Norges miljø- og biovitenskapelige universitet

Masteroppgave 2018 60 stp

Institute for chemistry, biotechnology and food science
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Characterization of cephalosporin-resistant *Escherichia coli* from Norwegian broiler production.

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Master's in Biotechology
Microbiology

Preface

The work presented in this Master thesis was performed under the Section for food Safety and Emerging Health Threats at the Norwegian Veterinary Institute (NVI) in Oslo. This study was part of the NoResist project, which main objective is to "to combat antimicrobial resistance in the Norwegian food chain".

First, I would like to thank my supervisor Solveig Sølverød Mo for giving me the opportunity to work on this project and giving me some guidance during the laboratory work. Second, I would like to thank Marianne Sunde, another co-supervisor for giving me some insightful advices regarding my thesis. Moreover, I would like to show my appreciation to Camilla Sekse and Jannice Schau Slettemeås for assisting me with the bioinformatics analysis. Another thanks to all the laboratory engineers at the Microbiology section at NVI, especially Bjørg Kvitle for sharing your valuable time with me and knowledge. Finally, I would like to show a great appreciation to my mum for her prayers and support. Finally, a big thanks to Clay Gouin at the NMBU writing center for giving me some guidance on my writing and my roommate Bastien Pierre Bissaro for all the help.

Abstract

Recently, extended spectrum beta lactamase (ESBL)-producing *E. coli* with a new genotype (*bla*$_{\text{CTX-M-1}}$) emerged in the Norwegian broiler production pyramid. Occurrence of ESBL-producing bacteria such as *E. coli* is a global concern because they resist highly important antimicrobials known as third-generation cephalosporins used in human medicine. Development of ESBL-producing bacteria have resulted from the use and misuse of antimicrobials in humans and livestock production. However, Norway hardly use antimicrobials in its broiler production but still detected ESBL-producing *E. coli* with *bla*$_{\text{CTX-M-1}}$. Thus, the goal of this study was to examine three questions regarding ESBL-producing *E. coli* emergence in the Norwegian broiler production pyramid. First, was this emergence a clonal spread of a specific ESBL-*E. coli* variant with one plasmid? Second, could it have been the clonal spread of several genetically unrelated *E. coli* variants with different plasmids and third, did a horizontal transfer of *bla*$_{\text{CTX-M-1}}$-harboring plasmids occur between different *E. coli* STs? Together 35 ESBL-producing *E. coli* isolates were investigated.

To examine these questions, molecular typing methods including PCR-based phylotyping and pulsed-field gel electrophoresis were used to analyze genetic relatedness between the ESBL-producing *E. coli* isolates. Isolates' phenotypic resistance was determined by minimum inhibitory concentration -and disk-diffusion tests. Plasmids associated with *bla*$_{\text{CTX-M-1}}$ were identified through conjugation experiments and plasmid replicon typing. For further characterization of the isolates, whole genome sequencing was performed to determine multi-locus sequence types (MLSTs), serotypes, virulence genes, acquired antimicrobial resistance genes, and genetic relatedness based on single nucleotide polymorphisms. Using the whole genome data, *bla*$_{\text{CTX-M-1}}$-carrying plasmids were identified, and one *bla*$_{\text{CTX-M-1}}$-IncI1α plasmid re-constructed.

Results showed most of the *E. coli* isolates were genetically related and grouped into a large cluster represented by the phylogroup D/ST-57-O140:H25 clonal lineage. Moreover, *E. coli* isolates from parents seemed more genetically diverse than the broiler isolates. IncI1α grouped into plasmid multi-locus sequence types (pMLST) 3 and 7 were identified as the main *bla*$_{\text{CTX-M-1}}$-carrying plasmids. IncI1α plasmids from this study shared close homology with other IncI1α plasmids detected in broilers from France and Switzerland. Genetic characteristics of the isolates and plasmids identified in this study were similar to previous reports in broilers from several European countries. Thus, the results demonstrated both clonal dissemination and horizontal transfer of the IncI1α plasmids disseminated cephalosporin resistant *E. coli* in the Norwegian broiler production. The *bla*$_{\text{CTX-M-1}}$-IncI1α plasmid characterized carried a toxin component, *hok* gene that could have maintained IncI1α plasmid in *E. coli* in the broiler production pyramid.

Norwegian Abstract

Nylig ble det gjort funn av ESBL-produserende *E. coli* med en ny genotype (*bla*$_{CTX-M-1}$) i den norske slaktekyllingproduksjonen. Forekomst av ESBL-produserende bakterier som *E. coli* er en global bekymring fordi de er motstandsdyktige mot kritisk viktige antimikrobielle midler som tredje generasjons-cefalosporiner, som brukes til behandling av infeksjoner hos mennesker. Utviklingen av ESBL-produserende *E. coli* skyldes bruk og misbruk av antimikrobielle midler hos mennesker og dyr. Imidlertid bruker Norge knapt antimikrobielle i sin slaktekyllingproduksjon, men allikevel påvises ESBL-produserende *E. coli* med *bla*$_{CTX-M-1}$. Hensikten med denne oppgaven var derfor å undersøke tre problemstillinger angående forekomsten av ESBL-produserende *E. coli* i den norske slaktekyllingproduksjonen. Først, kunne forekomsten ha vært en klonal spredning av en ESBL-produserende *E. coli* variant med ett plasmid? For det andre, kunne det ha vært en klonal spredning av flere genetiske ulike *E. coli* varianter med ulike plasmider, og sist, skjedde det en horisontal overføring av *bla*$_{CTX-M-1}$-bærende plasmider mellom forskjellige *E. coli* sekvenstyper? Til sammen ble 35 ESBL-*E. coli* med *bla*$_{CTX-M-1}$ undersøkt.

For å undersøke disse problemstillingene, ble det anvendt molekylære metoder som PCR-basert fylotyping og puls-felt gelelektroforese for å fastslå det genetiske slektskapet mellom de ESBL-produserende *E. coli* isolatene. Isolatenes fenotypiske resistens ble bestemt ved minste hemmende konsentrasjon og lappediffusjonstest. Plasmider assosiert med *bla*$_{CTX-M-1}$ ble identifisert gjennom konjugasjonsforsøk og plasmid-replikon-typing. For videre karakterisering av isolatene, ble de helgenomsekvensert for å bestemme MLST, serotype, virulensgener, antibiotika resistensgener og genetisk slektskap basert på enkletnukleotidpolymorfier (SNP). Ved bruk av helgenomdata ble *bla*$_{CTX-M-1}$-bærende plasmider identifisert, og ett *bla*$_{CTX-M-1}$-IncIl-plasmid karakterisert.

Resultatene viste at de fleste av *E. coli*-isolatene var genetiske relaterte og gruppert i et stort kluster representert av fylogruppe D/ST-57-O140: H25 klonen. I tillegg så det ut som at det var en større genetisk variasjon i *E. coli*-isolatene fra foreldredyr enn isolatene fra slaktekylling. IncI1α/ST3 og IncI1α/ST7 ble identifisert som de viktigste *bla*$_{CTX-M-1}$-bærende plasmidene. IncI1α plasmider fra denne studien var i større grad likt andre IncI1α plasmider funnet i slaktekylling fra Frankrike og Sveits. De genetiske egenskapene til isolatene og plasmidene funnet i denne studien lignet på isolater og plasmider fra slaktekylling i flere europeiske land. Stort sett indikerte resultatene at både klonal utbredelse og horisontal overføring av IncI1α plasmidene spredte cefalosporin resistent *E. coli* i den norske slaktekyllingproduksjonen. Det karakteriserte *bla*$_{CTX-M-1}$-IncI1α plasmidet uttrykte en toksinkomponent, *hok*-genet, som kunne ha opprettholdt IncIl-plasmid i *E. coli* i slaktekyllingproduksjonen.

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1. Introduction

1.1 Norwegian broiler production

Broiler (*Gallus gallus*) is a chicken raised specifically for meat production. In Europe, the broiler production follows a breeding pyramid structure where purebred animals such as great grandparents are on top followed by the breeders (grandparent and parent stocks) in the middle and the broilers at the bottom of the pyramid (Figure. 1) (Mo, 2016). In Norway, the parent animals and broilers are raised, with the number of broilers produced in 2017 around 65.5 million according to the Statistics Norway (SSB, 2017)

Broiler production in Norway starts with the import of hatching eggs from grandparent animals, exported from Scotland to Sweden. The imported eggs from Sweden are hatched in Norway into day-old parent animals and sent to rearing farms where they are kept until 18 weeks old. Eggs from the parent animals that are 18 weeks or older are hatched into day-old broilers at a hatchery and further delivered to the broiler farms. Here, broilers are raised until 28-32 days before being slaughtered for meat production (Mo, 2016).

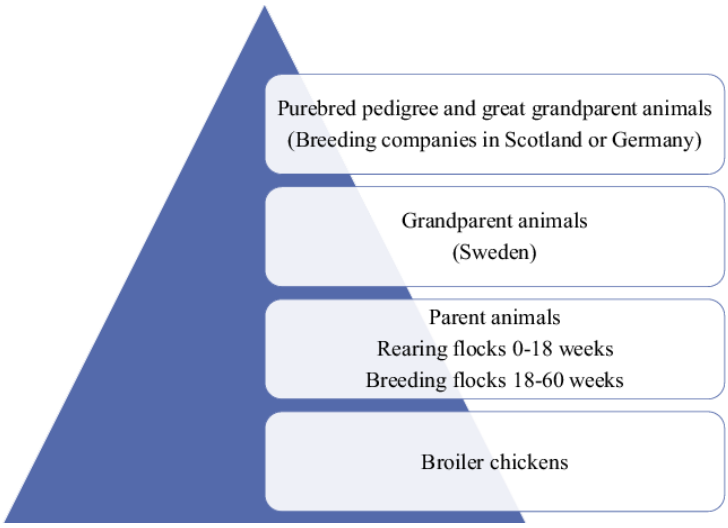


Figure 1. The pyramidal structure of broiler production. Adapted from (Mo, 2016).

1.2 Antimicrobial agents:

Antimicrobial agents used in animal production to prevent risk of infections and treat diseases are the same drugs classes used in human medicine. These drugs function by targeting different structures in the bacteria and are classified based on their ability to inhibit

cell growth (bacteriostatic) or induce cell death (bactericidal) (Kohanski et al., 2010). To achieve the bacteriostatic or bactericidal effects, antimicrobial agents interfere with the reactions that synthesize structures that bacteria depend on to survive and develop. Thus, the cell wall, ribosomes, and nucleic acids are the main targets of these antimicrobial agents, particularly the bactericidal. On the other hand, the bacteriostatic drugs prevent bacteria from carrying out their metabolism (Kohanski et al., 2010).

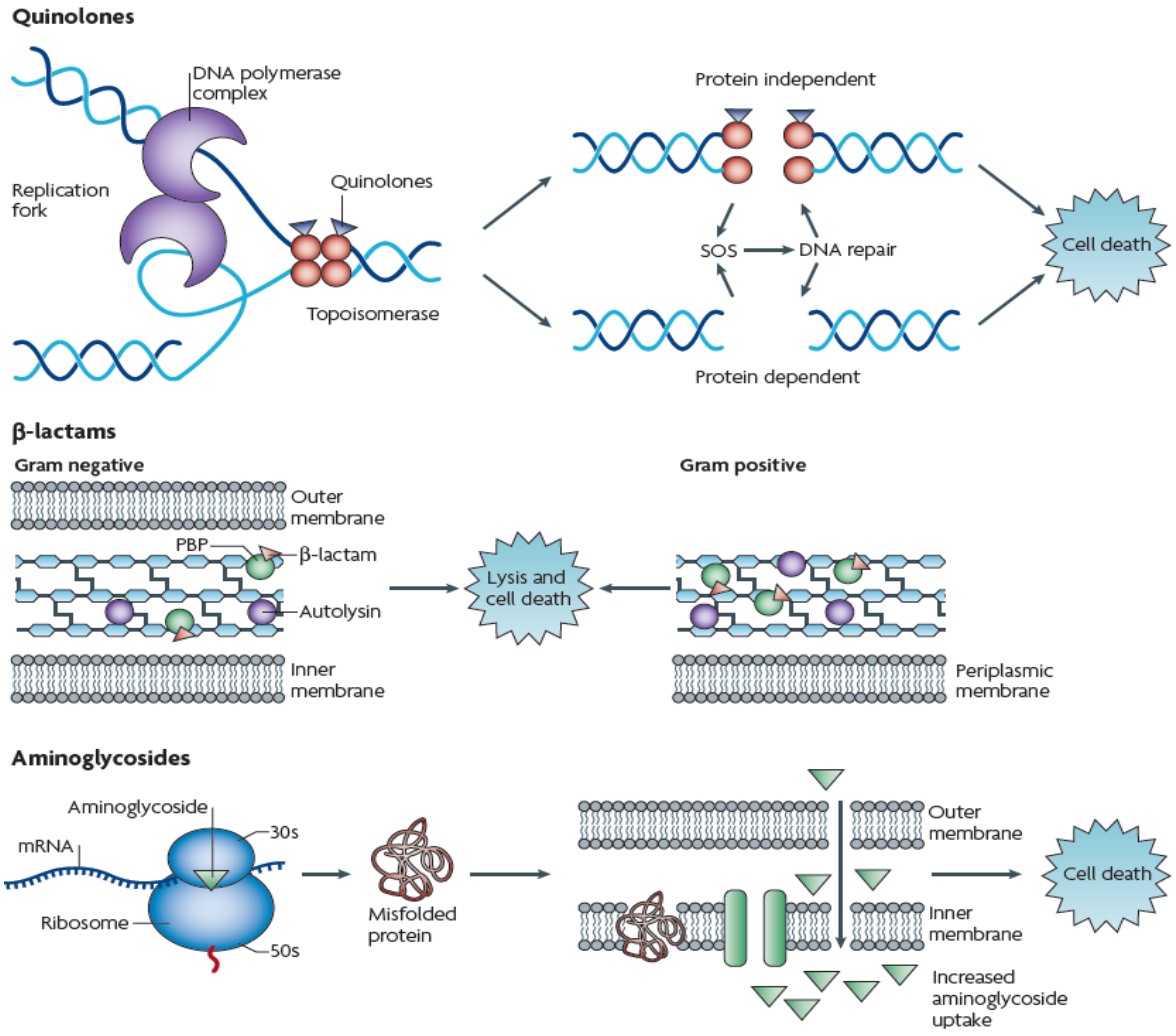


Figure 2. Schematic representation of bactericidal antimicrobial agents and its mechanism leading to cell death. Adapted from (Kohanski et al., 2010).

1.2.1 Inhibition of cell wall synthesis:

The cell wall is the most vital structure that surrounds bacteria. It strengthens and protects the cell against stress and damage. Without this structure, bacteria are more susceptible to attack by various toxic compounds such as antimicrobials. The cell wall is the primary target of the beta-lactam antimicrobials. In the presence of beta-lactams, bacteria are unable to synthesize new cell wall as the enzymes (transpeptidases) involved are repressed (Kohanski et

al., 2010). As a result, the cell wall structure weakens leading to lysis and cell death (Figure 2).

1.2.2 Inhibition of protein synthesis

The site of protein synthesis in bacteria, ribosomes, are the main target of antimicrobial agents such aminoglycosides and chloramphenicol. Ribosomes consist of two subunits: 50S and 30S that are involved in protein synthesis (Figure 2). Once antimicrobial agents compromise ribosomes, reactions involved in the protein production stops or the bacteria generate non-functional proteins. For instance, chloramphenicol prevents peptide bond formation that result in a functional protein. On the other hand, aminoglycoside interacts with the 30S ribosome subunit and causes the tRNA to carry the incorrect amino acids to the ribosomes (Kohanski et al., 2010, Willey et al., 2014b)

1.2.3 Inhibition of nucleic acid synthesis

Synthesizing nucleic acids (DNA) is a fundamental process in all life forms, including bacteria. To initiate this process in bacteria, it requires a "relaxed" DNA where the double strands are broken, and twists removed. The DNA gyrase (topoisomerase II) and topoisomerase IV are the main enzymes that bind to relax the DNA (Willey et al., 2014b). Thus, antimicrobial agents called quinolones target these enzymes to inhibit their function and, in the process, DNA synthesis.

