25%)	7/26 (27%)
	Adult	4/15 (27%)	4/12 (33%)	8/27 (30%)
	Unknown	0/0	8/37 (22%)	8/37 (22%)
Cefotaxime	Young	0/10 (0%)	0/16 (0%)	0/26 (0%)
	Adult	0/15 (0%)	0/12 (0%)	0/27 (0%)
	Unknown	0/0	0/37 (0%)	0/37 (0%)
Total with AMR	Young	3/10 (30%)	5/16 (31%)	8/26 (31%)
	Adult	5/15 (33%)	5/12 (42%)	10/27 (37%)
	Unknown	0/0	8/37 (22%)	8/37 (22%)

Table 2: The carriage rate of antimicrobial resistant strains of *E. coli* according to phenotype, age, and host species. Samples collected in 2019.

Screening for polymyxin resistant isolates in samples from 2019

All the samples collected in 2019 (n=90) were additionally screened for polymyxin resistant *E. coli*. A total of 22 isolates, with growth on agar plates infused with polymyxin, were obtained from 21 faecal samples. This screening method was suspected to have a low specificity and so all 22 isolates would later be tested with MIC in order to qualitatively confirm the expression of resistance.

Antimicrobial susceptibility testing (AST)

A collection of 101 *E. coli* isolates were chosen based on positive screening results for resistance against NAL, TET or Polymyxin and were investigated further. Thirty-three isolates originated from samples collected in 2015, 9 of which were NAL resistant and 24 of which were TET resistant on screening. Of these 33 isolates only two originated from Greylag geese and the remaining 31 from Pink-footed geese. The majority, 58 isolates, originated from samples collected in 2019, of which 36 isolates were resistant to NAL (n=13) and TET (n=23), 22 isolates were resistant against polymyxin and the distribution between Greylag- and Pink-footed geese were 36 and 22, respectively. Antimicrobial susceptibility testing, using the disk-diffusion method, included the following antibiotic groups; aminopenicillins, aminoglycosides, ESC, trimethoprim, sulphonamides, tetracyclines and quinolones.

AMR in *E. coli* isolates originating from geese samples (2015)

From the 62 *E. coli* isolates, that were successfully obtained through selective screening, a subset of 33 isolates with NAL or TET resistance were chosen for antimicrobial susceptibility testing. The remaining 29 isolates were not investigated further with AST. Screening for tetracycline resistance demonstrated the highest reliability with 23 out of 24 isolates, that had growth on tetracycline screening plates, expressing phenotypic resistance against the same antibiotic using the disk-diffusion method. The ratio was a bit lower for nalidixic acid screening, where 6 out of 9 isolates also expressed resistance on disc-diffusion, indicating a lower specificity (Table 3). Still, the total number of TET resistant isolates were 24, matching the number of isolates from screening. The reason being that isolate 2015-3869-3-NAL, resistant to NAL from screening, also expressed TET resistance on disc-diffusion. It also expressed the same resistance pattern as isolate 2015-3869-3-TET, indicating that they are part of the same strain as they both originated from the same sample. The total number of NAL resistant isolates from AST (n=14) exceeded that of screening by 5 isolates, all of which were TET resistant isolates from screening, but expressed combined resistance against TET and NAL on disc-diffusion. The most prevalent resistance profiles detected were combined resistance against TET and NAL, 7 isolates, and ampicillin (AMP), trimethoprim/sulfamethoxazole (SXT) and NAL, 5 isolates. Two isolates originating from the same young, pink-footed goose expressed multi-drug resistance against ampicillin, trimethoprim/sulfamethoxazole, tetracycline, narrow-spectrum quinolone and fluroquinolone. No resistance against ESC or aminoglycosides were observed from the 33 isolates.

			Screening		Disk diffusion (AST)				
Isolate name	**species**	**Age**	**TET**	**NAL**	**AMP**	**SXT**	**TET**	**NAL**	**CIP**
2015-3810-11-NAL	Pink-footed	Young			R	R		R	
2015-3810-12-NAL	Pink-footed	Adult			R	R		I	
2015-3810-12-TET	Pink-footed	Adult					R	I	
2015-3810-13-TET	Pink-footed	Adult					R	I	
2015-3810-14-NAL	Pink-footed	Young			R	R		R	
2015-3810-3-NAL	Pink-footed	Young			R	R		I	
2015-3810-5-NAL	Pink-footed	Young			R	R		R	
2015-3810-6-NAL	Pink-footed	Young			R	R		I	
2015-3810-6-TET	Pink-footed	Young			R	R		I	
2015-3824-10-NAL	Pink-footed	Young			R	R		R	
2015-3824-10-TET	Pink-footed	Young					R	R	
2015-3824-11-TET	Pink-footed	Young					R	I	
2015-3824-12-TET	Pink-footed	Young					R	R	
2015-3824-13-TET	Pink-footed	Young					R	I	
2015-3824-18-TET	Pink-footed	Young				R	R	I	
2015-3824-19-TET	Pink-footed	Young					R		
2015-3824-21-NAL	Pink-footed	Young			R	R		R	
2015-3824-21-TET	Pink-footed	Young				R	R	I	
2015-3824-27-TET	Pink-footed	Young					R	R	
2015-3824-32-TET	Pink-footed	Adult					R	I	
2015-3824-36-TET	Pink-footed	Adult					R	I	
2015-3824-37-TET	Greylag	Young					R	I	
2015-3824-7-TET	Pink-footed	Young					R	I	
2015-3869-3-NAL	Pink-footed	Young			R	R	R	R	R
2015-3869-3-TET	Pink-footed	Young			R	R	R	R	R
2015-3869-4-TET	Pink-footed	Young				R	R	I	
2015-3869-5-TET	Pink-footed	Young					R	I	
2015-3869-7-TET	Pink-footed	Young					R	I	
2015-3992-13-TET	Greylag	Young					R	R	
2015-3992-20-TET	Pink-footed	Adult					R	R	
2015-3992-6-TET	Pink-footed	Young					R	R	
2015-3992-8-TET	Pink-footed	Young					R	R	
2015-4279-1-TET	Pink-footed	Young					R	I	

Table 3: Overview of 33 screening resistant *E. coli* isolates originating from greylag- and pink-footed geese hunted in Norway in 2015. Presence of phenotypic resistance shown as a matrix. Method of detection indicated at the top of the matrix underscored by the antibiotics (TET: tetracycline, NAL, nalidixic acid, AMP: ampicillin, SXT: trimethoprim/sulfamethoxazole, CIP: ciprofloxacin). Gentamicin and cefotaxime have been excluded from the figure since no resistance was found. Grey squares indicate presence of resistance, light grey indicate intermediate zone on disc-diffusion and white indicates no resistance found. R stands for resistance and I stand for intermediate.

AMR in *E. coli* isolates originating from geese samples (2019)

All 58 isolates obtained from selective screening underwent further antimicrobial susceptibility testing. For every isolate, with phenotypic resistance on MacConkey agar infused with the antibiotics TET and NAL, screening results were confirmed with disc diffusion, indicating high reliability and specificity of screening (Table 4). Out of 23 TET resistant screening isolates 5 (22%) also expressed resistance against NAL, while 3/13 (23%) NAL resistant screening isolates also expressed resistance against TET. The most prevalent phenotypic resistance profiles detected were combined resistance against AMP, TET and NAL, 4 isolates, and AMP and TET, 3 isolates. Two isolates originating from the same greylag goose expressed resistance against tetracycline, narrow-spectrum quinolone and fluroquinolone. These two isolates could be suspected to be of the same strain. None of the isolates with growth on Superpolymyxin plates showed resistance towards any of the antibiotics that were tested. Only 5 polymyxin resistant isolates had intermediate zones around NAL disc on AST. No resistance against ESC or aminoglycosides were observed from the 58 isolates.