Quinolones restrict the DNA gyrase and topoisomerase IV by forming a stable complex structure with them (Figure 2). In this structure, the enzymes become trapped and are unable to relax the condensed DNA. As a result, the DNA synthesis is blocked and cell growth prevented. Furthermore, quinolones are bactericidal and cell death occurs when the drugs inhibit a DNA repair system known as the SOS response in bacteria (Figure 2).

1.2.4 Inhibition of metabolic processes

Some antimicrobial agents are known as "anti-metabolites" because they interfere with metabolic pathways that are essential to the bacteria (Scholar and William, 2000). Folic acid synthesis is essential in bacteria because the process generate folic acid, which bacteria use to synthesize DNA, RNA, and other cell components such as ATP. Sulfonamides and trimethoprim are well-known anti-metabolites that disrupt the folic acid synthesis in bacteria (Willey et al., 2014b).

1.3 Antimicrobial resistance

According to the World Health Organization (WHO), antimicrobial resistance (AMR) remains one of the major threats to global health (WHO, 2014). Antimicrobial resistance is a natural phenomenon in microorganisms, such as bacteria, where antimicrobials designed to treat diseases in humans and animals become futile. Over the years, antimicrobial resistance has spread worldwide, and one major cause involves the inappropriate use of antimicrobials in human and veterinary medicine (Ventola, 2015). As a result, these actions have driven the emergence and spread of antibiotic resistant bacteria destroying important antimicrobials (ECDC & EFSA, 2018). Of the antimicrobials, resistance to third-generation cephalosporins greatly concerns the WHO as they prioritize these drugs as "critically important" in human and animal therapy (WHO, 2017).

In general, antimicrobial resistance in bacteria develops by two main mechanisms- intrinsic and acquired resistance (Smith and Lewin, 1993). Intrinsic resistance occurs when bacteria naturally resist antimicrobials due to its structure or functional processes (Blair et al., 2015). For instance, the *Mycoplasma spp*. are "intrinsically resistant" to beta-lactams because they target the cell wall, which these bacteria do not possess (Thenmozhi et al., 2014b). Another example of intrinsic resistance involves the vancomycin resistance in Gram-negative bacteria (Thenmozhi et al., 2014b). The outer membrane structure in these bacteria acts as a barrier against the drug entry and prevent it from reaching its target site (Cox and Wright, 2013). In contrast, acquired resistance occurs when a susceptible bacterium becomes resistant to an antimicrobial (WHO, 2011).

Of the two resistance mechanisms, acquired resistance is clinically relevant because it can spread among different bacteria species and reduce antimicrobial treatment options (Munita and Arias, 2016) . Furthermore, acquired resistance develops in two ways, i.e., when there is a genetic mutation associated with the antimicrobial' actions or when a bacterium obtains foreign DNA consisting of resistance genes from other bacteria (Munita and Arias, 2016)

1.3.1 Main resistance mechanisms

1.3.1. i Enzymatic inactivation of antimicrobials

Bacteria can produce enzymes that either destroy or modify the antimicrobials. One example of antimicrobial inactivation due to enzymes is the production of beta-lactamases that destroy beta-lactams. One the other hand, bacteria can generate modifying enzymes (acetylases, phosphorylases, and adenylase) that cause a steric hindrance in the antimicrobial

molecule. The steric hindrance effect reduces the drug's affinity for its the target sites (Munita and Arias, 2016).

1.3.1. ii Efflux pumps

Antimicrobials unable to enter the cell to perform its actions can be due to efflux pump production in bacteria (Cox and Wright, 2013). Efflux pumps are proteins that transport antimicrobials or other toxic compounds out of the cell (Figure 3). Genes encoding efflux proteins are located on chromosomes or mobile genetic elements (MGEs) (Thenmozhi et al., 2014a). Tetracycline resistance in certain bacteria illustrates an efflux-mediated because the tetracycline efflux protein (TetA) pumps out the drug before reaching its target in the cell (Munita and Arias, 2016).

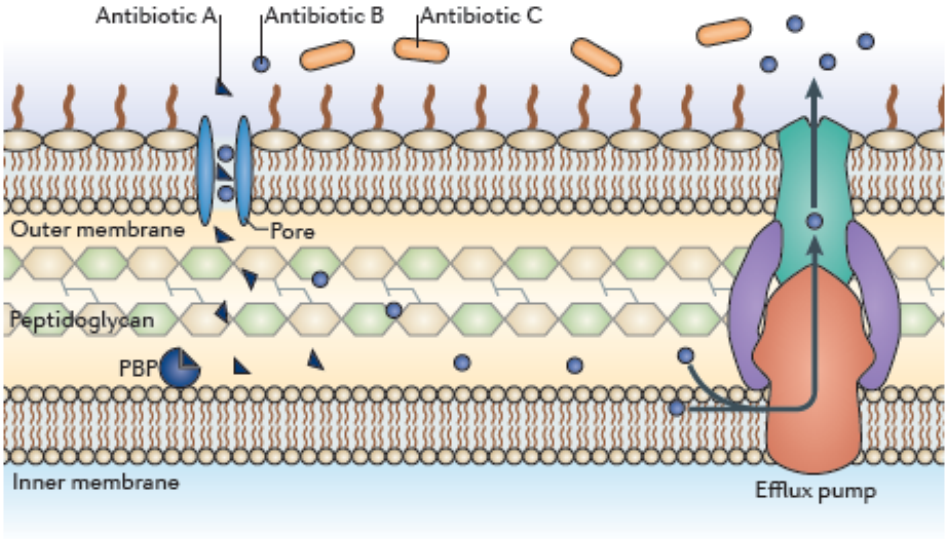


Figure 3. Representation of an efflux-mediated resistance. Both antimicrobials A and B enter the cell membrane, but antimicrobials B is transported out from the cell by efflux pump Adapted from (Blair, 2014).

1.3.1.iii Alteration in target sites

For antimicrobials to exert its functions, they need to interact with their target sites in the bacteria. However, bacteria can prevent this interaction by protecting or modifying these target sites (Munita and Arias, 2016). One way a bacterium modifies an antimicrobial target site is to mutate the genes encoding that site. In turn, the genes will encode abnormal targets sites that cannot interact with the antimicrobials. Rifamycin resistance due to point mutations in the *rpoB* gene shows an example of mutational resistance (Munita and Arias, 2016).

1.4 Acquired resistance via horizontal gene transfer

In addition to genetic mutation, exchange of genetic material between bacteria is the process where they can acquire an antimicrobial resistance. This process is generally referred to as horizontal gene transfer and occurs via three main mechanisms: transformation, transduction, and conjugation (Pepper et al., 2014).

1.4.1 Transformation

In the transformation mechanism, bacteria take up DNA from their external environment and integrate into its genome via homologous recombination (Pepper et al., 2014). The DNA is released by dead cells referred to as donors. Bacteria that can take up the DNA and transform into the donor cell are referred to as competent bacteria.

1.4.2 Transduction

Transduction, on the other hand, rely on bacteriophages to transfer DNA between bacteria. Bacteriophages are viruses that infect bacteria and further use them as host to multiply and produce more bacteriophages. After viruses multiply, they assemble into mature virions and at this stage, they can take up DNA fragments containing antimicrobial resistance genes from the bacteria (Willey et al., 2014a). When these bacteriophages infect new bacterial host cells, they inject piece of the DNA from the previous bacterial host cell into the genome of a new bacteria host (Pepper et al., 2014).

1.4.3 Conjugation

Of the three mechanisms, conjugation is most effective in transferring antimicrobial resistance between bacteria. This mechanism requires a direct cell-to-cell contact between two cells, i.e. a donor and recipient (Figure 4). During conjugation, the donor cell (F^+) that harbours the resistance genes on their mobile genetic elements (MGEs) or conjugative elements, transfer them to the recipient cell (F^-). The gene transfer from the donor cell to recipient is mediated by transfer *(tra)* genes that form sex pili to connect the two cells. After receiving the MGEs containing the resistance genes, the recipient cell becomes a transconjugant that in turn can transfer the resistance genes to other recipient cells (Wiley et al., 2014). Bacteria such as *E. coli* carry out conjugation to transfer genes to other bacteria, as seen with case of cephalosporin resistance (Mo et al., 2017).

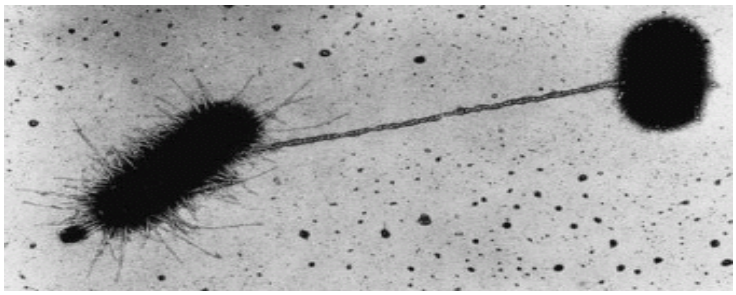


Figure 4. Illustration of a conjugation process. DNA is transferred from donor cell (F^+, left) to recipient cell (F^-, right) through the sex pilus. Adapted from (Willey et al., 2014a).

1.5 Mobile genetic elements (MGEs):

Mobile genetic elements, i.e. plasmids, transposons, and integrons are DNA segments in bacteria that move from parts of a genome to another or between genomes (Bennett, 2008). These elements are easily transferred between bacteria via the horizontal gene transfer mechanisms, and can harbour AMR encoding genes (Cantón et al., 2012).

1.5.1 Plasmids

Plasmids, seen as circular and double-stranded in bacteria, are additional DNA that can replicate independently from the chromosomal DNA. Furthermore, some plasmids are referred to as conjugative plasmids because they contain genes necessary for conjugation functions, i.e. the formation of sex pilus to enable horizontal gene transfer. Some plasmids have developed mechanisms, such as toxin-antitoxin (TA) systems, to ensure their maintenance in the bacterial cell. The toxin-antitoxin system maintains plasmids in the cell by eliminating daughter cells that have lost the plasmids during cell division (Bennett, as cited in Brolund, 2014).

1.5.2 Transposons

Transposons (*Tn*), often known as “jumping genes”, are DNA sequences that move from one location on the genome to another. Like the conjugative plasmids, they contain genes that encode for conjugation and antimicrobial resistance. However, unlike the plasmids, they do not have their own origin of replication (*oriT*) region and for this reason, they integrate themselves into chromosomes or plasmids for maintenance. *Tn10* and *Tn3* are known transposons that resist tetracycline and beta-lactams, respectively (Bennett, 2008).

1.5.3 Integrons and gene cassettes

Integrons are genetic elements that capture and carry antimicrobial resistance genes. They consist of three elements including (1) an integrase-encoding gene, *int*, that is required for site-specific recombination (2) a *attI* region, where DNA sequences known as gene cassettes insert, and (3) a promoter region located upstream of the *attI* where the gene cassettes are transcribed and expressed (Bennett, 2008). One common integron found on plasmids, chromosomes, and transposons is the class 1 integron (Figure 5).

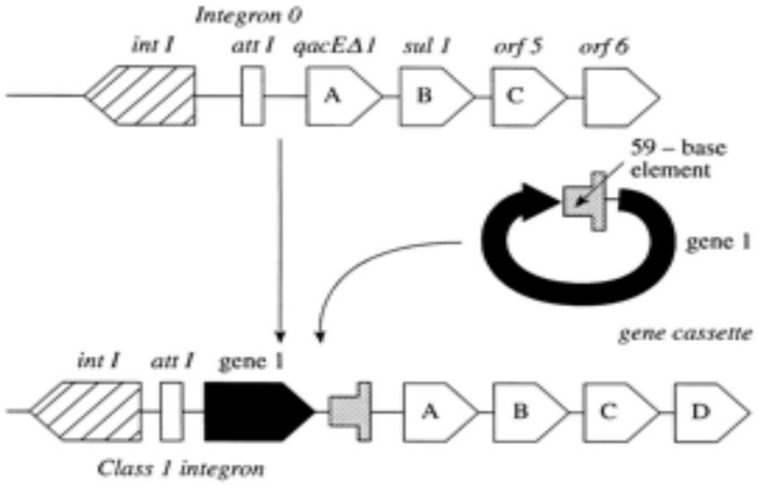


Figure 5. Illustration of the class 1 resistance integron element. Integron 0 is considered the most basic integron without gene cassette. The class I integron contains an inserted gene cassette in addition to the four genes: sulphonamide resistance, *sul1*; quaternary ammonium compound resistance, *qacE*Δ1 and the two *orf* 5 and 6 with unknown functions. Adapted from Adapted from (Bennett, 1999).

1.5.4 Insertions sequences

Of the mobile genetic elements, insertion sequences are the simplest due to its size (1 kb). These elements are involved in the over-expression of *bla* genes and integrate within conjugative plasmids present in bacteria (Cantón et al., 2012). For instance, in the *Kluyvera spp.*, the movement of the $bla_{\text{CTX-M}}$ genes has been associated with insertion sequence elements (ISEs) located upstream. Among the ISEs, the IS*Ecp1* is most frequent at the upstream of different $bla_{\text{CTX-M}}$ genes such as the $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-9}}$ etc. (Figure 6) (Lartigue et al. 2004).

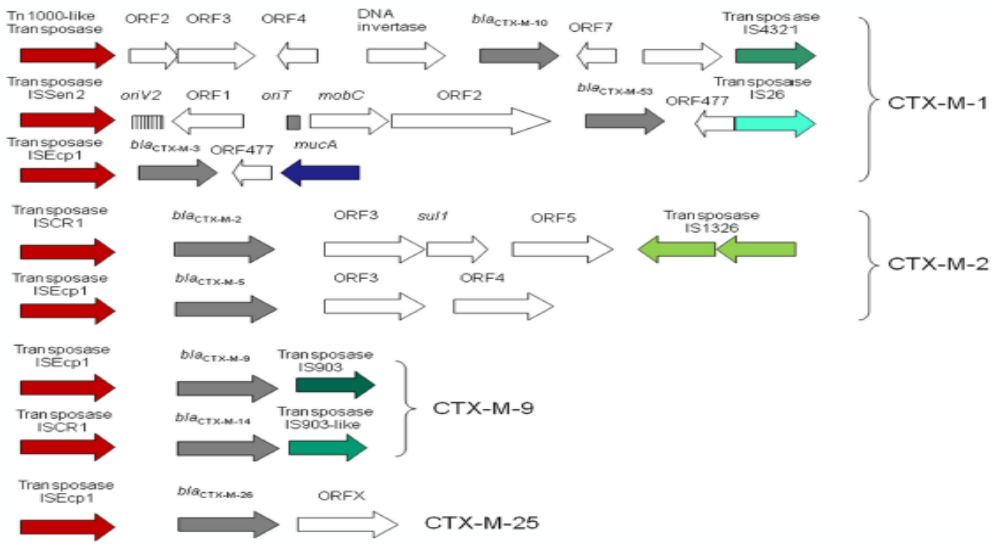


Figure 6. Genetic environments of the different *bla*$_{\text{CTX-M}}$ genes: *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-9}}$, and *bla*$_{\text{CTX-M-25}}$. IS*Ecp1* and other insertion sequence elements (red arrow) are present in all four genes. Adapted from (Canton et al., 2012).

1.6 Beta-lactamases

Beta-lactamases are group of enzymes produced by bacteria that degrade beta-lactam antimicrobials. These enzymes destroy beta-lactam drugs by hydrolysing the most active part, which is the beta-lactam ring. In the process, the drugs become ineffective to kill the bacteria.