			Screening			Disk diffusion (AST)				
Isolate name	Species	Age	TET	NAL	POL	AMP	SXT	TET	NAL	CIP
F01N	Graylag	Young							R	
F01P	Graylag	Young							I	
F04T	Graylag	Young				R		R	R	
F11N	Graylag	Young							R	
F11T	Graylag	Young						R		
F14N	Graylag	Young							R	
F14T	Graylag	Young				R		R	R	
F16T	Graylag	Young						R		
F22T	Graylag	Adult						R		
F24N	Graylag	Adult							R	
F25T	Graylag	Adult						R		
F26T	Graylag	Adult				R	R	R	I	
F27N	Graylag	Adult							R	
F27T	Graylag	Adult				R		R	R	
F29N	Pink-footed	Adult							R	
F29T	Pink-footed	Adult						R	I	
F31P	Pink-footed	Adult							I	
F32T	Pink-footed	Adult						R	I	
F34N	Pink-footed	Adult							R	
F37N	Pink-footed	Adult							R	
F37T	Pink-footed	Adult				R		R		
F41T	Pink-footed	Adult						R	I	
F45T	Pink-footed	Young						R	I	
F50T	Pink-footed	Young				R		R		
F51P	Pink-footed	Young							I	
F53N	Pink-footed	Young							R	
F53T	Pink-footed	Young						R	I	
FU253N	Graylag	Unknown				R			R	
FU253T	Graylag	Unknown				R		R	I	
FU258T	Graylag	Unknown				R		R	I	
FU260T	Graylag	Unknown						R	I	
FU261N	Graylag	Unknown				R		R	R	
FU261T	Graylag	Unknown						R	I	
FU266T	Graylag	Unknown						R	I	
FU271N	Graylag	Unknown						R	R	
FU271T	Graylag	Unknown						R	R	
FU275T	Graylag	Unknown						R	I	
FU279N	Graylag	Unknown						R	R	R
FU279T	Graylag	Unknown						R	R	R
FU282P1	Graylag	Unknown							I	
FU282P2	Graylag	Unknown							I	

Table 4: Overview of 41 screening resistant *E. coli* isolates originating from greylag- and pink-footed geese hunted in Norway in 2019. 17 isolates have not been included on this figure because no resistance was detected. Presence of phenotypic resistance shown as a matrix. Method of detection indicated at the top of the matrix underscored by the antibiotics (TET: tetracycline, NAL: nalidixic acid, AMP: ampicillin, SXT: trimethoprim/sulfamethoxazole, CIP: ciprofloxacin). Gentamicin and cefotaxime have been excluded from the figure since no resistance was found. Grey squares indicate presence of resistance, light grey indicate intermediate zone on disc-diffusion and white indicates no resistance found. R stands for resistance and I stand for intermediate.

Minimum inhibitory concentration (MIC)

A total of 34 isolates, with resistance against NAL (n=13), TET (n=19) or SUL (n=2) on screening, were selected from samples obtained in 2015 and underwent additional MIC testing (Table 5). For almost every isolate, with phenotypic resistance on MacConkey agar infused with antibiotics, screening results were confirmed in MIC testing, demonstrating the high specificity and reliability of screening. The inhibitory concentration of the respective compounds was above the breakpoint for 31 (91%) of the isolates, 13, 16 and 2 of which were NAL, TET and SUL resistant, respectively. Leaving a discrepancy of 3 TET positive isolates that did not have their screening results confirmed.

Additional MIC testing on 22 isolates from samples obtained in 2019, that had growth on polymyxin screening media, revealed two colistin resistant isolates, F10P and F37P, with minimum inhibitory concentrations of 16, well above the breakpoint. This discrepancy between screening and MIC can be explained by the low specificity of polymyxin agar, making this type of screening less representative of the actual prevalence of polymyxin resistance.

Isolate name	species	Age	SMX	TMP	CIP	TET	MERO	AZI	NAL	FOT	CHL	TGC	TAZ	COL	AMP	GEN
2015-3810-11-NAL	Pink-footed	Adult	>1024	>32	0,25	<=2	<0,03	4	128	<=0,25	<=8	<0,25	<0,5	<1	>64	2
2015-3810-12-NAL	Pink-footed	Young	<8	0,5	0,25	4	<0,03	16	128	<=0,25	<=8	<0,25	<0,5	<1	2	<0,5
2015-3810-13-TET	Greylag	Young	<8	0,5	<=0,015	32	<0,03	8	<=4	<=0,25	<=8	<0,25	<0,5	2	<=1	<0,5
2015-3810-14-NAL	Pink-footed	Young	>1024	>32	0,25	<=2	<0,03	8	128	<=0,25	<=8	<0,25	<0,5	<1	>64	<0,5
2015-3810-5-NAL	Pink-footed	Young	>1024	>32	0,25	<=2	<0,03	8	>128	<=0,25	<=8	<0,25	<0,5	<1	>64	<0,5
2015-3824-10-NAL	Pink-footed	Adult	>1024	>32	0,25	<=2	<0,03	4	128	<=0,25	<=8	<0,25	<0,5	<1	>64	1
2015-3824-11-TET	Pink-footed	Young	>1024	<=0,25	<=0,015	>64	<0,03	4	<=4	<=0,25	<=8	<0,25	<0,5	2	2	<0,5
2015-3824-12-TET	Pink-footed	Adult	>1024	1	<=0,015	64	<0,03	8	<=4	<=0,25	>128	<0,25	<0,5	<1	4	<0,5
2015-3824-13-TET	Pink-footed	Young	>1024	1	<=0,015	>64	<0,03	8	<=4	<=0,25	>128	0,5	<0,5	<1	4	<0,5
2015-3824-18-TET	Pink-footed	Young	>1024	>32	<=0,015	64	<0,03	8	<=4	<=0,25	<=8	<0,25	<0,5	<1	4	<0,5
2015-3824-19-TET	Pink-footed	Young	<8	<=0,25	<=0,015	32	<0,03	4	<=4	<=0,25	<=8	<0,25	<0,5	<1	<=1	<0,5
2015-3824-21-NAL	Pink-footed	Adult	>1024	>32	0,25	<=2	<0,03	4	>128	<=0,25	<=8	<0,25	<0,5	<1	>64	<0,5
2015-3824-27-TET	Pink-footed	Young	>1024	1	<=0,015	64	<0,03	8	<=4	<=0,25	>128	0,5	<0,5	<1	4	<0,5
2015-3824-30-SUL	Pink-footed	Young	>1024	0,5	<=0.015	64	<=0.03	8	<=4	<=0.25	<=8	<=0.25	<=0.5	<=1	>64	<=0.5
2015-3824-32-TET	Pink-footed	Young	>1024	1	<=0,015	64	<0,03	8	<=4	<=0,25	>128	<0,25	<0,5	<1	4	<0,5
2015-3824-36-TET	Pink-footed	Young	>1024	1	<=0,015	>64	<0,03	8	<=4	<=0,25	>128	<0,25	<0,5	<1	4	<0,5
2015-3824-37-TET	Greylag	Young	<8	<=0,25	0,03	64	<0,03	8	16	<=0,25	<=8	<0,25	<0,5	2	2	1
2015-3824-39-NAL	Pink-footed	Young	>1024	>32	>8	>64	<=0.03	64	>128	<=0.25	>128	<=0.25	<=0.5	<=1	>64	1
2015-3824-7-TET	Greylag	Adult	>1024	1	<=0,015	64	<0,03	8	<=4	<=0,25	>128	0,5	<0,5	<1	4	1
2015-3824-9-NAL	Pink-footed	Adult	>1024	>32	0,25	<=2	<0,03	4	128	<=0,25	<=8	<0,25	<0,5	<1	>64	<0,5
2015-3869-3-NAL	Pink-footed	Young	>1024	>32	8	>64	<=0.03	64	>128	<=0.25	>128	<=0.25	<=0.5	<=1	>64	<=0.5
2015-3869-4-TET	Pink-footed	Adult	>1024	>32	<=0,015	64	<0,03	8	<=4	<=0,25	<=8	0,5	<0,5	<1	4	<0,5
2015-3869-5-TET	Pink-footed	Adult	>1024	0,5	<=0,015	>64	<0,03	4	<=4	<=0,25	<=8	<0,25	<0,5	<1	2	1
2015-3869-7-SUL	Pink-footed	Young	>1024	<=0.25	<=0.015	>64	<=0.03	4	<=4	<=0.25	<=8	<=0.25	<=0.5	<=1	2	<=0.5
2015-3869-7-TET	Pink-footed	Adult	>1024	0,5	<=0,015	>64	<0,03	4	<=4	<=0,25	<=8	<0,25	<0,5	<1	2	<0,5
2015-3992-13-TET	Pink-footed	Young	<8	0,5	<=0,015	64	<0,03	8	<=4	<=0,25	<=8	<0,25	<0,5	<1	2	1
2015-3992-16-NAL	Pink-footed	Young	>1024	>32	0,25	<=2	<0,03	4	128	<=0,25	<=8	<0,25	<0,5	<1	>64	<0,5
2015-3992-20-TET	Pink-footed	Young	<8	0,5	0,03	64	<0,03	8	<=4	<=0,25	<=8	<0,25	<0,5	2	2	<0,5
2015-3992-21-NAL	Pink-footed	Young	>1024	>32	0,25	<=2	<0,03	4	128	<=0,25	<=8	<0,25	<0,5	<1	>64	<0,5
2015-3992-5-NAL	Pink-footed	Young	<8	<=0.25	0,25	<2	<=0.03	4	128	<=0.25	<=8	<=0.25	<=0.5	<=1	2	<=0.5
2015-3992-6-TET	Pink-footed	Young	<8	0,5	0,25	64	<0,03	4	8	<=0,25	128	<0,25	<0,5	<1	2	<0,5
2015-3992-7-NAL	Pink-footed	Young	>1024	>32	>8	>64	<=0.03	32	>128	<=0.25	>128	<=0.25	<=0.5	<=1	>64	<=0.5
2015-3992-8-TET	Pink-footed	Young	>1024	1	<=0,015	64	<0,03	8	<=4	<=0,25	128	0,5	<0,5	<1	4	<0,5
2015-4279-1-TET	Pink-footed	Young	<8	0,5	<=0,015	32	<0,03	8	<=4	<=0,25	<=8	<0,25	<0,5	<1	2	<0,5
2019-F10P	Pink-footed	Young	<8	1	0,03	4	<0,03	8	<4	<0,25	<8	<0,25	<0,5	16	4	<0,5
2019-F37P	Pink-footed	Adult	<8	1	<0,015	4	<0,03	8	<4	<0,25	<8	<0,25	<0,5	16	4	<0,5

Table 5: MICs for screening-resistant *E. coli* isolated from greylag- and pink-footed geese in Norway. Bottom two isolates obtained from samples collected in 2019 and are the only colistin resistant isolates. Antibiotics indicated at the top of the matrix (SMX: sulfamethoxazole, TMP: trimethoprim, CIP: ciprofloxacin, TET: tetracycline, MERO: meropenem, AZI: azithromycin, NAL: nalidixic acid, FOT: cefotaxime, CHL: chloramphenicol, TGC: tigecycline, TAZ: ceftazidime, COL: colistin, AMP: ampicillin, GEN: gentamicin). Grey squares indicate MIC-value above cut-off and white indicates MIC-value below cut-off.

WGS data

A collection of 40 isolates were chosen for further analysis through DNA extraction and whole genome sequencing, in order to identify the mechanism behind phenotypic resistance (AMR genes, mutations), virulence factors, plasmids, sequence types and phylogenetic grouping. Four isolates were discarded because of contamination in the process of DNA extraction. The isolates originating from samples collected in 2015 had resistance against NAL (n=13), TET (n=19) and SUL (n=2). An additional two isolates from the samples collected in 2019 were included in further analysis as they expressed high MIC values against colistin.

Phenotypic and genotypic correlation of resistance in *E. coli*

AMR gene detection using ResFinder 3.2 identified a total of 11 different AMR genes in 32 of the 36 *E. coli* isolates that were whole genome sequenced (Table 6). The genes found belonged to the following groups of antibiotics; aminoglycosides, narrow-spectrum beta lactams, quinolones, sulphonamides, tetracyclines and trimethoprim. The genotypic results correlated well with the phenotype test results for most of the AMR genes. Only three isolates with *tetA* and one isolate with sul2 did not have phenotypic resistance against tetracyclines and sulphonamides, respectively. In addition, none of the 25 isolates with *aph(3")-lb* or *aph(6)-ld*, coding for resistance against aminoglycosides, showed high MIC-values against the aminoglycosides (gentamicin) that were included. Excluding aminoglycosides, the most prevalent AMR genes were *sul1* and *sul2* coding resistance against sulphonamides. Tetracycline resistance was identified in 19 isolates with two different genes, *tetA* (15 isolates) and *tetB* (4 isolates). *blaTEM-1B*, beta-lactam encoding gene, was detected in 11 isolates, *mphA*, macrolide-resistance gene, was detected in 3 isolates and *dfrA17/dfrA5*, encoding resistance against trimethoprim, was detected in 13 isolates. In one isolate, *qnrS1*,

which encodes for quinolone resistance, was detected and also expressed MIC-value against CIP (fluroquinolone) above the cut-off, but not for nalidixic acid (narrow-spectrum quinolone). None of the 13 isolates, with combined NAL and TET resistance on MIC, had any AMR gene to explain the resistance. In addition, 5/25 (20%) of the isolates with sulfamethoxazole resistance, 5/21 (24%) of the isolates with tetracycline resistance and the two isolates with colistin resistance did not have a matching AMR gene to explain the phenotypic resistance that was observed.

			aph(3'')-Ib	aph(6)-Id	blaTEM-1B	mph(A)	qnrS1	sul1	sul2	tet(A)	tet(B)	dfrA17	dfrA5	MIC								
Isolate name	**Species**	**Age**												GEN	AMP	AZI	NAL	CIP	SMX	TET	TMP	COL
2015-3810-11-NAL	Pink-footed	Young												2	>64	4	128	0,25	>1024	<=2	>32	<1
2015-3810-12-NAL	Pink-footed	Adult												<0,5	2	16	128	0,25	<8	4	0,5	<1
2015-3810-13-TET	Pink-footed	Adult												<0,5	<=1	8	<=4	<=0,015	<8	32	0,5	2
2015-3810-14-NAL	Pink-footed	Young												<0,5	>64	8	128	0,25	>1024	<=2	>32	<1
2015-3810-5-NAL	Pink-footed	Young												<0,5	>64	8	>128	0,25	>1024	<=2	>32	<1
2015-3824-10-NAL	Pink-footed	Young												1	>64	4	128	0,25	>1024	<=2	>32	<1
2015-3824-11-TET	Pink-footed	Young												<0,5	2	4	<=4	<=0,015	>1024	>64	<=0,25	2
2015-3824-12-TET	Pink-footed	Young												<0,5	4	8	<=4	<=0,015	>1024	64	1	<1
2015-3824-13-TET	Pink-footed	Young												<0,5	4	8	<=4	<=0,015	>1024	>64	1	<1
2015-3824-18-TET	Pink-footed	Young												<0,5	4	8	<=4	<=0,015	>1024	64	>32	<1
2015-3824-19-TET	Pink-footed	Young												<0,5	<=1	4	<=4	<=0,015	<8	32	<=0,25	<1
2015-3824-21-NAL	Pink-footed	Young												<0,5	>64	4	>128	0,25	>1024	<=2	>32	<1
2015-3824-27-TET	Pink-footed	Young												<0,5	4	8	<=4	<=0,015	>1024	64	1	<1
2015-3824-30-SUL	Pink-footed	Adult												<=0.5	>64	8	<=4	<=0.015	>1024	64	0,5	<=1
2015-3824-32-TET	Pink-footed	Adult												<0,5	4	8	<=4	<=0,015	>1024	64	1	<1
2015-3824-36-TET	Pink-footed	Adult												<0,5	4	8	<=4	<=0,015	>1024	>64	1	<1
2015-3824-37-TET	Greylag	Young												1	2	8	16	0,03	<8	64	<=0,25	2
2015-3824-39-NAL	Greylag	Adult												1	>64	64	>128	>8	>1024	>64	>32	<=1
2015-3824-7-TET	Pink-footed	Young												1	4	8	<=4	<=0,015	>1024	64	1	<1
2015-3824-9-NAL	Pink-footed	Young												<0,5	>64	4	128	0,25	>1024	<=2	>32	<1
2015-3869-3-NAL	Pink-footed	Young												<=0.5	>64	64	>128	8	>1024	>64	>32	<=1
2015-3869-4-TET	Pink-footed	Young												<0,5	4	8	<=4	<=0,015	>1024	64	>32	<1
2015-3869-5-TET	Pink-footed	Young												1	2	4	<=4	<=0,015	>1024	>64	0,5	<1
2015-3869-7-SUL	Pink-footed	Young												<=0.5	2	4	<=4	<=0.015	>1024	>64	<=0.25	<=1
2015-3869-7-TET	Pink-footed	Young												<0,5	2	4	<=4	<=0,015	>1024	>64	0,5	<1
2015-3992-13-TET	Greylag	Young												1	2	8	<=4	<=0,015	<8	64	0,5	<1
2015-3992-16-NAL	Pink-footed	Adult												<0,5	>64	4	128	0,25	>1024	<=2	>32	<1
2015-3992-20-TET	Pink-footed	Adult												<0,5	2	8	<=4	0,03	<8	64	0,5	2
2015-3992-21-NAL	Pink-footed	Adult												<0,5	>64	4	128	0,25	>1024	<=2	>32	<1
2015-3992-5-NAL	Pink-footed	Young												<=0.5	2	4	128	0,25	<8	<2	<=0.25	<=1
2015-3992-6-TET	Pink-footed	Young												<0,5	2	4	8	0,25	<8	64	0,5	<1
2015-3992-7-NAL	Pink-footed	Young												<=0.5	>64	32	>128	>8	>1024	>64	>32	<=1
2015-3992-8-TET	Pink-footed	Young												<0,5	4	8	<=4	<=0,015	>1024	64	1	<1
2015-4279-1-TET	Pink-footed	Young												<0,5	2	8	<=4	<=0,015	<8	32	0,5	<1
2019-F10-P	Pink-footed	Young												<0,5	4	8	<4	0,03	<8	4	1	16
2019-F37-P	Pink-footed	Adult												<0,5	4	8	<4	<0,015	<8	4	1	16

Table 6: Resistance genes and MICs for 36 *E. coli* isolates resistant against NAL, TET or SUL from initial screening and colistin from MIC-testing. Top bar indicates resistance genes and antibiotics used for MIC (SMX: sulfamethoxazole, TMP: trimethoprim, CIP:

ciprofloxacin, TET: tetracycline, AZI: azithromycin, NAL: nalidixic acid, COL: colistin, AMP: ampicillin, GEN: gentamicin. Coloured squares indicate the presence of resistance gene or phenotypic resistance. Each colour represents a different group of antibiotics, making comparison between genotype and phenotype easier to assess. Bottom two isolates obtained from samples collected in 2019 and are the only colistin resistant isolates. In this table only antibiotics where resistance was observed have been included.