According to the Ambler's Molecular classification, beta-lactamases are grouped into four main classes: A-D (Bradford, 2001) . The most important class A beta-lactamase involves the extended spectrum beta-lactamases (ESBLs), whereas in class C, the AmpC beta-lactamases are prevalent (Bradford, 2001). Since this study is about the ESBLs, emphasis on AmpC-enzymes is limited.

1.6.1 Extended Spectrum Beta-Lactamase (ESBLs)

Extended spectrum beta-lactamases (ESBLs) are usually plasmid-mediated beta-lactamases that resist third- and fourth-generation cephalosporins, and monobactams. In contrast, they are sensitive to cefoxitin, carbapenems, and the beta-lactamase inhibitors such as clavulanic acid. ESBLs are classified into different families including the TEM-, SHV-, and CTX-M (Bradford, 2001).

During the 1990s, SHV and TEM were the most common ESBL families found in *E. coli* and *Klebsiella spp* (EFSA, 2011). However, in the early 2000s, the CTX-M families emerged, and now are the most dominant genotype found in ESBL-producing *E. coli* from humans, food-producing animals, food, and the environment in Europe (Seiffert et al., 2013).

1.6.2 AmpC beta-lactamases: chromosomal AmpC (cAmpC) and plasmid-mediated AmpC (pAmpC)

AmpC beta-lactamases are cephalosporinases produced by some Gram-negative bacteria that resist third-generation cephalosporins, cefoxitin and the beta-lactam inhibitors. In contrats, they are sensitive to fourth-generation cephalosporins and carbapenems (Seiffert et al., 2013). Some Enterobacteriaceae have genes encoding AmpC enzymes in their chromosomes. Genes encoding cAmpC beta-lactamases are found on weak promoters. Here, the bla_{AmpC} genes are expressed in low numbers and as result cannot contribute to cephalosporin resistance (Jacoby, 2009). However, mutations in the weak promoters or attenuator regions of the chromosomal *ampC* gene can result in hyper-production of the cAmpC beta-lactamases and hence, resistance to third-generation cephalosporins (Shaheen et al., 2011). Over the past years, several studies have reported the movement of some AmpC-producing genes, particularly $bla_{\text{CMY-2}}$, from chromosomes to plasmids, where it expresses the cephalosporin-resistance (Jacoby, 2009).

1.7 Characterization of cephalosporin-resistant *E. coli* isolates

In general, methods used to characterize *E. coli* strains possess a high discriminatory power where it distinguishes between two closely related bacterial strains (EFSA, 2011, Farber, 1996).

1.7.1 Phylogenetic grouping of *E. coli* isolates

Phylogenetic analysis shows *E. coli* is divided into four main phylogroups: A, BI, B2, and D (Picard et al., 1999). Phylogrouping of *E. coli* strains involves the combination of the four genetic markers: *gadA, chuA, yjaA,* and the TSPE.C4 DNA fragment. A multiplex PCR is the strategy used to categorize *E. coli* into the four main phylogroups (Doumith et al., 2012). *E. coli* strains that fall into the phylogroups B2 and D are considered extra-intestinal pathogenic (ExPEC) because they possess virulence traits that cause extra-intestinal infections such as bacteremia, sepsis, urinary tract infections, and meningitis in humans (Picard et al., 1999, Smith et al., 2007). The commensal *E. coli* strains, on the other hand, are the phylogroups A and B1 (Picard et al., 1999).

1.7.2 Molecular typing of *E. coli* isolates: PFGE

Pulsed-field gel electrophoresis (PFGE) is a standard fingerprinting method used to investigate the genetic relatedness of several bacterial strains such as *E. coli*. Compared to

other molecular typing methods, PFGE possess a higher discriminatory power and yields more reproducible results (Sabat et al., 2013). In principle, the bacterial DNA embedded in agarose plugs is lysed and digested with a restriction enzyme, and separated on an agarose gel to generate a set of fingerprints that show the similarity or differences between the isolates (EFSA, 2011).

1.7.3 Plasmid characterization

Characterizing plasmids harboring ESBL/pAmpC genes is crucial in studying how they disseminate between different reservoirs (EFSA, 2011). Plasmids are characterized into incompatibility (Inc) groups, where incompatibility refers to the inability of two plasmids belonging to the same Inc goup to exist stably in the same bacterial cell (Thomas, 2014). The Inc groups IncF, IncI1α, IncN, IncA/C, IncK, and IncHI12 are the major plasmids that disseminate ESBL/AmpC genes (Carloni et al., 2017). These plasmids are epidemic and have been identified in ESBL/AmpC-producing bacteria from animals, food products and humans (EFSA, 2011). The IncI1α and IncN plasmids particularly have been linked to the spread of the *bla*$_{\text{CTX-M-1}}$ gene among *E. coli* from poultry (Zurfluh et al., 2014, EFSA, 2011).

1.8 Epidemiology of cephalosporin-resistant *E. coli* and CTX-M genes

In the past years, third-generation cephalosporin resistance has increased in several bacteria (ECDC & EFSA, 2016). This has been demonstrated in *E. coli* strains from different reservoirs including food-producing animals, water, soils and human clinical samples (Hu et al., 2013). Production of the ESBLs/AmpC beta-lactamases by bacteria have resulted in higher rates of third-generation cephalosporin resistance (EARS-NET, 2016). Among food-producing animals, broilers are highly contaminated by ESBL/AmpC-producing *E. coli* and the cause of this has been linked to the massive use of antimicrobial agents in the production (WHO,2011). Further analysis shows *bla*$_{\text{CTXM-1}}$ and *bla*$_{\text{CMY-2}}$ as the main genes facilitating this resistance in poultry (Dierikx et al., 2013, Leverstein-van Hall et al., 2011).

On the contrary, despite low antimicrobial usage, AmpC -producing *E. coli* with *bla*$_{\text{CMY-2}}$ have been detected in the Norwegian broiler production pyramid since selective screening was initiated in 2011 (NORM/NORM-VET 2011). First ESBL-producing *E. coli* found in broiler in Norway was reported in 2006 (NORM/NORM-VET, 2006). However, the identified ESBL-producing *E. coli* had a different genotype, which was *bla*$_{\text{TEM-20}}$. Since then AmpC-producing *E. coli* have been prevalent until the discovery of the ESBL-producing *E. coli* with *bla*$_{\text{CTX-M-1.}}$ in 2016.

Worldwide, cephalosporin resistance among *E. coli* has been associated with the horizontal transfer of *bla*$_{\text{CTX-M-1}}$-carrying plasmids (Cantón et al., 2012). For the past years, the study on plasmid-encoded ESBL genes has been prioritized due its spread of antimicrobial resistance between bacteria. Thus, studying the epidemiology of these plasmids in *E. coli* from food-producing animals can reveal if they both share common plasmid(s).

In this study, cephalosporin-resistance in *E. coli* from broilers in Norway and Europe are examined. In several European broiler-producing countries, ESBL/AmpC-producing *E. coli* have been identified (ECDC & EFSA, 2016). Countries such as France, Spain, the Netherlands, Belgium, Italy and Switzerland have reported ESBL-producing *E. coli* with *bla*$_{\text{CTX-M-1}}$ as most prevalent in broilers (EFSA, 2011), whereas in Norway, AmpC-producing *E. coli* with *bla*$_{\text{CMY-2}}$ is the dominant type (Mo et al., 2017). However, in 2016, ESBL-producing *E. coli* with *bla*$_{\text{CTX-M-1}}$ was detected in broilers and parent flocks from Norway (unpublished data).

1.8.1 Epidemiology in Norwegian broiler production pyramid and Europe

The first detection of an ESBL-producing *E. coli* with *bla*$_{\text{CTX-M-1}}$ in poultry occurred during a Spanish antimicrobial resistance surveillance program in 2000-2001 (Briñas et al., 2003). Fast forward to today, the CTX-M-1 enzymes are the common ESBLs in *E. coli* isolated from both sick and healthy broilers in several European countries (EFSA, 2011).

In Norway

Norway is one of the few European countries with a low third-generation cephalosporin resistance rate in *E. coli* from broilers. In general, the rate of third-generation cephalosporin resistant *E. coli* from broilers has ranged from 0-1.5% (ECDC & EFSA, 2016). However, this rate dropped to 0 % in 2016 (ECDC &EFSA, 2018). Norway achieves this low resistance level due to negligible use of antimicrobials in its broiler production pyramid and maintaining high biosecurity level at the broiler farms (NORM/NORM-VET, 2006, 2009, 2012).

In Europe

Unlike Norway, third-generation cephalosporin resistance in *E. coli* from broilers is higher in most European countries, with the exception of Sweden, Denmark, and Iceland (Mo, 2016). In 2014, the overall resistance rate ranged from 0-32.2% with countries like Malta, Slovakia, and Spain showing moderate levels of resistance between 12.9-15% (Figure 7). High

resistance levels (>30%) were seen in countries including Cyprus, Latvia and Lithuania (EFSA and ECDC, 2016). Compared to 2014, data from 2016 showed that the majority of the countries had significant decrease in resistance to third-generation cephalosporin (cefotaxime) in *E. coli* from broilers (Figure 8). However, in Lithuania, the level increased from 31.8% in 2014 to 52% in 2016 (EFSA and ECDC, 2016; EFSA and ECDC, 2018).

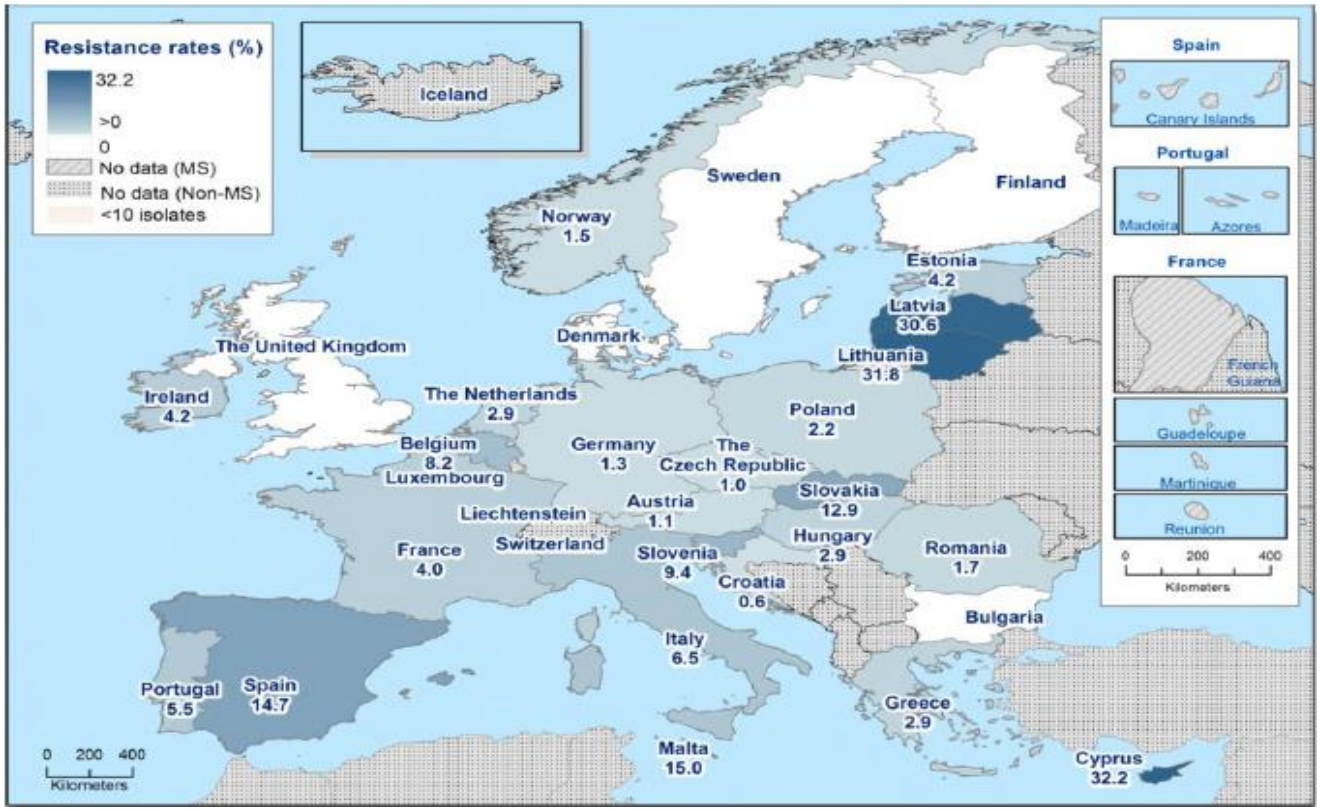


Figure 7. Prevalence of cephalosporin resistance in indicator *E. coli* isolated from European broiler in 2014. Adapted from (EFSA and ECDC, 2016).

Figure 8. Occurrence of cephalosporin-resistant *E. coli* isolates from broilers in European Union in 2016. Adapted from (EFSA and ECDC, 2018).

1.9 Public health: ramifications of ESBL-producing *E. coli*

ESBL-producing *E. coli* can cause infections in humans such as bacteremia, sepsis, and urinary tract infections (Collignon et al., 2013). Owing to their resistance, these bacteria reduce the effectiveness of third-generation cephalosporins. Thus, this can lead to longer hospital stays and higher risks of nosocomial and community-acquired infections (Seiffert et al., 2013). Since ESBL-producing *E. coli* occurs in food and food producing animals, food as a possible transmission route for ESBL-producing bacteria to humans remains an open question. However, there has been some inconclusive evidence concerning the direct transmission of ESBL-producing bacteria from food-producing animals to humans, through the food chain (Collignon et al., 2013). This transmission is linked to handling or consumption of contaminated food, which may serve as a reservoir for ESBL-producing bacteria (Coque et al., 2008).

Leverstein-van Hall et al., (2011) and Overdevest et al., (2011) discovered identical *E. coli* strains from chicken meat and humans. However, results from both studies were based on molecular typing methods that have low discriminatory power. By contrast, de Been et al.,

(2014) and Berg et al., (2017) performed a whole genome sequencing analysis (WGS) to study *E. coli* strains from humans and chicken meat. This method generally has the highest level of resolution to discriminate between isolates and yields more reliable results. de Been et al., (2014) found no close relationship between the *E. coli* strains from chicken meat and humans as previously reported by Leverstein-van Hall et., (2011). However, identical plasmids, i.e. IncI1α/ST3 and IncI1α/ST7 that disseminate the $bla_{\text{CTX-M-1}}$ gene was discovered (de Been et al., 2014). On the other hand, WGS analysis applied in a Norwegian study showed the *E. coli* strains from patients suffering from UTI infection and chicken meat were closely-related, including an IncK plasmid with $bla_{\text{CMY-2}}$ (Berg et al., 2017)**.** Hence, by considering the results from Berg et al., (2017), clonal transfer of cephalosporin-resistant *E. coli* from chicken meat to humans can be possible to some extent.

1.10 Aim of study

The aim of this study was to investigate how ESBL-producing *E. coli* with $bla_{\text{CTX-M-1}}$ emerged and disseminated in the Norwegian broiler production. Three hypotheses regarding the emergence and dissemination of these bacteria were investigated;

(1) a possible clonal spread of a specific *E. coli* variant with one $bla_{\text{CTX-M-1}}$ harbouring plasmid (clonal spread)

(2) more *E. coli* clones with different $bla_{\text{CTX-M-1}}$ harbouring plasmids, and

(3) horizontal transmission of a specific $bla_{\text{CTX-M-1}}$-harboring plasmid to different *E. coli* strains from broilers in Norway.

2 Materials and methods.

All laboratory experiments were performed at the Norwegian Veterinary Institute (NVI) in Oslo. Bacterial isolates included in the study were previously from boot swabs collected in all broiler flocks sampled the Norwegian Salmonella control program from May-October 2016, and available for further characterization.