Screening for virulence genes in resistant *E. coli*

Of the 36 isolates, screening for virulence genes (VG) through VirulenceFinder identified at least one or more VGs in 30 (83%) isolates. A total of 9 different VGs were identified; *iss* (increased serum survival), *iroN* (enterobactin siderophore receptor protein), *ipfA* (long polar fimbriae), *gad* (glutamate decarboxylase), *cma* (structural gene for colicin M), *mchF* (ABC transporter protein), *iha* (IrggA homologue adhesin), *pic* (colonization associated protein), *capU* (cap locus protein hexosyltransferase). The most prevalent VG was *iss*, identified in 25 (69%) of the isolates (Table 7). Long polar fimbriae (*ipfA)* was identified in 17 (47%) of the isolates, *iroN* was identified in 15 (42%) of the isolates, *gad* was identified in 15 (42%) of the isolates, *cma* was identified in 9 (25%) of the isolates, *mchF* was identified in 8 (22%) of the isolates, *pic* was identified in 4 (11%) of the isolates, *iha* was identified in 3 (8%) of the isolates and *capU* was identified in only one (3%) of the isolates.

Isolate name	Species	Age	Sequence type	Phylogroup	capU	cma	gad	iha	lpfA	iroN	iss	mchF	pic
2015-3810-11-NAL	Pink-footed	Adult	162|469	B1					■	■	■		
2015-3810-14-NAL	Pink-footed	Young	162|469	B1			■		■	■	■		
2015-3824-10-NAL	Pink-footed	Adult	162|469	B1			■		■	■	■		
2015-3824-21-NAL	Pink-footed	Adult	162|469	B1			■		■	■	■		
2015-3810-5-NAL	Pink-footed	Young	162|469	B1					■	■	■		
2015-3824-30-SUL	Pink-footed	Young	88|23	C					■	■	■	■	
2015-3869-7-SUL	Pink-footed	Young	117	B2/F					■	■	■	■	■
2015-3824-9-NAL	Pink-footed	Adult	162|469	B1					■	■	■		
2015-3992-16-NAL	Pink-footed	Young	162|469	B1		■			■	■	■		
2015-3992-21-NAL	Pink-footed	Young	162|469	B1		■			■	■	■		
2015-3824-12-TET	Pink-footed	Adult	1126	B1		■	■		■		■		
2015-3824-13-TET	Pink-footed	Young	1126	B1		■	■		■		■		
2015-3824-27-TET	Pink-footed	Young	1126	B1		■	■		■		■		
2015-3824-32-TET	Pink-footed	Young	1126	B1		■	■		■		■		
2015-3824-36-TET	Pink-footed	Young	1126	B1		■	■		■		■		
2015-3824-7-TET	Greylag	Adult	1126	B1		■	■		■		■		
2015-3992-8-TET	Pink-footed	Young	1126	B1		■	■		■		■		
2015-3824-11-TET	Pink-footed	Young	117	B2/F				■		■	■	■	■
2015-3869-5-TET	Pink-footed	Adult	117	B2/F				■		■	■	■	■
2015-3869-7-TET	Pink-footed	Adult	117	B2/F				■		■	■	■	■
2015-3824-18-TET	Pink-footed	Young	540	A						■	■	■	
2015-3869-4-TET	Pink-footed	Adult	540	A						■	■	■	
2015-3992-5-NAL	Pink-footed	Young	95|95	B2							■	■	
2015-4279-1-TET	Pink-footed	Young	10|10	A							■		
2015-3810-13-TET	Greylag	Young	10|10	A			■				■		
2015-3992-6-TET	Pink-footed	Young	2165	B1			■		■				
2015-3824-19-TET	Pink-footed	Young	10|10	A			■						
2015-3824-37-TET	Greylag	Young	34|10	A			■						
2015-3810-12-NAL	Pink-footed	Young	1695	A	■								
2015-3824-39-NAL	Pink-footed	Young	744	A									
2015-3869-3-NAL	Pink-footed	Young	744	A			■						
2015-3992-13-TET	Pink-footed	Young	2064										
2015-3992-20-TET	Pink-footed	Young	34|10	A									
2015-3992-7-NAL	Pink-footed	Young	744	A									
2019-F10-P	Pink-footed	Young	720	D									
2019-F37-P	Pink-footed	Adult	720	D									

Table 7: Virulence genes, sequence types and phylogroups for 36 *E. coli* isolates resistant against NAL, TET or SUL from initial screening and colistin from MIC-testing. Top bar indicates virulence genes. Grey squares indicate the presence of virulence gene and white indicates no gene detected.

Screening for mutations in quinolone resistance-determining regions (QRDR)

Detection of QRDR mutations by ResFiner identified a total of 4 different mutations, two in *gyrA* and two in *parC*, in 12/36 isolates that were whole genome sequenced (Table 8). All the isolates with mutations were positive on initial screening for resistance against nalidixic acid and had no resistance gene identified. The *gyrA* single mutation (S83L) was the most prevalent one identified in 12 isolates and three of those had the *gyrA* double mutation (S83L plus D87N) Only three isolates were identified with the parC double mutation (A56T plus S80I) and all of them also harboured *parC* double mutation (A56T plus S80I). The combination of double mutation in both *gyrA* and *parC* had an increased effect on fluroquinolone resistance as high MIC-values against CIP of 8 or higher were only detected in those isolates. Those three isolates also had high MIC-values against NAL, but this was also found in two other isolates with only a single *gyrA* mutation (S83L).

Isolate name	Species	Age	Sequence type	Phylogrpoup	MIC		Quinolone mutations			
							gyrAp.		parCp.	
					CIP	NAL	D87N	S83L	A56T	S80I
2015-3810-11-NAL	Pink-footed	Adult	162|469	B1	0,25	128				
2015-3810-12-NAL	Pink-footed	Young	1695	A	0,25	128				
2015-3810-13-TET	Pink-footed	Adult	10|10	A	<=0,015	<=4				
2015-3810-14-NAL	Pink-footed	Adult	162|469	B1	0,25	128				
2015-3810-5-NAL	Pink-footed	Young	162|469	B1	0,25	>128				
2015-3824-10-NAL	Pink-footed	Young	162|469	B1	0,25	128				
2015-3824-11-TET	Pink-footed	Young	117	B2/F	<=0,015	<=4				
2015-3824-12-TET	Pink-footed	Adult	1126	B1	<=0,015	<=4				
2015-3824-13-TET	Pink-footed	Young	1126	B1	<=0,015	<=4				
2015-3824-18-TET	Pink-footed	Young	540	A	<=0,015	<=4				
2015-3824-19-TET	Pink-footed	Adult	10|10	A	<=0,015	<=4				
2015-3824-21-NAL	Pink-footed	Young	162|469	B1	0,25	>128				
2015-3824-27-TET	Pink-footed	Young	1126	B1	<=0,015	<=4				
2015-3824-30-SUL	Pink-footed	Young	88|23	C	<=0.015	<=4				
2015-3824-32-TET	Pink-footed	Young	1126	B1	<=0,015	<=4				
2015-3824-36-TET	Greylag	Adult	1126	B1	<=0,015	<=4				
2015-3824-37-TET	Pink-footed	Young	34|10	A	0,03	16				
2015-3824-39-NAL	Pink-footed	Young	744	A	>8	>128				
2015-3824-7-TET	Pink-footed	Adult	1126	B1	<=0,015	<=4				
2015-3824-9-NAL	Pink-footed	Adult	162|469	B1	0,25	128				
2015-3869-3-NAL	Pink-footed	Young	744	A	8	>128				
2015-3869-4-TET	Pink-footed	Adult	540	A	<=0,015	<=4				
2015-3869-5-TET	Pink-footed	Young	117	B2/F	<=0,015	<=4				
2015-3869-7-SUL	Pink-footed	Young	117	B2/F	<=0.015	<=4				
2015-3869-7-TET	Greylag	Young	117	B2/F	<=0,015	<=4				
2015-3992-13-TET	Pink-footed	Young	2064		<=0,015	<=4				
2015-3992-16-NAL	Pink-footed	Young	162|469	B1	0,25	128				
2015-3992-20-TET	Greylag	Young	34|10	A	0,03	<=4				
2015-3992-21-NAL	Pink-footed	Young	162|469	B1	0,25	128				
2015-3992-5-NAL	Pink-footed	Young	95|95	B2	0,25	128				
2015-3992-6-TET	Pink-footed	Young	2165	B1	0,25	8				
2015-3992-7-NAL	Pink-footed	Young	744	A	>8	>128				
2015-3992-8-TET	Pink-footed	Young	1126	B1	<=0,015	<=4				
2015-4279-1-TET	Pink-footed	Young	10|10	A	<=0,015	<=4				
2019-F10-P	Pink-footed	Young	720	D	0,03	<4				
2019-F37-P	Pink-footed	Adult	720	D	<0,015	<4				

Table 8: Mutations in QRDR, MIC-value for CIP and NAL, sequence types and phylogroups for 36. *E. coli* isolates resistant against NAL, TET or SUL from initial screening and colistin from MIC-testing. Grey and light-grey squared indicates MIC-value above cut-off or the presence of mutations.

Screening for mutations associated with colistin resistance

Detection of mutations in regions associated with colistin resistance, including the pmrAB and PhoPQ two-component regulatory systems, was done through annotation with OATRIC, identification of the appropriate genes and alignment with reference genes by MEGAx. A total of 6 genes were investigated for mutations; *MgrB, PhoP, PhoQ, pmrA, pmrB* and *pmrD*. The same mutations were found in both susceptible and resistant isolates, but the specific combination of multiple mutations detected in the colistin resistant isolates was not found in any of the susceptible isolates. This was the combination of mutations in *pmrb* (D283G plus H2R) and pmrD (K82T plus V83A).

						Colistin mutations																								
					MIC	MgrB		PhoP	PhoQ				pmrA			pmrB									pmrD				
Isolate name	Species	Age	Sequence type	Phylogrpoup	COL	I41L	V8A	Q21L	A482T	E464D	I175F	R6H	G144S	I128N	T31S	A242T	D283G	E123D	V351I	A360V	R341Q	H2R	Y358N	L19I	S71C	K82T	V83A
2015-3810-11-NAL	Pink-footed	Adult	162|469	B1	<1																						
2015-3810-12-NAL	Pink-footed	Young	1695	A	<1																						
2015-3810-13-TET	Pink-footed	Adult	10|10	A	2																						
2015-3810-14-NAL	Pink-footed	Adult	162|469	B1	<1																						
2015-3810-5-NAL	Pink-footed	Young	162|469	B1	<1																						
2015-3824-10-NAL	Pink-footed	Young	162|469	B1	<1																						
2015-3824-11-TET	Pink-footed	Young	117	B2/F	2																						
2015-3824-12-TET	Pink-footed	Adult	1126	B1	<1																						
2015-3824-13-TET	Pink-footed	Young	1126	B1	<1																						
2015-3824-18-TET	Pink-footed	Young	540	A	<1																						
2015-3824-19-TET	Pink-footed	Adult	10|10	A	<1																						
2015-3824-21-NAL	Pink-footed	Young	162|469	B1	<1																						
2015-3824-27-TET	Pink-footed	Young	1126	B1	<1																						
2015-3824-30-SUL	Pink-footed	Young	88|23	C	<=1																						
2015-3824-32-TET	Pink-footed	Young	1126	B1	<1																						
2015-3824-36-TET	Greylag	Adult	1126	B1	<1																						
2015-3824-37-TET	Pink-footed	Young	34|10	A	2																						
2015-3824-39-NAL	Pink-footed	Young	744	A	<=1																						
2015-3824-7-TET	Pink-footed	Adult	1126	B1	<1																						
2015-3824-9-NAL	Pink-footed	Adult	162|469	B1	<1																						
2015-3869-3-NAL	Pink-footed	Young	744	A	<=1																						
2015-3869-4-TET	Pink-footed	Adult	540	A	<1																						
2015-3869-5-TET	Pink-footed	Young	117	B2/F	<1																						
2015-3869-7-SUL	Pink-footed	Young	117	B2/F	<=1																						
2015-3869-7-TET	Greylag	Young	117	B2/F	<1																						
2015-3992-13-TET	Pink-footed	Young	2064		<1																						
2015-3992-16-NAL	Pink-footed	Young	162|469	B1	<1																						
2015-3992-20-TET	Greylag	Young	34|10	A	2																						
2015-3992-21-NAL	Pink-footed	Young	162|469	B1	<1																						
2015-3992-5-NAL	Pink-footed	Young	95|95	B2	<=1																						
2015-3992-6-TET	Pink-footed	Young	2165	B1	<1																						
2015-3992-7-NAL	Pink-footed	Young	744	A	<=1																						
2015-3992-8-TET	Pink-footed	Young	1126	B1	<1																						
2015-4279-1-TET	Pink-footed	Young	10|10	A	<1																						
2019-F10-P	Pink-footed	Young	720	D	16																						
2019-F37-P	Pink-footed	Adult	720	D	16																						

Table 9: Mutations in CRDR, MIC-values for COL, sequence type and phylogroup for 36 *E. coli* isolates resistant against NAL, TET or SUL from initial screening and colistin from MIC-testing. Grey and light-grey squared indicates MIC-value above cut-off or the presence of mutations.

Sequence type distribution within 36 *E. coli* isolates selected for WGS

The sequence types (STs) of all 36 *E. coli* isolates were identified using pubMLST, providing a total of 14 different STs. ST-162 was identified in 8 (22%) of the isolates, followed by ST-1126 in 7 (19%) of the isolates (Figure 4). ST-744, which was only found in young individuals, and ST-162 was associated with the most phenotypic resistance. Isolates of ST117 also originated only from young individuals. The two colistin resistant isolates were identified as ST-720.

Figure 4: Phylogeny of 35 resistant *E. coli* isolated from greylag- and pink-footed geese. Outer line indicates sequence type. Coloured lines indicate the presence of phenotypic

resistance and each colour represents a different antibiotic. The centre demonstrates a phylogenetic tree with maximum likelihood between the different isolates. A single isolate, 3810-11-NAL, has been misplaced in the phylogenetic tree and another isolate, 3869-7-SUL, is not present in the figure.

Isolates within the same sequence type expressed the same phenotypic resistance profiles (Table 10). The sequence types with resistance on MIC against the most antibiotics were ST-162 and ST-744. ST-162 had MIC-values above cut-off for SMX, TMP, CIP, NAL and AMP. ST-744 isolates were resistant against SMX, TMP, AZI, CIP, TET, NAL, chloramphenicol (CHL) and AMP and were the only ones exhibiting high MIC value against CIP, most likely due to the carriage of double mutations in *gyrA and parC*. One additional isolate of ST-1695 also expressed quinolone- and fluroquinolone resistance.