2.1 Selective isolation and confirmation of ESBL-producing *E. coli*

Boot swab samples were homogenized in peptone water and 100 µl inoculated on a MacConkey agar with 1 mg/mL cefotaxime to select cephalosporin resistant isolates. Plates were incubated overnight at 37 °C. Colonies with similar morphology and showing cefotaxime resistance were isolated from the MacConkey agar, sub-cultured onto blood agar and incubated at 37 °C overnight. A matrix laser desorption ionization-time of flight

(MALDI-TOF) technique was used to confirm the isolates from the blood agar plates as *E. coli* (MALDI-TOF, Bruker Daltonics).

The *E. coli* isolates were confirmed as ESBL producers based on the synergy test between third-generation cephalosporin and clavulanic acid. This was carried using the automated Sensititre^{TM} ESBL/AmpC MIC plates (TREK Diagnostic Systems), and results interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints. In addition, PCR confirmed the presence of $bla_{\text{CTX-M-1}}$ as the ESBL genotype in all the isolates. Isolation and identification of *E. coli* as ESBL producers was performed by the laboratory staff at NVI.

2.2 Characterization of ESBL-producing *E. coli* isolates

A total of 35 ESBL-producing *E. coli* with $bla_{\text{CTX-M-1}}$ were included in this study. Of these, 26 were from broiler flocks and seven from parent flocks. In addition, two ESBL-producing *E. coli* isolates from poultry in Iceland were included. The two isolates from Iceland were confirmed as ESBL-producers in this study. In addition to typing of the isolates, their susceptibility to different antimicrobial agents was determined.

2.2.1 DNA extraction

DNA was extracted from all 35 isolates by the boiling lysis method. Bacterial suspensions were prepared in 100 µl milli-Q water using a single colony from blood agar. Cells were lysed at 100 °C for 15 minutes and the suspension centrifuged for 5 minutes at 13200 rpm. The supernatants were transferred to new Eppendorf tubes and used as DNA template.

2.2.2 Phylogenetic grouping

A multiplex PCR method (Doumith, Day et al. 2012) was used to assign phylogenetic groups to the 35 *E. coli* isolates. Primers used to amplify conserved regions of the four phylogenetic markers: *gadA, chuA, yjaA,* and DNA fragment TSPE4.C2 are shown in Table 1. A phylogroup B2 *E. coli* strain was used as the positive control as it contains all four phylogenetic markers (Sunde et al., 2015). Table 2 illustrates the assignment of each isolate into the respective phylogenetic group based on the combinations of the four markers.

Table 1. Primer sequences used for the amplification of target regions and phylotyping of *E. coli* isolates.

Genetic marker	Primer sequence (5′→ 3′)	Base pair (bp) length
gadA	Forward: GATGAAATGGCGTTGGCGCAAG Reverse: GGCGGAAGTCCCAGACGATATCC	373
chuA	Forward: ATGATCATCGCGGCGTGCTG Reverse: AAACGCGCTCGCGCCTAAT	281
yjaA	Forward: TGTTCGCGATCTTGAAAGCAAACGT Reverse: ACCTGTGACAAACCGCCCTCA	216
TSPE4.C2	Forward: GCGGGTGAGACAGAAACGCG Reverse: TTGTCGTGAGTTGCGAACCCG	152

In an Eppendorf tube, 10 µL of each primer (10 µM) for the four markers were mixed together with 20 µL Milli-Q water to obtain a "primer mix". The PCR reaction of each isolate was carried out in a 25 µL reaction mixture containing 12.5 µL 1x Qiagen Multiplex PCR mix, 0.5 µL 0.2 µM primer mix, and 2 µL of DNA template. Next PCR was run using Sure cycler 8800 (Agilent Technologies) under the conditions shown in Table 3. Ten microliters of each PCR amplification product was visualized with 2.5 µL 6x DNA loading dye and separated on a 1% agarose gel stained with 10 µl GelRed nucleic acid dye (ThermoFisher Scientific).

Table 2. Interpretation of the phylogenetic grouping of the *E. coli* isolates based on the presence and/or absence of the four phylogenetic markers.

Phylogenetic group	*gadA*	*chuA*	*yjaA*	TSPE4.C2
A	+	-	+/-	-
B1	+	-	-	+
B2	+	+	+	+/-
D	+	+	-	+/-

Table 3. Multiplex PCR program

Hold for	4 minutes at 95 °C
30 cycles	30 seconds at 95 °C 30 seconds at 60 °C 30 seconds at 72 °C
Hold for	5 minutes at 72 °C
Infinity	8 °C

2.3 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the broth microdilution method and agar diffusion method.

2.3.1 Micro broth dilution for MIC determination

Minimum inhibitory concentration (MIC) values were determined for the 14 antimicrobial agents sulfamethoxazole (SMX), trimethoprim (TMP), ciprofloxacin (CIP), tetracycline (TET), meropenem (MERO), azithromycin (AZI), nalidixin acid (NAL), cefotaxime (FOT), chloramphenicol (CHL), tigecycline (TGE), ceftazidime (TAZ), colistin (COL), ampicillin (AMP), and gentamicin (GEN). The procedure was carried out using the Sensititre™ Gram Negative MIC Plate (ThermoFisher Scientific).

MIC values (mg/l) of the antimicrobials had been determined against 33 of the 35 *E. coli* isolates (personal communication, Solveig Sølverød Mo, NVI). Thus, in this study, MIC values of the 14 agents were determined against the two *E. coli* isolates from Iceland.

In brief, bacterial suspensions with a 0.3-0.5 McFarland were prepared separately for the isolates in a 5 mL distilled water. McFarland. Fifty-microliters of the suspension was inoculated into 11 mL Sensititre ® Cation Adjusted Mueller-Hinton Broth (CAMHBT) and 50 µl of the mixture automatically inoculated into each well in the microtiter plates using the

Sensititre® AIM™ pipetting robot. Wells in the microtiter plates are dosed with different concentrations of the above mentioned antimicrobial agents. Plates were incubated at 35 ± 1°C °C overnight. *E. coli* ATC25922 isolate was used as the quality control strain.

2.3.2 Agar disk diffusion method

Bacterial suspensions were made in a 5 mL 0.9% saline water and turbidity adjusted to 0.3-0.5 McFarland. A cotton swab dipped in the suspension was spread on a Mueller-Hinton agar plate (Oxoid™, ThermoScientific™) using an automatic plate rotator. Antimicrobial discs were placed onto the plates using the Oxoid™ Antimicrobial Susceptibility Disc Dispenser (ThermoFisher Scientific) and incubated at 35 ± 1°C overnight. The antimicrobial discs included: ampicillin (10 µg), amoxicillin/clavulanic acid (30 µg), sulfamethoxazole/trimethoprim (23.75 +1.25 µg), tetracycline (30 µg), cephalexin (30 µg), ciprofloxacin (5 µg), neomycin (30 µg), gentamicin (10 µg), polymixin/colistin (300 units), trimethoprim (5 µg), nalidixic acid (30 µg), clindamycin (2 µg), erythromycin (15 µg), and penicillin (1 unit). *E. coli* ATC25922 was used as a quality control strain. Inhibition zone diameters (mm) were interpreted according to the EUCAST Clinical breakpoints (version 7.1; 2017-03-10).

2.4 Pulsed-Field Gel Electrophoresis (PFGE)

The genetic relatedness between the 35 *E. coli* isolates was determined using the pulsed-field gel electrophoresis protocol described in (Caprioli et al., 2014). The procedure involves: 1) preparation of bacterial cell suspension 2) preparation of agarose plugs 3) plug lysis 4) plug washing 5) restriction enzyme digestion 6) gel electrophoresis 7) staining and visualization of the gel, and 8) data analysis.

2.4.1 Preparation of cell suspension

Cell suspensions was prepared for each of the 35 *E. coli* isoltes in 2 mL Tris-EDTA (TE) buffer and turbidity measured to an optical density (OD) of 0.7-0.79.

2.4.2 Gel plug preparation

Of each cell suspension, 400 µL was mixed with 20 µL proteinase K and 400 µl 1% melted PFGE agarose gel. The mixture was transferred into a PFGE disposable plug mold (Bio-Rad Laboratories) to generate agarose plugs.

2.4.3 Lysis and washing of plugs

Bacterial DNA embedded in the agarose plugs was lysed in a mixture of 25 µl Proteinase K and 5 mL cell lysis buffer (CLB 0.1 mg/mL) at 55 ˚C for two hours. After lysis, plugs were washed twice in 10 mL pre-heated milliQ water and four times in 10 mL pre-heated TE-buffer. Both milliQ water and TE-buffer were pre-heated at 50 ˚C. Between each wash, mixture was placed in a shaking incubator for 15 minutes at 50 ˚C. Gel plugs were transferred into 1 mL cold TE-buffer and stored at 4 ˚C.

2.4.4 Digestion of DNA in agarose plugs

Gel plugs were cut into 2-2.5 mm slices with a glass coverslip and each slice digested with 5 µL 10U/µL *XbaI* restriction enzyme (ThermoFisher Scientific) for 3.5 hours at 37 ˚C.

2.4.5 Gel electrophoresis

Digested plugs slices were loaded into wells of a 14 x 14 cm gel form and separated in a CHEF DR III system (Bio-Rad Laboratoratories, Hercules, CA) on a 1% SeaKem Gold PFGE grade agarose. Electrophoresis ran for 24 hours under the following conditions described in (Agersø et al., 2014). An *XbaI*-digested DNA from *Salmonella* enterica serovar Braenderup strain H9812 was used as the marker.

2.4.6 Staining and visualization of the gel

The gel was stained in a mixture of 120 µl GelRed + 400 mL Milli-Q water for 25 minutes and de-stained in 400 mL Milli-Q water for 15 minutes. A Bio-Rad Molecular Imager® Gel Doc™ XR+ imaging system (Bio-Rad Laboratories, Milan, Italy) was used to visualize the gel.

2.4.7 Data analysis of the gel image

The BioNumerics software v 6.6 (Applied Maths, …)was used to analyze the generated PFGE fingerprints. The similarities of fingerprints were compared using a Dice correlation coefficient at 1.5% tolerance and 1.5% optimization, and a dendogram constructed with the unweighted pair group method with arithmetic averages (UPGMA) clustering method using the BioNumerics program (Agersø et al., 2014). Isolates with similarity at ≥ 97% cut-off value were considered as clonally-related whereas isolates with similarity of ≥ 80% were grouped in the same PFGE cluster (Mo et al., 2016).

2.5 Conjugative transfer and characterization of plasmid replicons

2.5.1 Conjugation experiments

Transfer of *bla*$_{\text{CTX-M-1}}$-carrying plasmids was determined in a series of conjugation broth mating experiments as described in (Mo et al., 2016). The cephalosporin-resistant *E. coli* isolates, which were sensitive to nalidixic acid (naI^{S}) served as the donor strains whereas a plasmid-free *E. coli* DH5α (nalidixic acid resistant) was used as the recipient strain.

Overnight cultures of both donor and recipient strains were prepared separately in 4 mL Luria Bertani (LB) broth at 37°C. Next, 500 µL of recipient strain was mixed with 10 µL of each donor strain in a 4 mL LB broth and incubated at 37°C without shaking for four hours. After four hours, 100 µL of broth mating was plated on a Mueller-Hinton (MH) agar supplemented with 0.5 mg/L cefotaxime and 20 mg/L nalidixic acid to select the transconjugants. This step was repeated after 6 and 24 hour-broth mating if no transconjugants were obtained after the 4 hours.

2.5.2 PCR-detection of *bla*$_{\text{CTX-M-1}}$ in the transconjugants

To examine if *bla*$_{\text{CTX-M-1}}$ transferred from the donor strains to the recipient strain, PCR was used to confirm the presence of the *bla*$_{\text{CTX-M-}1}$ in the transconjugants. The *bla*$_{\text{CTX-M-1}}$ gene was targeted using the primers (forward: 5′ ATGTGCAGYACCAGTAARGTKATGGC 3′ and reverse: 5′ TGGGTRAARTARGTSACCAGAAYCAGCGG 3′) described in (Hasman et al., 2005).

Genomic DNA was extracted from transconjugants using the boiling lysis method. Extracted DNA was used as the template for the PCR reaction, which was carried out in a 25 µL mixture containing 2.5 µL 1x PCR buffer, 0.5 µL0.2 mM dNTP mix, 0.5 µL 0.2 µM of each *bla*$_{\text{CTX-M-1}}$ primer, 0.1 µL 0.5 U Taq DNA polymerase, 18.4 µL milli-Q water, and 2.5 µL of extracted DNA. Table 4 shows the amplification conditions for the PCR reactions (Agilent Surecycler 8800). To visualize the presence of the *bla*$_{\text{CTX-M-1}}$, PCR products and 6x loading dye (LD) were mixed in a 10 µL: 2.5 µL ratio and run on a 1% (w/v) agarose gel electrophoresis. *E. coli* K8-1 strain and MilliQ-water was used as positive and negative control, respectively.

Table 4. Thermal cycler conditions for PCR detection of *bla*$_{CTX-M-1}$.

Hold for	5 minutes at 95 °C
30 cycles	30 seconds at 95 °C 30 seconds at 60 °C 1 minute at 72 °C
Hold for	7 minutes at 72 °C
Infinity	8 °C

2.5.3 Plasmid typing: PCR-based replicon typing (PBRT) of transconjugants

Transconjugants positive for *bla*$_{CTX-M-1}$ were subjected to plasmid replicon typing using the commercial PCR-based replicon typing (PBRT) kit (Diahtheva, Italy). PBRT determines the Inc groups of major plasmid families in *Enterobacteriaceae* (Liebana et al., 2013). In principle, the PBRT kit is composed of eight specific standard PCR assays (M1-M8) (Figure 9). Primers in one PCR assay can target and amplify three to four amplicons that represent major plasmid Inc groups located on resistance plasmids among *Enterobacteriaceae* (Carattoli et al., 2005). In addition, the kit contains positive controls for each PCR mix.

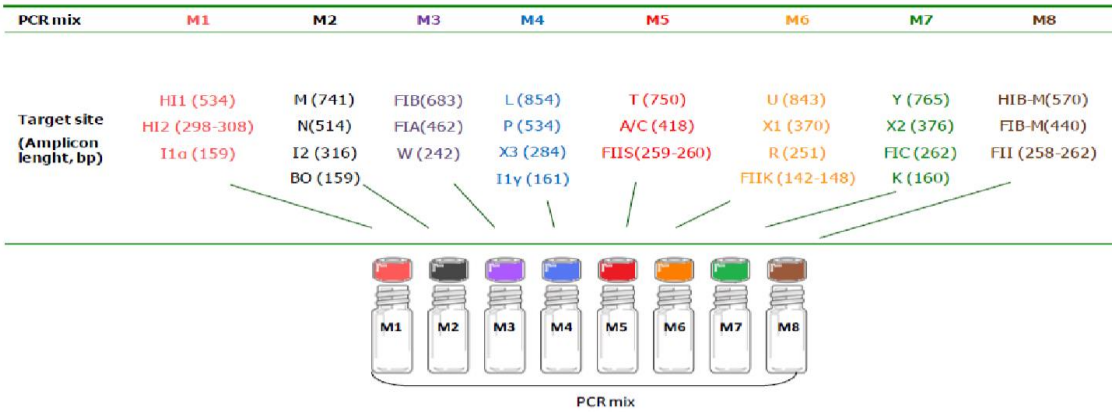


Figure 9. PCR organization and replication targets in the PBRT-KIT scheme. Each PCR mix is color-coded. Adapted from PBRT-kit (version 14/02/2017) (Diatheva, Fano, Italy).

The PBRT procedure was performed according to the manufacturer's instructions (Diatheva, Fano, Italy). First, a mastermix solution consisting of each PCR mix (i.e. M1) and DNA polymerase (5U/µL) was prepared and 24 µL of the mastermix aliquoted into PCR strip tubes. Next, 1 µL of positive control was added to one PCR strip tube and 1 µL of each transconjugant DNA template to the remaining tubes. The PCR reaction was run under the conditions shown in Table 5. All amplification products were visualized by gel electrophoresis on a 2.5% agarose gel stained with 20 µl GelRed. One microliter milliQ water was used as the negative control.

According to the PBRT manufacturer's protocol, IncK plasmids can react with both B/O and K primers in the M2 and M7 PCR mixes. To discriminate between Inc B/O and K plasmids, a PCR that specifically targets the IncB/O replicon was performed on isolates showing weak Inc K amplicon using the PBRT kit. The Inc B/O primers and PCR conditions used is described previously (Carattoli et al., 2005).

Table 5. Thermal cycler condition of the PBRT-kit

1 cycle	95℃ for 10 min
25 cycles	95℃ for 60 secs 60℃ for 30 secs 72℃ for 60 secs
1 cycle	72℃ for 5 min
	Cool down to 4℃

2.5.4 Suceptibility testing of transconjugants

Following conjugation was the phenotypic testing of the transconjugants to six antimicrobial agents by disk diffusion described in 2.3.2. This was done to detect the co-transfer of resistance genes other than the *bla*$_{\text{CTX-M-1}}$. The antimicrobial discs used included ampicillin (10 µg), tetracycline (30 µg), trimethoprim (5 µg), sulfamethoxazole, ceftazidime, and cefotaxime.

2.6 Whole genome sequencing (WGS)

All 35 isolates had previously been subjected to whole genome sequencing, and assembled sequences were available for this study. Following is a brief description of the methods used.

2.6.1 Bacterial DNA isolation with Qiasymphony

Genomic DNA isolation with QiAsymphony DSP DNA mini kit and QIAsymphony SP automated instrument (Qiagen® Sample &Assay Technologies) was performed by Solveig Sølverød Mo. The concentrations (ng/µL) of the extracted DNA were determined with a Qubit® dsDNA BR Assay Kit (ThermoFisher Scientific), and purity measured with a NanoDrop™ 2000 UV spectrophotometer (ThermoFisher Scientific).

2.6.2 Sequencing

Genomic DNA was sequenced on a NextSeq 500 Illumina platform, obtaining 150 bp paired-end reads. Sequencing of the isolates was performed at the Norwegian Sequencing Centre, Ullevål, Oslo.

Before sequencing, a Nextera XT DNA Library preparation kit (Illumina, USA) was used to prepare the sample libraries. In principle, the Nextera XT DNA Library preparation workflow occurs in six steps and involve: (1) tagging of genomic DNA, (2) cleaning the tagged DNA (3) amplification of libraries (i.e. tagged DNA fragments), (4) cleaning up the libraries, (5) checking the libraries, and (6) normalization and pooling of the libraries.

First, the extracted genomic DNA normalized to 0.2 ng/µl, are fragmented and tagged with adaptor sequences. Tagged DNA is purified through Zymo DNA binding buffer and Zymo DNA Wash Buffer. Purified tagged DNA is amplified using a 5-cycle PCR program and the DNA libraries produced are purified with an AMPure XB beads. Purified libraries are quality-controlled on an Agilent Technology 2100 Bioanalyzer. In the last step, the libraries are normalized to 2 nM and pooled, i.e. mixed together in a single tube, which is then diluted and de-natured before sequencing. See the Illumina Nextera® DNA Library Prep Reference Guide (1000000006836 v00, January 2016) for more information about library preparation protocol.

2.6.3 Pre-processing of raw sequence data: assembly of genomes

Raw reads generated after sequencing were pre-processed to yield high quality data for analysis. Reads were quality controlled using the FastQC tool and trimmed to remove duplicate reads and adaptor sequences using Trimmomatic (Bolger et al., 2014). Reads were *de novo* assembled into contigs using SPAdes version 3.9.0 (Bankevich et al., 2012) and assemblies evaluated with the QUAST assembly tool (Gurevich et al., 2013). The complete genome of the *E. coli* K-12 substr. MG1655 strain (Accession number NZ_CP027060.1) was used as a reference. Pre-processing of the raw sequence reads was performed by Camilla Sekse, a researcher at NVI.

2.7 *In silico* analysis of the whole genome sequencing:

2.7.1 Bacterial Analysis Pipeline:

The 35-assembled sequence reads in FASTA files were uploaded to the web-based Bacterial Analysis Pipeline (BAP) for data analysis. The BAP is an automatic and robust tool

that analyses bacterial genomes (Thomsen et al., 2016). This pipeline, with its default settings, executes a series of workflow involved in the analysis of bacterial isolates (Figure 10).

During the workflow, if uploaded sequences are unassembled, the BAP begins with a draft *de novo* assembly of the sequence reads into contigs (Thomsen et al., 2016). The KmerFinder tool, which is run in parallel to the Assembly, identifies species. The ContigAnalyzer tool, analyzes assembled contigs by calculating the number of contigs, total number of bases, and the N50 value, which is defined as the shortest contig (sequence length) that represent 50% of the whole genome assembly (Larsen et al., 2012)**.** After the ContigAnalyzer service, the assembled contigs are submitted to ResFinder for identification of acquired resistance genes (Joensen et al., 2014). BAP gradually performs the remaining services, which includes multi-locus sequence types (MLST) (Larsen et al., 2012) PlasmidFinder and pMLST (Carattoli et al., 2014, Thomsen et al., 2016), and VirulenceFinder (Joensen et al., 2014). All these services, available at the Center for Genomic Epidemiology, DTU, Denmark: https://cge.cbs.dtu.dk/services/ are run based on their databases that relate to *E. coli*.

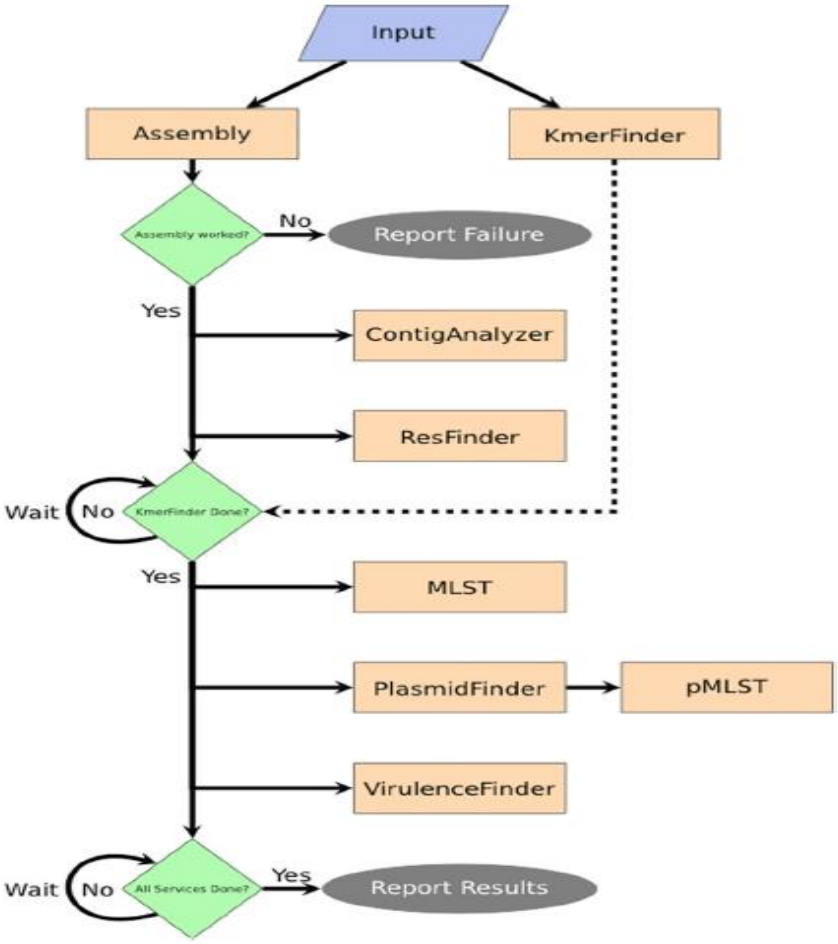


Figure 10. Bacterial Analysis Pipeline workflow. Adapted from (Thomsen et al., 2016).

2.7.2 Phylogenetic analyses: CSI-phylogeny

Phylogenetic analysis of the isolates was based on the single nucleotide polymorphisms (SNPs) difference between the whole genome sequenced data. SNPs were determined using the CSI Phylogeny v 1.4 web tool available at https://cge.cbs.dtu.dk/services/CSIPhylogeny/. The assembled WGS data were uploaded and analysis run with the default parameters of the pipeline as described in (Kaas et al., 2014). The reads from each genome was mapped against the reference genome: *E. coli* str. K.12 substr. MG1655 (Accession number NZ_CP027060.1). A Newick file generated after the SNP analysis was used to construct a phylogenetic tree on Figtree v1.4.3.

2.7.3 Serotyping (SerotypeFinder)

Serotypes of the sequenced *E. coli* isolates were identified with the SerotypeFinder v 1.1 web tool: https://cge.cbs.dtu.dk/services/SerotypeFinder/ (Joensen et al., 2014)

2.7.4 *bla*$_{\text{CTX-M-1}}$-harboring plasmid characterization

In addition to plasmid typing and subtyping, one *bla*$_{\text{CTX-M-1}}$-carrying plasmid was reconstructed to determine its nucleotide sequence. As several studies have reported the prevalence of highly similar IncI1α plasmids carring *bla*$_{\text{CTX-M-1}}$ in the broiler production pyramid (Touzain et al., 2018, Wang et al., 2014), it was hypothesized that the *bla*$_{\text{CTX-M-1}}$-carrying plasmids from this study share similarities to plasmids found in broilers from other European countries.

To characterize the plasmid, the contig sequence that contained the *bla*$_{\text{CTX-M-1}}$-harboring plasmid was extracted from the WGS data using CLC Genomics (CLC Bio, Qiagen, Aarhus, Denmark). The contig containing the *bla*$_{\text{CTX-M-1}}$-harboring plasmid was aligned with two plasmids from Switzerland (accession no. KM377238 and KM377239) and the comparisons visualized using BLAST Ring Image Generator (BRIG) (Alikhan et al., 2011). Another plasmid (accession: SAMN07197432) from France was also aligned with the contig containing the *bla*$_{\text{CTX-M-1}}$-harboring plasmid. Primers were designed in CLC Genomics to determine sequences of gaps pointing outward from the ends of the contigs.

Using the primers, a gradient PCR was performed to amplify the target sequences followed by Sanger sequencing of the amplified PCR products. In general, gradient PCR is a series of individual PCR reactions of the same content (i.e. DNA, primers, enzymes and buffers) performed at different annealing temperature ranges. The idea here was to generate

overlap sequences that can close the gaps and obtain a complete sequence of the contig with the *bla*$_{\text{CTX-M-1}}$-harboring plasmid.

2.7.4 i DNA extraction with QiaAMP DNA kit

DNA was extracted from donor and the corresponding transconjugants of the 2016-40-14272 isolate using QiaAMP DNA mini kit according to the manufacture's protocol (Qiagen®). DNA concentrations was quantified using Qubit® dsDNA BR Assay Kit (ThermoFisher Scientific), and purity measured with a NanoDrop™ 2000 UV spectrophotometer (ThermoFisher Scientific). The extracted DNA were used as template for the PCR reaction.

2.7.4 ii PCR and Sanger sequencing

The PCR reaction was carried out in a 25 µL mixture containing 2.5 µL Qiagen 10x buffer, 0.5 µl dNTP mix, 1 µL 0.4 µM forward primer, 1 µL 0.4 µM reverse primer, 0.1 µL Qiagen Taq polymerase, 18.9 µL milliQ water, and 1 µL of template DNA. Table 6 shows the amplification conditions for the gradient PCR reaction run on the T100™ thermal cycler (BIO-RAD). To visualize the products formed, 10 µl of each PCR product was mixed with 2.5 µL 6x loading dye (LD) and run on a 1% (w/v) agarose gel electrophoresis. Two PCR products with high gel band intensity were sent for Sanger sequencing. Sanger sequencing of the PCR products was performed by the staff at the molecular biology section at NVI.

Table 6. Program for the gradient PCR set-up

Hold for	5 minutes at 95 °C
25 cycles	30 seconds at 95 °C *30 seconds at 50-60 °C 1 minute at 72 °C
Hold for	2:30 minutes at 72 °C
Infinity	8 °C

* Annealing temperature ranges: 50-60 °C

2.7.5 Annotation of the plasmid

The nucleotide sequence of the contig with *bla*$_{\text{CTX-M-1}}$-plasmid was annotated automatically using the Online Rapid Annotation Subsequencing Technology (RAST; Aziz et al., 2008) and manually in CLC Main Workbench visualized in CLC genomics.

3. Results

3.1 Genotypic and phenotypic characterization of the *E. coli* isolates

3.1.1 Phylogenetic grouping

The 35 *E. coli* isolates were assigned to three phylogenetic groups, i.e. A, B1, and D. Of these, 17 isolates (48%) belonged to the virulent extra-intestinal *E. coli* group D. Ten (29%) and eight (23%) isolates were classified into the commensal phylogenetic groups A and B1, respectively (Figure 11 B). Figure 12 depicts the assignment of nine selected isolates to their respective phylogenetic group.

In broilers, the phylogroup D was most represented among the *E. coli* isolates. Of the 26 isolates, 15 (58%) were classified as phylogroup D whereas eight (31%) and three (11%) isolates belonged to phylogroup B1 and A, respectively. All seven *E. coli* isolates from parent flocks, by contrast, belonged to phylogroup A (Figure 12 A and C). The two poultry samples from Iceland with unknown origin were assigned to the phylogenetic group D and included in Figure 12 B.

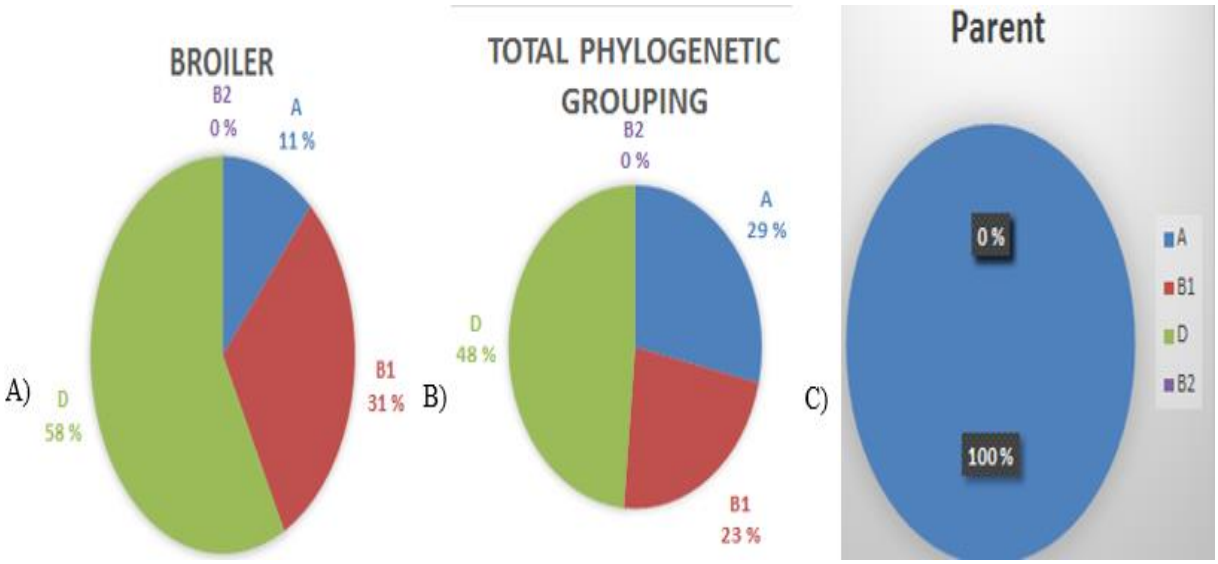


Figure 11. Distribution of ESBL-producing *E. coli* isolates from broilers and parent. All 35 isolates divided into the phylogenetic groups A (n= 10) , D (n= 17), and B1 (n= 8) is shown in the middle (B).