Isolate name	Species	Age	Sequence type	Phylogrpoup	MIC							
					GEN	AMP	AZI	NAL	CIP	SMX	TET	TMP
2015-3810-12-NAL	Pink-footed	Adult	1695	A	<0,5	2	16	128	0,25	<8	4	0,5
2015-3810-13-TET	Pink-footed	Adult	10|10	A	<0,5	<=1	8	<=4	<=0,015	<8	32	0,5
2015-3824-18-TET	Pink-footed	Young	540	A	<0,5	4	8	<=4	<=0,015	>1024	64	>32
2015-3824-19-TET	Pink-footed	Young	10|10	A	<0,5	<=1	4	<=4	<=0,015	<8	32	<=0,25
2015-3824-37-TET	Greylag	Young	34|10	A	1	2	8	16	0,03	<8	64	<=0,25
2015-3824-39-NAL	Greylag	Adult	744	A	1	>64	64	>128	>8	>1024	>64	>32
2015-3869-3-NAL	Pink-footed	Young	744	A	<=0.5	>64	64	>128	8	>1024	>64	>32
2015-3869-4-TET	Pink-footed	Young	540	A	<0,5	4	8	<=4	<=0,015	>1024	64	>32
2015-3992-20-TET	Pink-footed	Adult	34|10	A	<0,5	2	8	<=4	0,03	<8	64	0,5
2015-3992-7-NAL	Pink-footed	Young	744	A	<=0.5	>64	32	>128	>8	>1024	>64	>32
2015-4279-1-TET	Pink-footed	Young	10|10	A	<0,5	2	8	<=4	<=0,015	<8	32	0,5
2015-3810-11-NAL	Pink-footed	Young	162|469	B1	2	>64	4	128	0,25	>1024	<=2	>32
2015-3810-14-NAL	Pink-footed	Young	162|469	B1	<0,5	>64	8	128	0,25	>1024	<=2	>32
2015-3810-5-NAL	Pink-footed	Young	162|469	B1	<0,5	>64	8	>128	0,25	>1024	<=2	>32
2015-3824-10-NAL	Pink-footed	Young	162|469	B1	1	>64	4	128	0,25	>1024	<=2	>32
2015-3824-12-TET	Pink-footed	Young	1126	B1	<0,5	4	8	<=4	<=0,015	>1024	64	1
2015-3824-13-TET	Pink-footed	Young	1126	B1	<0,5	4	8	<=4	<=0,015	>1024	>64	1
2015-3824-21-NAL	Pink-footed	Young	162|469	B1	<0,5	>64	4	>128	0,25	>1024	<=2	>32
2015-3824-27-TET	Pink-footed	Young	1126	B1	<0,5	4	8	<=4	<=0,015	>1024	64	1
2015-3824-32-TET	Pink-footed	Adult	1126	B1	<0,5	4	8	<=4	<=0,015	>1024	64	1
2015-3824-36-TET	Pink-footed	Adult	1126	B1	<0,5	4	8	<=4	<=0,015	>1024	>64	1
2015-3824-7-TET	Pink-footed	Young	1126	B1	1	4	8	<=4	<=0,015	>1024	64	1
2015-3824-9-NAL	Pink-footed	Young	162|469	B1	<0,5	>64	4	128	0,25	>1024	<=2	>32
2015-3992-16-NAL	Pink-footed	Adult	162|469	B1	<0,5	>64	4	128	0,25	>1024	<=2	>32
2015-3992-21-NAL	Pink-footed	Adult	162|469	B1	<0,5	>64	4	128	0,25	>1024	<=2	>32
2015-3992-6-TET	Pink-footed	Young	2165	B1	<0,5	2	4	8	0,25	<8	64	0,5
2015-3992-8-TET	Pink-footed	Young	1126	B1	<0,5	4	8	<=4	<=0,015	>1024	64	1
2015-3992-5-NAL	Pink-footed	Young	95|95	B2	<=0.5	2	4	128	0,25	<8	<2	<=0.25
2015-3824-11-TET	Pink-footed	Young	117	B2/F	<0,5	2	4	<=4	<=0,015	>1024	>64	<=0,25
2015-3869-5-TET	Pink-footed	Young	117	B2/F	1	2	4	<=4	<=0,015	>1024	>64	0,5
2015-3869-7-SUL	Pink-footed	Young	117	B2/F	<=0.5	2	4	<=4	<=0.015	>1024	>64	<=0.25
2015-3869-7-TET	Pink-footed	Young	117	B2/F	<0,5	2	4	<=4	<=0,015	>1024	>64	0,5
2015-3824-30-SUL	Pink-footed	Adult	88|23	C	<=0.5	>64	8	<=4	<=0.015	>1024	64	0,5
2015-3992-13-TET	Greylag	Young	2064		1	2	8	<=4	<=0,015	<8	64	0,5
2019-F10-P	Pink-footed	Young	720	D	<0,5	4	8	<4	0,03	<8	4	1
2019-F37-P	Pink-footed	Adult	720	D	<0,5	4	8	<4	<0,015	<8	4	1

Table 10: MIC values, sequence type and phylogroup of 36 *E. coli* isolates. Top bar indicates the antibiotics that were used. Coloured square indicates MIC-value above cut-off and the different colours corresponds to groups of antibiotics.

Isolates with the same sequence type tended towards carrying the same set of AMR genes Table 11). The most resistance genes were detected in ST-162 and ST-744, which also had the most phenotypic resistance. More than five resistance genes were only detected in ST-744 and five resistance genes were detected in ST-162 and ST-88. *mphA*, which codes for resistance against macrolides was identified in the ST-744 isolates. ST-2165 carried the *qnrS1* gene, encoding quinolone resistance, but had no other resistance genes.

Isolate name	Species	Age	Sequence type	Phylogroups	aph(3")-Ib	aph(6)-Id	blaTEM-1B	mph(A)	qnrS1	sul1	sul2	tet(A)	tet(B)	dfrA17	dfrA5
2015-3824-37-TET	Greylag	Young	34|10	A											
2015-3992-20-TET	Pink-footed	Adult	34|10	A											
2015-3810-13-TET	Pink-footed	Adult	10|10	A											
2015-3824-19-TET	Pink-footed	Young	10|10	A											
2015-4279-1-TET	Pink-footed	Young	10|10	A											
2015-3810-12-NAL	Pink-footed	Adult	1695	A											
2015-3824-39-NAL	Greylag	Adult	744	A	Q	Q					Q				
2015-3869-3-NAL	Pink-footed	Young	744	A	Q	Q					Q				
2015-3992-7-NAL	Pink-footed	Young	744	A	Q	Q					Q				
2015-3824-18-TET	Pink-footed	Young	540	A						F/B		F/B			F/B
2015-3869-4-TET	Pink-footed	Young	540	A						F/B		F/B			F/B
2015-3810-11-NAL	Pink-footed	Young	162|469	B1	Q	Q					Q				B
2015-3810-14-NAL	Pink-footed	Young	162|469	B1	Q	Q					Q				F/B
2015-3810-5-NAL	Pink-footed	Young	162|469	B1	Q	Q					Q				B
2015-3824-10-NAL	Pink-footed	Young	162|469	B1	Q	Q					Q				F/B
2015-3824-21-NAL	Pink-footed	Young	162|469	B1	Q	Q					Q				F/B
2015-3824-9-NAL	Pink-footed	Young	162|469	B1	Q	Q					Q				B
2015-3992-16-NAL	Pink-footed	Adult	162|469	B1	Q	Q					Q				
2015-3992-21-NAL	Pink-footed	Adult	162|469	B1	Q	Q					Q				
2015-3992-6-TET	Pink-footed	Young	2165	B1											
2015-3824-12-TET	Pink-footed	Young	1126	B1								F			
2015-3824-13-TET	Pink-footed	Young	1126	B1								F			
2015-3824-27-TET	Pink-footed	Young	1126	B1								F			
2015-3824-32-TET	Pink-footed	Adult	1126	B1								F			
2015-3824-36-TET	Pink-footed	Adult	1126	B1								F			
2015-3824-7-TET	Pink-footed	Young	1126	B1								F			
2015-3992-8-TET	Pink-footed	Young	1126	B1								F			
2015-3992-5-NAL	Pink-footed	Young	95|95	B2											
2015-3824-11-TET	Pink-footed	Young	117	B2/F											
2015-3869-5-TET	Pink-footed	Young	117	B2/F											
2015-3869-7-SUL	Pink-footed	Young	117	B2/F											
2015-3869-7-TET	Pink-footed	Young	117	B2/F											
2015-3824-30-SUL	Pink-footed	Adult	88|23	C								B			
2015-3992-13-TET	Greylag	Young	2064												
2019-F10-P	Pink-footed	Young	720	D											
2019-F37-P	Pink-footed	Adult	720	D											

Table 11: Resistance genes, sequence type and phylogroup of 36 *E. coli* isolates. Top bare indicates resistance genes. Coloured square indicates the presence of gene and white indicates no gene present. Each colour corresponds to a specific group of antibiotics. The letters Q, F, B and F/B indicates that the resistance genes are located on plasmids with either IncQ1 (Q) replicon, IncFII (F) replicon, IncFIB(AP001918) (B) replicon or a combination of IncFII/IncFIB(AP001918) (F/B) replicons.

Like with MIC and AMR genes, the same sequence types carried similar virulence genes (VGs) (Table 7). The most resistant ST-744 isolates turned out to have the lowest number of virulence genes, while ST-162, also expressing broad resistance, had between 3 and 4 VGs. The most VGs was found in ST-117 and ST-1126 also carried a quite high (4) number of VGs.

Phylogenetic group distribution within 36 *E. coli* isolates selected for WGS

Bu utilizing phylogroup affiliation of sequence types we were able to identify the phylogenetic grouping in 35 of the 36. E coli isolates that were whole genome sequenced. The following groups were identified; A, B1, B2/F, B2, C and D. The majority of isolates belonged to the less pathogenic groups of A and B1. Seven isolates belonged to the groups of B2 (1 isolate), B2/F (4 isolates) and D (2 isolates) which are known to be more pathogenic. A single isolate was identified in phylogroup C.