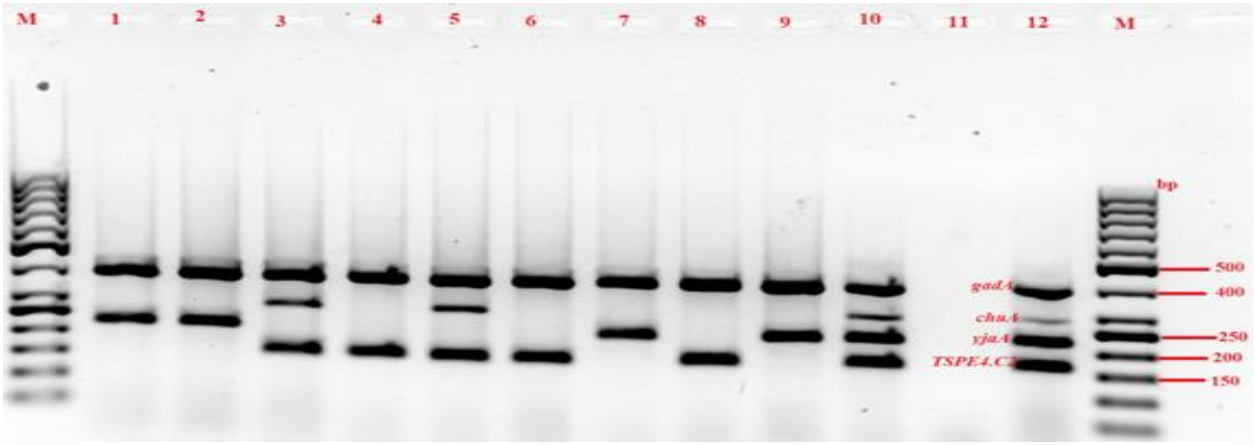


Figure 12. Multiplex PCR profile demonstrating phylogrouping of *E. coli* isolates from nine of the poultry samples. Lanes 1, 2, 7, and 9: **phylogroup A** [*gad*+, *chuA*-, *yjaA*+, TSPE4.C2-]; lanes 3 and 5: **phylogroup D** [*gad*+, *chuA*+, *yjaA*, TSPE4.C2+]; lanes 4, 6, and 8: **phylogroup B1** [*gadA*+, *chuA*-, *yjaA*-, TSPE4.C2+]. Lanes 10 and 12: **phylogroup B2**-*E. coli* as positive control [*gadA*+, *chuA*+, *yjaA*+, TSPE4.C2+]. Lane 11: negative control and lanes M: GeneRuler^{TM} 50 bp DNA ladder.

3.1.2 **Antimicrobial** resistance profile: phenotypic testing

Based on the MIC and disk diffusion tests, some isolates displayed multi-drug resistance (MDR) phenotype. MDR symbolizes bacteria that resist three or more different classes of antimicrobials (DANMAP, 2016).

3.1.2.i MIC determination

In the susceptibility testing of the isolates to the 14 antimicrobial agents, occurrence of resistance was highest to ampicillin cefotaxime, ceftazidime, and sulfamethoxazole. Table 7 shows distribution of the MIC values and level of antimicrobial resistance among the 35 ESBL-producing *E. coli* isolates. Overall, resistance to ampicillin (MIC= >64 mg/L) and cefotaxime (MIC= >4 mg/L) was 100% whereas ceftazidime (MIC= 1-2 mg/L) and sulfamethoxazole (MIC= >1024 mg/L) resistance was 97.1% and 91.4%, respectively. Six isolates (17%) were resistant to tetracycline (MIC= ≥64%) and three isolates (8.6%) to trimethoprim (MIC= >36 mg/L). None of the isolates showed resistance phenotype to the remaining antimicrobials: ciprofloxacin (MIC= ≤0.015-0.03 mg/L), nalidixic acid (MIC= ≤4mg/L), tigecycline (MIC= ≤0.25 mg/L), colistin (MIC= ≤1 mg/L), gentamicin (MIC= ≤0.5-1 mg/L), and the carbapenem drug- meropenem (MIC= ≤0.03 mg/L).

Table 7. Minimum Inhibitory Concentrations (MICs) and antimicrobial resistance in *Escherichia coli* resistant to extended spectrum cephalosporins (ESC) (n=35) and harboring the $bla_{CTX-M-1}$ gene isolated from parent and broiler flocks in Norway during May-October 2016.

Substance	Resistance (%)	Distribution (%) of MIC values (mg/L)*															
		0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	≥ 512
Ampicillin	100													100			
Ciprofloxacin	0	91.4	8.6														
Nalidixic acid	0									100							
Gentamicin	0						82.6	17.4									
Tetracycline	17.1								82.9					8.6	8.6		
Colistin	0							100									
Sulfamethoxazole	91.4											8.6					91.4
Trimethoprim	8.6					91.4							8.6				
Chlorampenicol	0										100						
Cefotaxime	100.0									100							
Ceftazidime	97.1						2.9	20	77.1								

*Bold vertical lines denote epidemiological cut-off values for resistance. White fields denote range of dilutions tested for each antimicrobial agent. MIC values higher than the highest concentration tested are given as the lowest MIC value above the range. MIC values equal to or lower than the lowest concentration tested are given as the lowest concentration tested.

3.1.2.ii Agar Disc diffusion test

Results from the disk diffusion tests are presented in Figure 13. Ampicillin, cefotaxime, ciprofloxacin, nalidixic acid, gentamicin, tetracycline, and trimethoprim, tested in both disc diffusion and MIC testing had the same resistance rates. In addition, all 35 isolates were resistant to clindamycin, erythromycin, and penicillin. Six isolates (one from broilers and five from parents) and three isolates (all from parents) showed moderate resistance to the antimicrobial combination disks amoxicillin-clavulanic acid and sulfa-trimethoprim, respectively. On the other hand, cefalexin resistance was observed in 10 isolates. None of the isolates, in contrast, were resistant to the polymyxin/colistin disk**.**

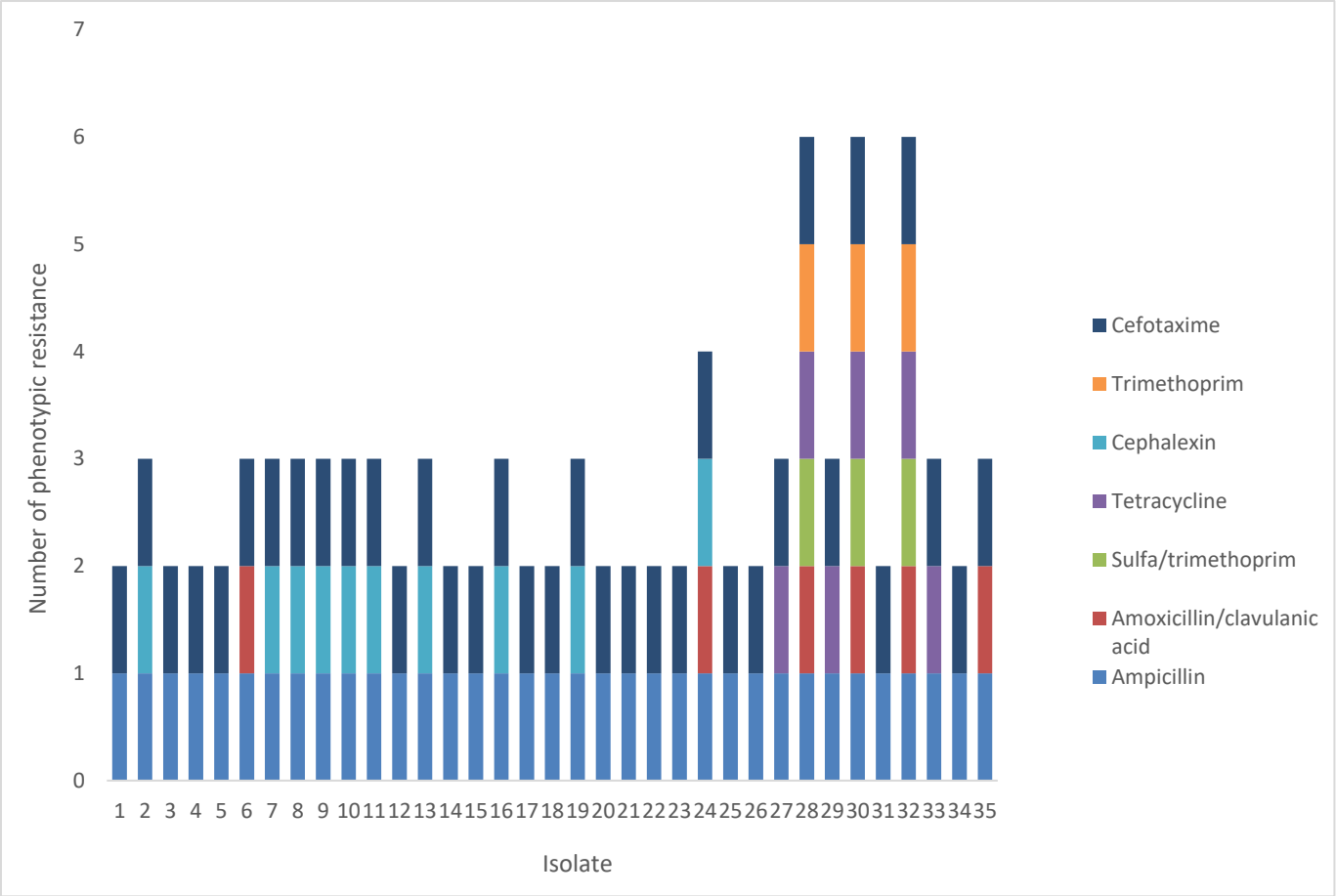
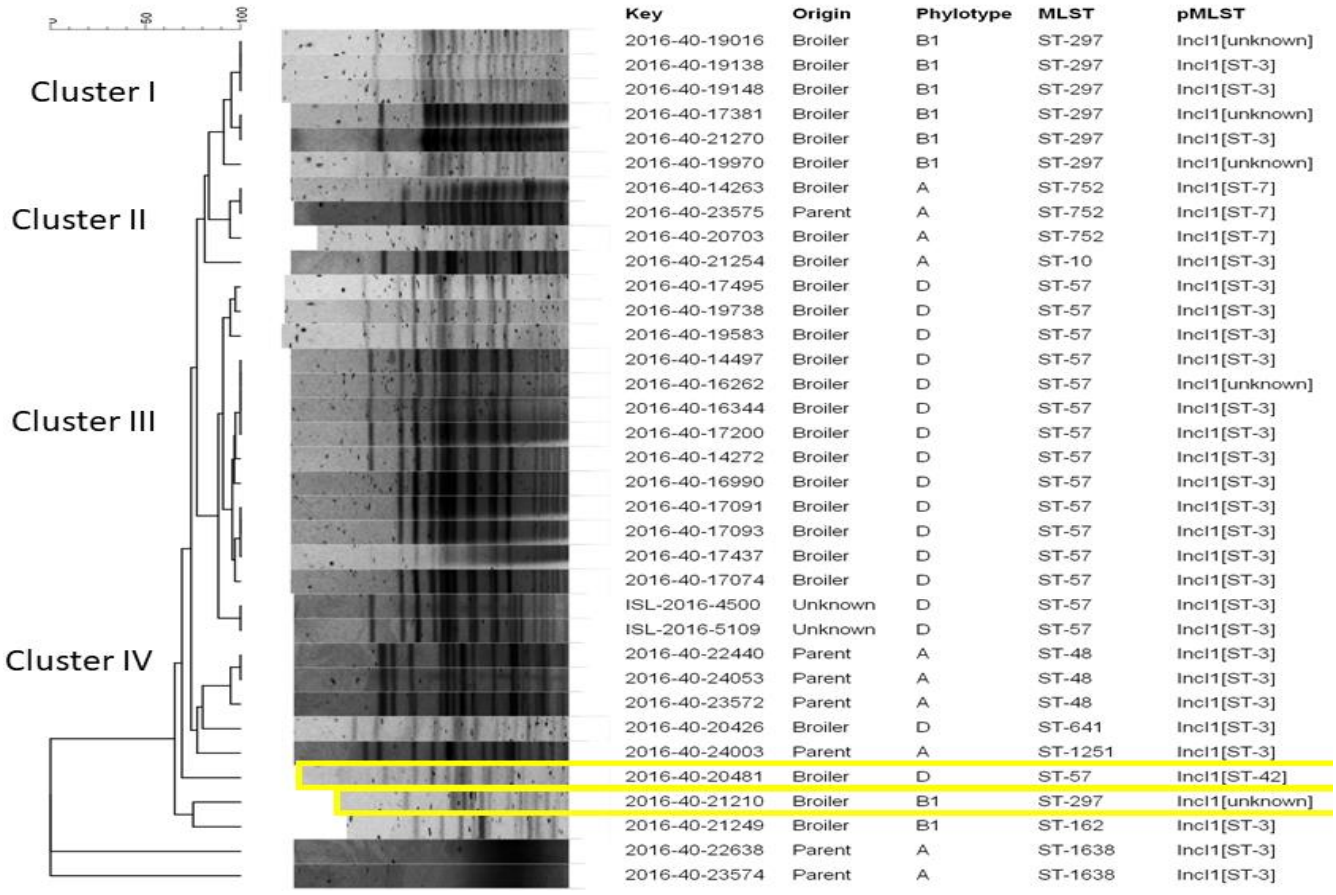


Figure 13. Total phenotypic resistance of all 35 isolates based on the disk diffusion tests. Refer to Table 6 for Isolate ID. Isolates 28, 30, and 32 in addition to the beta-lactams (cefotaxime, cephalexin, amoxicillin/clavulanic acid, and ampicillin) display multi-resistance to three non-beta lactams (sulfa-trimethoprim, trimethoprim, and tetracycline). The antimicrobials where isolates showed 100% susceptibility are excluded, i.e., gentamicin, ciprofloxacin, polymixin/colistin and nalidixic acid. Similarly, clindamycin, erythromycin, and penicillin are excluded due to their intrinsic resistance in *E. coli.*

3.1.3 PFGE

PFGE typing of all 35 *E. coli* isolates revealed 13 distinct pulsotypes. Isolates considered as clones or with indistinguishable PFGE patterns were based on ≥ 97 % similarity, whereas those with ≥ 80 % were grouped in the same PFGE cluster. Together, four different PFGE clusters were defined (Figure 14). Cluster II included the three isolates that were non-transferrable by the conjugation experiments. The two isolates; 2016-40-14263 and 2016-40-23575 had indistinguishable PFGE patterns (97.3 % similarity) whereas 2016-40-20703 was grouped into the same cluster. Six phylogroup B1 isolates in cluster I showed identical PFGE patterns and were defined as clones. Cluster III, which was the largest cluster, was composed of 15 phylogroup D isolates from the broiler samples including the two poultry samples from Iceland. Majority of the isolates within this cluster had ≥ 97% identity in their

PFGE profiles. Cluster IV contained three phylogroup A isolates from parents that showed ≥ 97% % identity. The remaining four isolates: 2016-40-21254 (phylogroup A), 2016-40-24003 (phylogroup A), 2016-40-20426 (phylogroup D), and 2016-40-20481 (phylogroup B1) had unique pulsotypes and were unrelated to the clusters. The last two isolates in the dendogram; 2016-40-22638 and 2016-40-23574 were non-typeable by PFGE and generated a smear after the *XbaI*-digestion.



Key	Origin	Phylotype	MLST	pMLST
2016-40-19016	Broiler	B1	ST-297	IncI1[unknown]
2016-40-19138	Broiler	B1	ST-297	IncI1[ST-3]
2016-40-19148	Broiler	B1	ST-297	IncI1[ST-3]
2016-40-17381	Broiler	B1	ST-297	IncI1[unknown]
2016-40-21270	Broiler	B1	ST-297	IncI1[ST-3]
2016-40-19970	Broiler	B1	ST-297	IncI1[unknown]
2016-40-14263	Broiler	A	ST-752	IncI1[ST-7]
2016-40-23575	Parent	A	ST-752	IncI1[ST-7]
2016-40-20703	Broiler	A	ST-752	IncI1[ST-7]
2016-40-21254	Broiler	A	ST-10	IncI1[ST-3]
2016-40-17495	Broiler	D	ST-57	IncI1[ST-3]
2016-40-19738	Broiler	D	ST-57	IncI1[ST-3]
2016-40-19583	Broiler	D	ST-57	IncI1[ST-3]
2016-40-14497	Broiler	D	ST-57	IncI1[ST-3]
2016-40-16262	Broiler	D	ST-57	IncI1[unknown]
2016-40-16344	Broiler	D	ST-57	IncI1[ST-3]
2016-40-17200	Broiler	D	ST-57	IncI1[ST-3]
2016-40-14272	Broiler	D	ST-57	IncI1[ST-3]
2016-40-16990	Broiler	D	ST-57	IncI1[ST-3]
2016-40-17091	Broiler	D	ST-57	IncI1[ST-3]
2016-40-17093	Broiler	D	ST-57	IncI1[ST-3]
2016-40-17437	Broiler	D	ST-57	IncI1[ST-3]
2016-40-17074	Broiler	D	ST-57	IncI1[ST-3]
ISL-2016-4500	Unknown	D	ST-57	IncI1[ST-3]
ISL-2016-5109	Unknown	D	ST-57	IncI1[ST-3]
2016-40-22440	Parent	A	ST-48	IncI1[ST-3]
2016-40-24053	Parent	A	ST-48	IncI1[ST-3]
2016-40-23572	Parent	A	ST-48	IncI1[ST-3]
2016-40-20426	Broiler	D	ST-641	IncI1[ST-3]
2016-40-24003	Parent	A	ST-1251	IncI1[ST-3]
2016-40-20481	Broiler	D	ST-57	IncI1[ST-42]
2016-40-21210	Broiler	B1	ST-297	IncI1[unknown]
2016-40-21249	Broiler	B1	ST-162	IncI1[ST-3]
2016-40-22638	Parent	A	ST-1638	IncI1[ST-3]
2016-40-23574	Parent	A	ST-1638	IncI1[ST-3]

Figure 14. Dendogram analysis of *XbaI*-digested PFGE profiles for 35 ESBL-producing *E. coli* isolates. Isolate ID, samples origin, and phylogenetic grouping, MLST and pMLST profiles are also shown.

MLST based on wgs data and SNP analysis indicated the two isolates: 2016-40-20481 and 2016-40-21210 (in yellow) respectively belonged to cluster I and III in the PFGE dendogram as their isolates (D-ST-57 and B1-ST297). However, *XbaI*-digestion of the chromosomal DNA was not optimal for the two isolates and generated faint PFGE fingerprints, which BioNumerics analysed as distinct. As a result, PFGE was repeated for these two isolates by preparing new gel plus. For comparison, new plugs were prepared for two more isolates representing cluster I and III as indicated in Figure 14b. The new PFGE gel also included old plugs for some of the isolates (Figure 14b). As expected the phylogroup D

isolate (*2016-40-20481) had 97.1% similarity to the other phylogroup D/ST-57 isolates whereas the phylogroup B1 isolate (*2016-40-21210) had identical PFGE patterns as the other phylogroup B1/ST-297 isolates (Figure 14b).

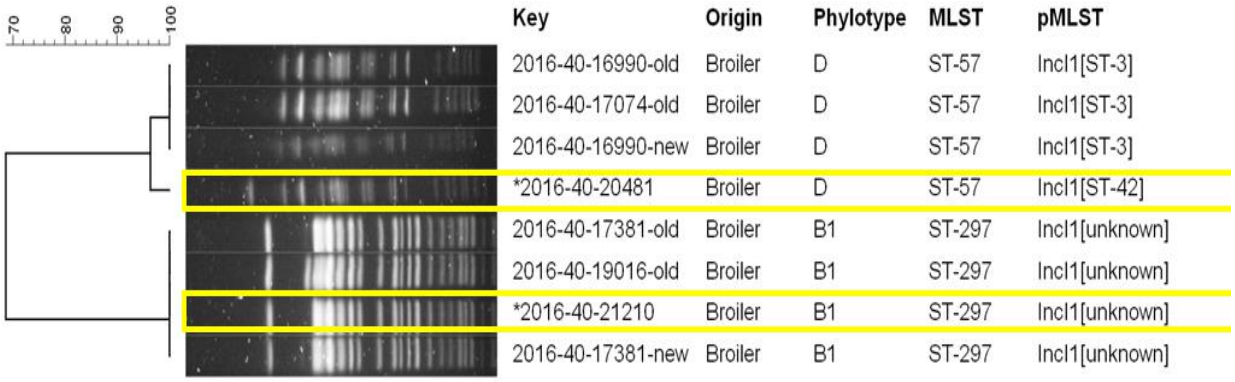


Figure 14b. PFGE dendrogram of the phylogroup B isolate (*2016-40-21210) and D isolate (*2016-40-20482) clustering with their respective highly related isolates.

3.2 Characterization of *bla*$_{\text{CTX-M-1}}$-carrying plasmids

3.2.1 Conjugative transfer of *bla*$_{\text{CTX-M-1}}$ and plasmid replicon typing

Each of the 35 *bla*$_{\text{CTX-M-1}}$ positive *E. coli* isolates was tested for its ability to transfer the third-generation cephalosporin resistance by conjugation. Of the 35 isolates, 32 (91%) transferred the *bla*$_{\text{CTX-M-1}}$ to the *E. coli* DH5α recipient. For the three isolates that failed to transfer the *bla*$_{\text{CTX-M-1}}$ gene, the conjugation experiment was repeated and performed at 4, 6, and 24 hours. Still, PCR did not detect *bla*$_{\text{CTX-M-1}}$ in the presumptive transconjugants, suggesting non-transferrability of the plasmids. Table 8 shows the summary of the conjugation experiments.

3.2.2 PBRT

After plasmid replicon typing., three plasmid Inc-groups: IncI1αα, IncFIB, and IncB/O were detected among the 32 transconjugants. However, some of the isolates gave weak bands of IncK replicon but they were confirmed as Inc B/O replicons using a single PCR reaction designed for IncB/O (Carattoli et al., 2005).

Of the 32 transconjugants, IncI1α was identified in 16 (50%), followed by a combination of IncI1α and FIB in 13 (41%) transconjugants, and IncI1α and IncB/O in three (9%) (Table 5). Thus, of the three Inc groups, IncI1αα was the dominant type appearing in all 32 transconjugants.

Table 8. Transferability of *bla*$_{CTX-M-1}$ in 35 ESBL-producing *E. coli* isolates. PBRT of transconjugants is also shown.

Donors			**Transconjugants (donor →*E. coli* DH5α)**		
Isolate	Resistance phenotype	Resistance gene	Transferability	Resistance gene	Plasmid replicon type
2016-40-14263	cefotaxime	*bla*$_{CTX-M-1}$	-	*bla*$_{CTX-M-1}$	***N.T**
2016-40-14272	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α, FIB
2016-40-14497	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α, FIB
2016-40-16262	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α, FIB,
2016-40-16344	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α, FIB
2016-40-16990	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α FIB
2016-40-17074	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α FIB
2016-40-17091	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α FIB
2016-40-17093	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α FIB
2016-40-17200	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α FIB
2016-40-17381	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α B/O
2016-40-17437	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α FIB
2016-40-17495	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α FIB
2016-40-19016	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-19138	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-19148	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α B/O
2016-40-19583	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α FIB
2016-40-19738	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α FIB
2016-40-19970	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α B/O
2016-40-20426	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-20481	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-20703	cefotaxime	*bla*$_{CTX-M-1}$	-	*bla*$_{CTX-M-1}$	***N.T**
2016-40-21210	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-21249	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-21254	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-21270	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-22440	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-22638	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-23572	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-23574	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-23575	cefotaxime	*bla*$_{CTX-M-1}$	-	*bla*$_{CTX-M-1}$	***N.T**
2016-40-24003	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
2016-40-24053	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
ISL-2016-40-4500	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α
ISL-2016-40-5109	cefotaxime	*bla*$_{CTX-M-1}$	+	*bla*$_{CTX-M-1}$	I1α

*****N.T= excluded from PBRT due to non-transferability of plasmids.

3.2.3 Susceptibility testing of transconjugants

Transconjugants obtained after the conjugation experiments displayed somewhat similar antimicrobial resistance patterns as their donor strains (Table 9). All 32 transconjugants were resistant to both cefotaxime and ampicillin, as previously observed in their donor strains. Likewise, as observed earlier in their corresponding donors, six (19%) transconjugants and three (9%) transconjugants also showed resistance to tetracycline and trimethoprim, respectively. Similarly, all transconjugants but one was susceptible to sulfamethoxazole. In contrast, ceftazidime resistance, differed significantly between the donor strains and transconjugants. Whereas 34 (97%) donor strains were resistant to ceftazidime, all 32 (100%) transconjugants were susceptible.

Table 9. Resistance phenotypes of *E. coli* donors and their transconjugants.

	ESBL-*E. coli* donor strains n=35		Transconjugants n= 32	
Antimicrobial agents	no. (%) of resistant donor	no. (%) of susceptible donors	no. (%) of resistant transconjugants	no. (%) of susceptible transconjugants
Ampicillin	32 (100%)	0	32 (100%)	0
Cefotaxime	32 (100%	0	32 (0%)	0
Sulfamethoxazole	32 (100%)	0	31 (97%)	1 (3%)
Trimethoprim	3 (9%)	32 (91%)	3 (9%)	29 (91%)
Tetracycline	6 (17%)	29 (83%)	6 (19%)	26 (81%)
Ceftazidime	34 (97%)	1 (3%)	0	32 (100%)

3.3 Whole genome data analysis: *in silico* characterization of the *E. coli* isolates

3.3.1 Multi-locus sequence typing (MLST)

MLST analysis revealed the 35 *E. coli* isolates belonged to nine different sequence types (STs): ST-57, ST-48, ST-1251, ST-752, ST-1638, ST-297, ST-10, ST-162, and ST-641 (Figure 15). Of the 17 phylogroup D isolates (in blue), 16 were assigned as ST-57 whereas the distinct isolate belonged to ST641. Similarly, seven of the eight phylogroup B1 isolates (in yellow) were typed as ST-297 and the one as ST-162. In contrast, the phylogroup A isolates (in red) were represented by five different MLSTs.

Of the 10 phylogroup A isolates, three belonged to ST-48 whereas the three isolates with non-transferrable plasmids were typed as ST-752. ST1-638 represents the two isolates not typeable by PFGE. The two remaining isolates belonged to ST-10 and ST-1251, respectively.

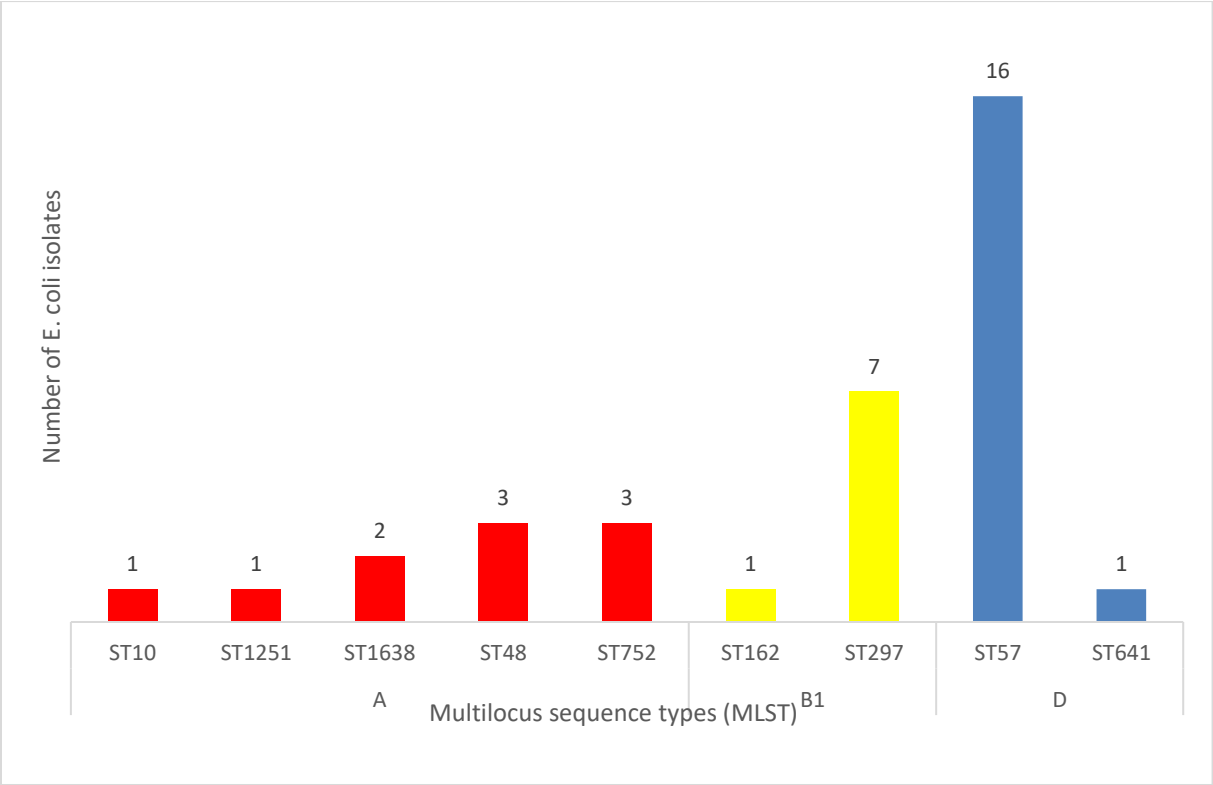


Figure 15. WGS-based MLST results for all 35 ESBL-producing *E. coli* isolates. Their corresponding phylogenetic groups: A (red), B1 (yellow), and D (blue) are also shown.

3.3.2 Phylogenetic analysis of the *E. coli* isolates

Phylogenetic tree constructed after the whole genome SNP (wgSNP) analysis is shown in Figure 16 with all the isolates divided into four main clusters (Cluster I, II, III, and IV). Sixteen of the 35 genomes were grouped into the phylogenetic cluster 1 and included the phylogroup D/ST-57 isolates from broilers. These isolates, displaying a clonal relationship, were separated by 23-79 SNPs within this cluster. Cluster II represent the seven phylogroup B1/ST-297 isolates separated by 23-93 SNPs. Cluster III represents the other phylogroup A isolates assigned as ST-48 and showing a close clonal relationship as they also differed by 20-35 SNPs. In cluster IV are the phylogroup A/ST-752 isolates that had non-transferrable plasmids by conjugation. These isolates only differed by 24-35 SNPs suggesting they are closely related. Furthermore, the two phylogroup A/ST-1638 isolates that were non-typeable by PFGE differed by only 31 SNPs and were considered as clones. The remaining isolates:

2016-40-21254 (phylogroup A/ST-10), 2016-40-24003 (phylogroup A/ ST-1251), 2016-40-21249 (phylogroup B1/ST-162), and 2016-40-20426 (phylogroup D/ ST-641), were distinct and unrelated to their cluster isolates as they differed by more than 3000 SNPs. SNP variations between each isolate from their respective phylogroup are shown in S6, S7, and S8 Tables.