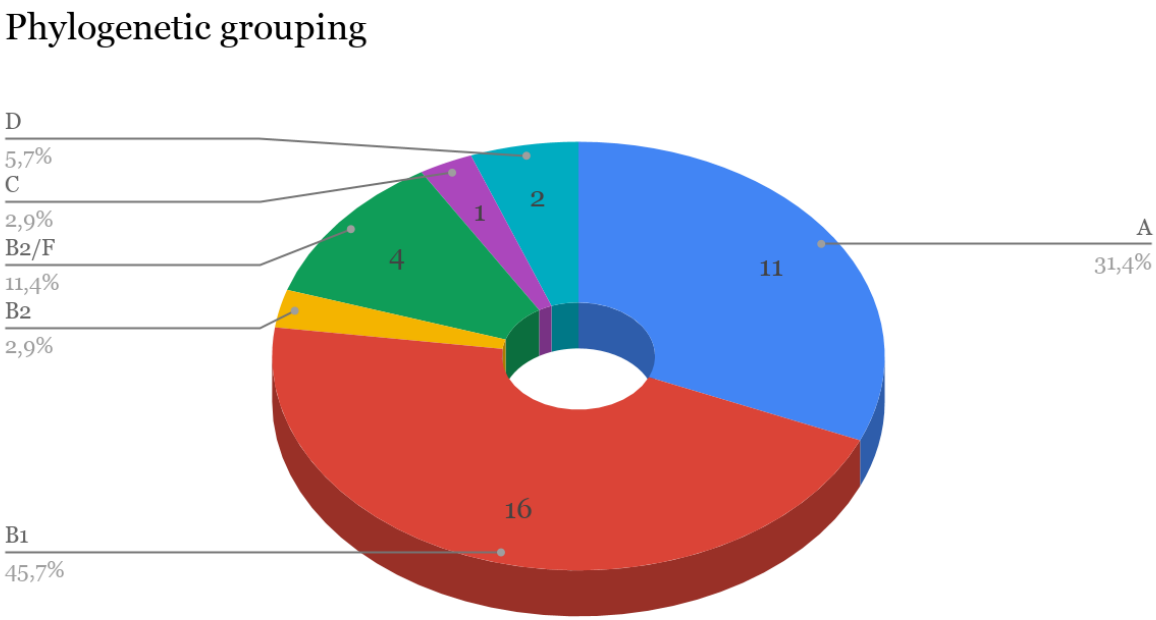


Figure 5: Sector diagram demonstrating the distribution of phylogroups among the 35 *E. coli* isolates that had resistance from screening and were investigated further with whole genome sequencing.

The less pathogenic phylogenetic groups of A and B1 expressed resistance against considerably more antibiotics than the more pathogenic groups of B2, B2/F and D (Table 10). Only resistance against SMX and TET was observed within the last three groups mentioned. A and B1 had, in addition to resistance against SMX and TET, high MIC values above cut-off for AMP (Ampicillins), AZI (Macrolides), NAL (Quinolones), CIP (Fluroquinolones), and TMP (Trimethoprim's). This was expected as previous research has demonstrated that the less pathogenic phylogroups tend to be more resistant.

There was a clear difference in the number of AMR genes identified in the different phylogroups (Table 11). Again, the less pathogenic groups of A and B carried far more resistance genes than that of the more pathogenic groups (B2, B2/F, D). The B2/F group only carried *aph(3'')-lb* and *aph(6)-ld*, encoding for macrolide resistance, while the B2 and D group didn't carry any resistance genes at all.

The more pathogenic phylogroups are associated with a wide range of virulence factors contributing to their pathogenicity (Table 7). From the 36 *E. coli* that were analysed, a clear difference could not be determined. For phylogroup B1, a less pathogenic one, 12/16 isolates carried as many as 4 virulence genes, while B2/F, a more pathogenic group, carried up to five virulence genes. The other pathogenic groups like B2 and D, only carried 2 and 0 VGs, respectively. The other less pathogenic group, A, carried less than three VGs, as expected.

Through PlasmidFinder we were able to identify 11 different plasmid replicons in 33 of our 36 whole genome sequenced *E. coli* isolates (Table 11). The plasmid replicons detected were; IncFIB AP001918 (23 isolates), IncFII (19 isolates), IncQ1 (12 isolates), Col156 (6 isolates), p0111 (1 isolate), IncFIC FII (4 isolates), ColRNAI (5 isolates), IncX1 (2 isolates), IncY (2

isolates), Col pHAD28 (4 isolates)9999 and IncFII pRSB107 (2 isolates). By looking at the annotation of genes we were able to identify replicons located at the same contig and made the conclusion that they were part of the same plasmid. We concluded the same for resistance genes located on the same contig as plasmids that were identified. Of the 33 isolates with plasmids identified, 5 isolates had 4 plasmids, 5 isolates had 3 plasmids, 13 isolates had 2 plasmids and 9 isolates had 1 plasmid. The most common replicons to be located on the same plasmid were IncFIB AP001918 and IncFII replicons. Plasmid-mediated resistance genes were found in 21 *E. coli* isolates. Three highly resistant ST-744 isolates were identified with *aph(3'')-lb, aph(6)-ld* and *sul2* genes located on IncQ1 plasmids. ST-162 had, in addition to the genes mentioned, *dfrA5* located on IncF plasmids. Two ST-520 isolates had *sul1, tetA* and *dfrA5* AMR genes on IncF plasmids with IncFIB AP001918/IncFII replicons. The TET resistant ST-1126 isolates, as well as a single isolate of phylogroup C, carried the *tetA* gene on IncF plasmids.

Discussion

Phenotypical analysis

Carriage rate of quinolone-, tetracycline-, and extended-spectrum cephalosporin resistant *E. coli*

Our gathered AMR prevalence from the 2015 and 2019 samples, tells us that around one third of our samples are resistant to TET, NAL and/or COL. However, the prevalence variance between the years is 10%. The reason for this gap in prevalence, is thought to be linked to the randomization of the selection of geese, and the near impossible chance of shooting the same subpopulation of birds in 2019, as in 2015. Furthermore, if hunting was systemized into hunting the geese in the same period, at the same time each year, randomization would still occur. If we could implement a surveillance program, using a combination of bird ringing and MLST, we would be able to track the bird's habitat of origin and do a possible connection between geographical location of the host and the bacterial sequence type. To get a good statistical overview of the average AMR prevalence, continuous sampling during a set period of time, should be considered.

Minimum inhibitory concentration (MIC) of polymyxin screening

A low number of isolates, only 2 out of 22. with growth on superpolymyxin plates, had their screening results confirmed on MIC-testing for colistin resistance. This high discrepancy between screening and actual expression of resistance is somewhat surprising considering previous reports estimating the superpolymyxin to have a sensitivity and specificity close to 100% (57).

Genotypical analysis

Mutations in QRDR and CRDR

The genotypical analysis for mutations in QRDR revealed that a single gyrA mutation was present in almost every isolate with quinolone resistance. In addition, the presence of double mutations in gyrA together with double mutations in parC conferred high-level resistance against fluroquinolones (ciprofloxacin MID, >8 microg/ml). These results correlate well with previous reports of high-level fluroquinolone resistance in *E. coli* isolates with both gyrA and parC mutations present (58).

To figure out the reason for the high MIC values of our colistin resistant isolates, without detecting plasmid mediated colistin resistance (PMCR), a closer investigation of the CRDR was indicated. Several mutations were found in all of the isolates that was genotypical tested. However, none of the mutations found gave any indications of being the reason for the increased MIC in our colistin resistant isolates. Furthermore, one isolate, ST95, expressed SNPs in 15 different locations in the CRDR. No resistance towards colistin was found in this isolate, despite these findings. This leads us to believe that further global research of the CRDR is required before any conclusions can be drawn, regarding our results.

Sequence type identification

Some of the STs in our study were of particular interest, as they are known to be connected to diseases in humans and livestock. ST744, which carried resistance to multiple types of antibiotics, was found in three of our samples. This ST has been found in human bloodstream infections and imported chick meat (59), as well as being a carrier of mcr-1 and blaKPC-3 resistance genes (60). There has also been reports of ST744 isolates carrying mcr-3 and $bla_{CTX\text{-}M\text{-}55}$ (61).

Our most predominant multi-resistant ST was ST162. This ST has been found in both broiler chickens and red foxes in Norway (62), and despite low usage of quinolone antibiotics on a national basis, a high prevalence of *gyrA and parC* mutations have been detected in our ST162 isolates. By detecting a rise in quinolone resistance in both livestock and wildlife in Norway, further studies are required to find out the reason for this increase in prevalence.

ST95, a well-known ST in human medicine, was found in one of our samples. This ST has been reported as a human pathogenic strain, more specifically known as neonatal meningitis (63). Furthermore, the same ST has been reported as an extra intestinal pathogen *E. coli* (ExPEC), which are found in poultry as reservoirs (64). This sample also had the most mutations in the CRDR in the two-component system, without expressing colistin resistance, and became a baseline for debunking articles mentioning specific mutations for vertical transmission for colistin resistance.

ST720, which amounted to two of our samples, were carriers of phenotypical colistin resistance. We found no known genotypical evidence for transmission of resistance through plasmid-mediation or chromosomal mutations. Further analysis of the CRDR in the two-component system gave no evidence of mutations in base-pairs known to express phenotypical resistance. Here, it seems like chromosomal mutations in CRDR are yet to be discovered, and mapping of potential mechanisms for vertical transmission is still to be determined. Even though ST720 is considered to be in phylogroup D, and therefore human pathogenic, no reports indicating colistin resistance in this ST has been reported.

Phylogenetic analysis

Our results indicate that B1 is the main phylogroup of *E. coli* isolated from wild migratory geese followed by phylogroup A. The same distribution of phylogroups has also been previously described in domestic animals (65). Strains within these two groups are known to be commensal and less pathogenic, but have been reported to express more resistance (66). Our results demonstrate the same as the less pathogenic groups of A and B1 expressed resistance against considerably more antibiotics than the other groups. The more pathogenic groups of B2 and D, associated with extraintestinal pathogenic *E. coli* and known to carry a waste variety of virulence factors (67), expressed sparse resistance against antibiotics and did not carry more virulence genes than the other groups in our study. The exception being phylogroup B2/F, a sister group to B2, carrying the most virulence genes. With the number of virulence genes being associated with higher pathogenicity (68), one could hypothesise that these isolate may pose a threat to animal and human health. On the other hand, these isolates are also the most susceptible ones making treatment failure less likely.

Plasmid-mediated resistance genes

In this study we were able to identify a range of different incompatibility groups of plasmid replicons, of which several carried resistance gene. The IncQ1 group found in 12 isolates, is a relatively small plasmid found in a broad range of bacterial hosts and known to carry resistance against streptomycin and sulphonamide. In our study, we found the plasmids to harbour the *sul2* gene, but did not identify the *tetA* gene which recent research have reported (69). This kind of resistance is not of major concern and is known to be present within this plasmid group. The IncF plasmids represent one of the most prevalent incompatibility types worldwide in Enterobacteriaceae and were found in the most isolates in this study. Our investigation identified three different resistance genes, *sul1*, *dfrA5* and *tetA*, on the IncF plasmids that are known to commonly carry multi resistance determinants (70). Although no

resistance genes against more important groups of antibiotics like cephalosporins and quinolones were identified, this demonstrates the potential for transfer of resistance genes both to, within and from *E. coli* isolates in geese, should the dissemination of AMR continue to increase within wild animals.

Further discussion

The global AMR problem

On a global basis, the world is experiencing a rise in antimicrobial resistance both in human- and veterinary practice. The reason for this, is thought to be an overly liberal use of antibiotics in the treatment of various infectious diseases, and as growth promotors in the animal industry. The latter, in which the veterinary field is uniquely responsible, has a huge impact factor when it comes to resistance drive. The volumes, concentrations and dosages of antibiotic administration through feed and veterinary practice, has a huge impact on the transfer of antibiotics through food for human consumption and into the environment. The extent of AMR impact on wildlife is yet to be determined in more details, and further research is required to get a bigger overview of the spillover from possible anthropogenic sources. However, our study shows that there is evidence of resistance mechanisms in *E. coli* in wildlife, which is thought to origin from human- and veterinary practice. By using the long-distance migratory geese as a model for AMR vectors, we were able to use a model that migrates between habitats in countries with a vast difference in antibiotic therapy recommendations. In addition to the geographical variable, the geese, as an avian species, are known to be carriers of other zoonotic diseases like avian influenza, and extra-intestinal *E. coli* (ExPEC). Finally, the geese also live in areas quite close to both human- and wildlife populations, which means that an eventual spillover of AMR has a potential of contaminating areas that would otherwise not be affected. All of these factors combined, makes the geese a

sufficient model for transferring AMR in a One Health perspective. To further evaluate the impact of this contamination, further sampling of the cloaca of the geese, the environment and the livestock near the habitats is required.

The connection between AMR, MLST and wildlife

The importance of multi-locus sequencing in *E. coli* isolates from wildlife is essential in this type of research, as it tells us the connection between host species and the environment in a bigger perspective. Wildlife, though underestimated, is of great importance to the global resistance picture, as it in theory should be unaffected by human- and veterinary practices regarding antibiotic therapies. By determining MLST in our isolates, we were able to find human- and animal pathogenic isolates, which can be found in articles regarding AMR in human- and veterinary practices. The diversity of clonal complexes also tells us that the origin of the geese may differ. However, our findings indicate that both the greylag geese and the Pink-footed geese carries multi-resistant and colistin resistant *E. coli*, often with the same type of clonal complexes. This means that there is some spillover between the host species, which most likely origins from their converging habitats.

Anthropogenically relevant factors in wildlife management

Reasons for antibiotic resistance drive in wildlife may be caused by runoff by sewers, livestock farming or other types of anthropogenic factors in the environment. Sewage and human waste are important factors, as they usually have their runoff into a watery environment. The sheer number of geese populations as it is today, reduces the sustainability of their natural habitats, and migration towards human sources is of essence to survive. Our study indicates the geese's need for rest and feeding in an area known to be close to human

sources, as the geese feed on farmland during their migrating flight. Further studies around these anthropogenic sources is of essence to determine the runoff and spillover capability in a One Health perspective.

MLST as a geographical fingerprint

In addition to determining the pathogenicity of our isolates, MLST might be used to determine the origins of the possible AMR spillover. In our study, there was no possible way to determine their habitat of origin before heading out for their mating flight. By further conducting research in areas known to be highly concentrated of the host species, a statistical overview of the most predominant clonal complex might be of importance to determine a geographical fingerprint of the bacteria. This way, we might get an understanding of a possible origin of spillover from anthropogenic sources.

Introduction of AMR to "pure" environments

The Pink-footed geese has its end of flight in Svalbard in their mating period. The archipelagos of Svalbard are known to be "pure" in the sense of human contamination and should not be affected by AMR drive due to anthropogenic sources. In our study, the geese carried resistant *E. coli*, most likely caused by human interactions, in a predetermined attempt to reach Svalbard for mating. This way, introduction of resistant *E. coli* to Svalbard is highly likely to occur through this route of transmission.

Wildlife and the One Health perspective

One Health as a concept focuses on the different factors involved in the process of disease transmission between humans and animals alike. For the most, research has been conducted within human- and veterinary practices, due to availability and importance. However, our study shows that the wildlife factor in the One Health perspective functions as a vector for transmissible diseases through geographical borders, and the possible interaction between wildlife and anthropogenic sources. To further strengthen the theory of wildlife impact in a One Health perspective, further research and implementation of research into microbiological surveillance programs is required.

Conclusion

Around one third of the birds sampled contained *E. coli* resistant against one or more antimicrobial agents, indicating that migrating birds can act as vectors for resistant bacteria. Genomic analysis suggests both horizontal and vertical transmission of resistance genes closely related to those commonly occurring in human isolates. To figure out the zoonotic capability of *E. coli* found in migratory birds, further testing of anthropogenic sources living closely to wildlife should be performed. Of these sources, the environmental component where migratory patterns in different species and subspecies converge is of special interest to evaluate any potential microbial spill over.

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Sammendrag

Tittel: Multiresistent *E. coli* i langdistansetrekkende gås. Hvordan grågås (*Anser anser*) og kortnebbgås (*Anser brachyrhynchus*) kan fungere som vektorer for antimikrobiell resistens.

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Trekkfugler kan bære antimikrobiell resistent *E. coli* over store avstander. Nyere studier har avdekket antibiotikaresistente bakterier fra ville dyr som bærer resistensgener mot antibiotika som er oppført på "WHO's List of Essential Medicines". I løpet av høsten 2015 og 2019 ble det samlet inn 201 kloakkprøver fra grågås og kortnebbgås som lander nær gårdsområder midt i Norge under flyturen mot deres overvintringsområde. Disse prøvene ble screenet for kinolon-, tetrasyklin- og cefalosporinresistent *E. coli*. Totalt førti-tre prøver screenet i 2015 (39%) inneholdt *E. coli* som uttrykte resistens mot en eller flere antibiotika, mens 26 av prøvene fra 2019 (29%) inneholdt resistente stammer med nesten identiske resistensmønstre. Vi fant isolater resistente mot kinoloner, tetrasykliner og andre typer antibiotika. Flere isolater hadde multiresistente mønstre. Isolater resistente mot colistin tilhørte ST720, som er identifisert som en mulig patogen sekvenstype gjennom fylogenetisk identifikasjon (fylotype D). Rundt en tredjedel av prøvene fra fugler inneholdt *E. coli* resistent mot ett eller flere antimikrobielle midler, noe som indikerer at trekkfugler kan fungere som vektorer for resistente bakterier.

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