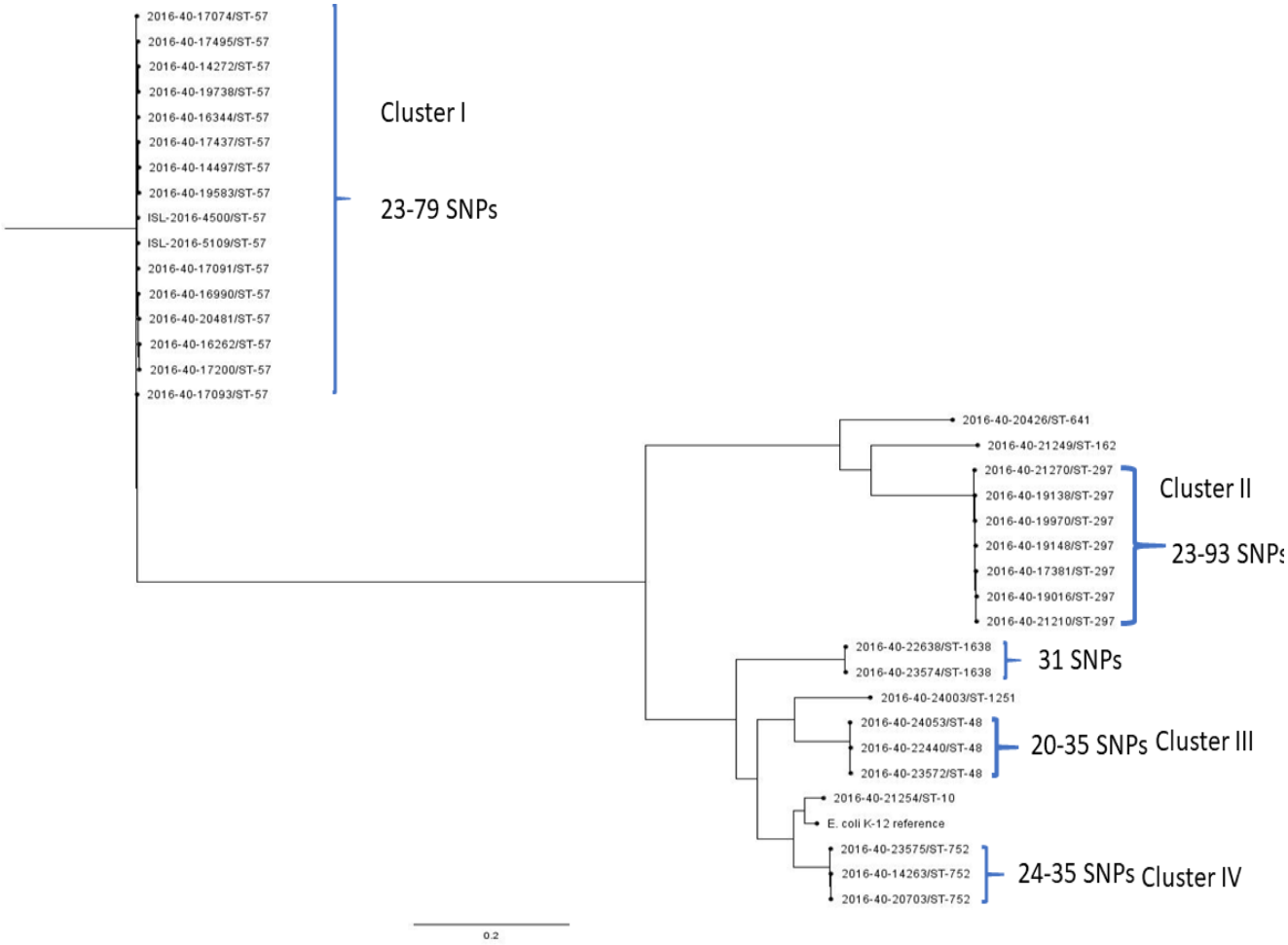


Figure 16. CSI Phylogeny SNP analysis of the 35 ESBL-producing *E. coli* isolates, visualized in Fig Tree v1.4.3. The phylogenetic tree includes the reference *E. coli* K-12 strain MG165 (Accession number NZ_CP027060.1). Sequence types (ST) are shown after /.

3.3.3 Detection of antimicrobial resistance genes

Isolates" resistance phenotypes were verified with the identification of acquired resistance genes based on their WGS data. None of the isolates contained resistance genes to antimicrobials where they showed phenotypic susceptibility. As expected, the ResFinder detected the *bla*$_{\text{CTX-M-1}}$ gene in all 35 isolates. Other resistance genes identified were *sul1* and *sul2* (sulphonamide resistance), *strA*, *strB*, and *aadA1* (aminoglycoside resistance); *tetA*

(tetracycline resistance)*;* *drfA1* (trimethoprim resistance), and *bla*$_{TEM-1B}$, which is gene encoding resistance to ampicillin. Overall, the ResFinder results were in accordance with the phenotypic resistance profile of the *E. coli* isolates.

Of the 35 isolates, 26 had identical ResFinder profile with the *bla*$_{CTX-M-1}$ and *sul2* resistance genes. These isolates belonged to both the phylogenetic groups B1 and D. On the other hand, the phylogroup A isolates contained several acquired antimicrobial resistance genes in their ResFinder profiles as shown in Figure 17.

Three isolates: 2016-40-14263; 2016-40-20703, and 2016-40-23575 had a ResFinder profile with the four resistance genes: *bla*$_{TEM-1B}$, *strB*, *strA*, and *bla*$_{CTX-M-1.}$ Moreover, three different isolates (2016-40-22638; 2016-40-23574; and 2016-40-24003) detected seven resistance genes including *bla*$_{CTX-M-1}$, *sul1*, *sul2*, *strB*, *strA*, *tetA*, and *drfA1*. The remaining three isolates (2016-40-22440; 2016-40-23572; and 2016-40-24053) had the *bla*$_{CTX-M-1,}$ *sul2*, and *tetA* resistance genes. The isolate, 2016-40-21254, had an identical ResFinder profile as the phylogroup B and D isolates

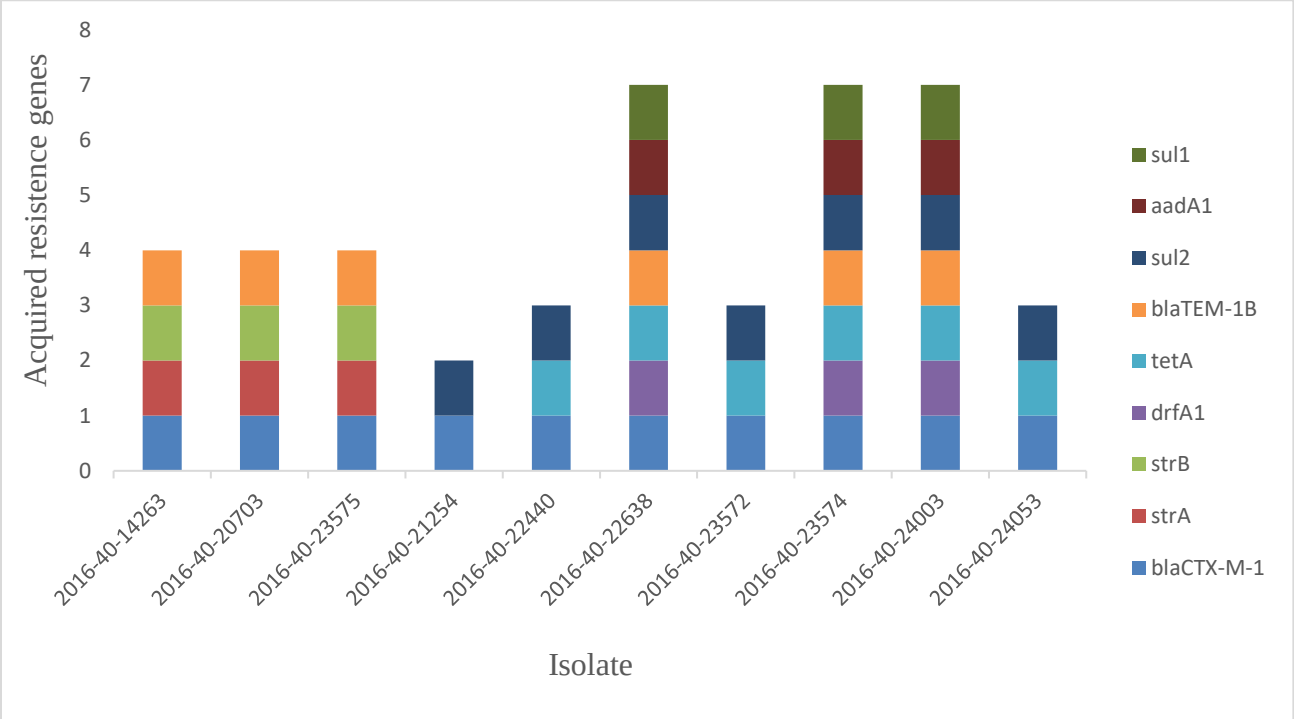


Figure 17. Identified antimicrobial resistance genes in the 10 phylogroup A *E. coli* isolates.

3.3.4 Detection of virulence genes

TheVirulenceFinder tool described in (Joensen et al., 2014) detected 18 virulence genes based on the WGS data of the 35 *E. coli* isolates (Figure 18). The identified virulence

genes included *cma* (colicin M), *gad* (glutamate decarboxylase), *iroN* (enterobactin siderophore receptor protein), *iss* (increased serum survival), *astA* (heat-stable enterotoxin 1), *cif* (type III secreted effector), *espA* (type III secretion system), *espB* (secreted protein B), *mchB* (ABC transporter protein MchB), *mchC* (ABC transporter protein MchC), *mchF* (ABC transporter protein MchF), *nleB* (non-lee-encoded effector B), *tir* (translocated intimin receptor protein), *tsh* (serine protease autotransporters), *IpfA* (long polar frimbriae), and *tccP* (tir cytoskeleton coupling protein).

Of the 18 genes, 13 were identified in the three phylogroup A/ST-752 isolates, followed by nine genes in the seven phylogroup B1/ST-297 isolates, eight genes in the two phylogroup A/ST-1638 isolates, and five virulence genes in the phylogroup A/ST-162 isolate. On the other hand, the phylogroup A/ST-48 and ST-1251, and phylogroup D/ST-57 isolates shared a similar virulence gene profile with a combination of *cma, gad, iroN, and iss*. However, one phylogroupD/ ST-57 isolate lacked the *cma* gene and detected the three virulence genes: *gad, iroN, and iss*. Likewise, three virulence genes were identified in phylogroup A/ST-10 isolate whereas the phylogroup B1/ST-641 isolate detected only two virulence genes. Interestingly, the *gad* gene was detected in all isolates of the different MLST types. Furthermore, *iss* and *iroN* were among the most detected virulence genes, appearing in all but the phylogroup B1/ST-641 isolate.

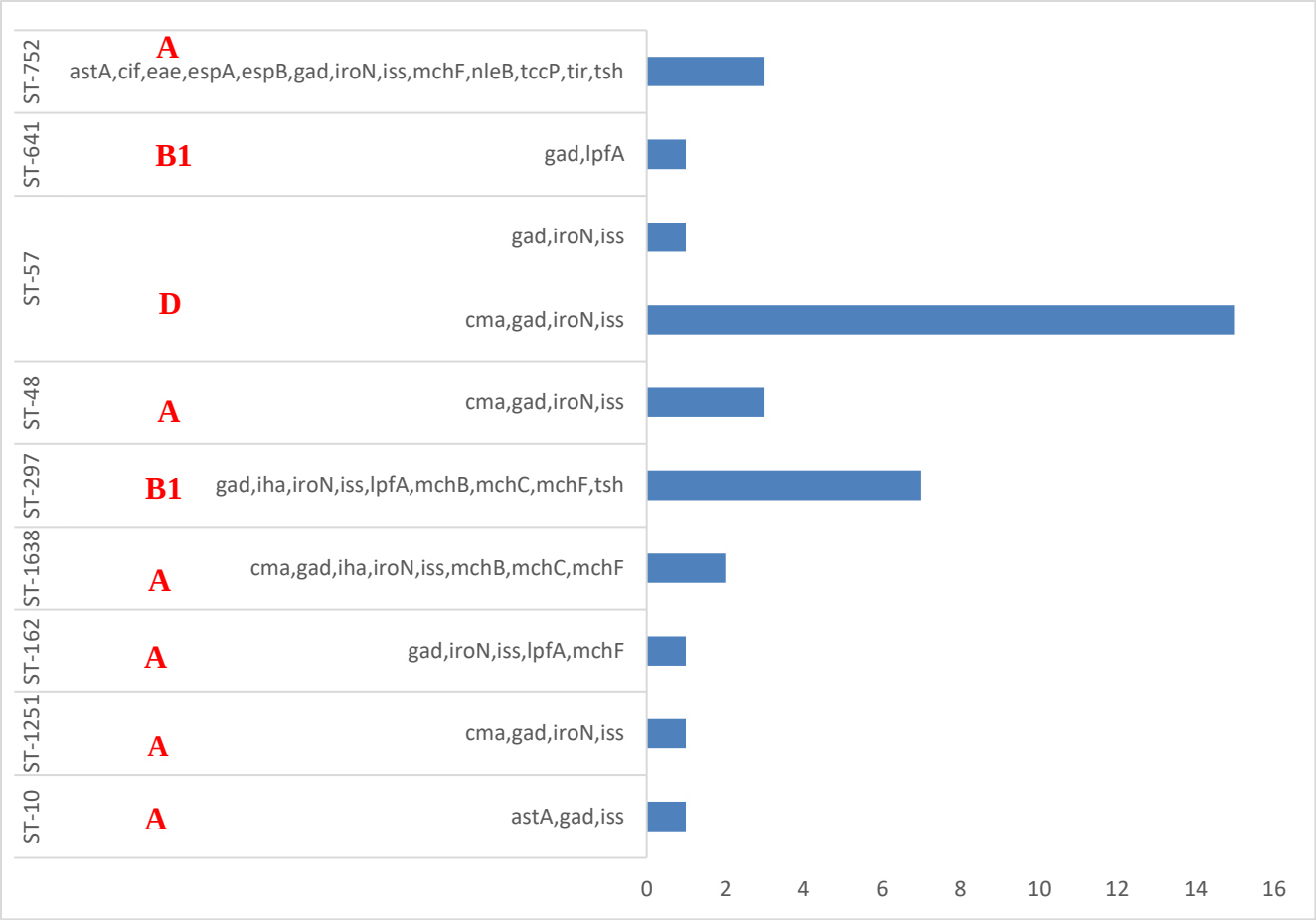


Figure 18. VirulenceFinder profile of the 35 ESBL-producing *E. coli* isolates displayed as their MLST profile. Phylogenetic group each MLST is shown in red.

3.6.5 Serotyping of the *E. coli* isolates

Based on their WGS data, the 35 *E. coli* isolates belonged to 10 distinct serotypes (Figure 19). All 16 phylogroup D/ST-57 isolates in cluster I in the SNP phylogenetic tree were identified as serotype O140:H25. The O37:H40 serotype was assigned to the distinct phylogroup D/ST-641 isolate. Similarly, the seven phylogroup B1/ST-297 isolates considered as clonally-related belonged to serotype O45:H8 whereas the one phylogroup B1 isolate was serotyped as O8:H28. The phylogroup A isolates, in contrast, were classified into five different serotypes.

The O53:H18 serotype represents the two isolates (ST-1638) that were non-typeable by PFGE. Moreover, the three ST48 isolates were classified as the O8:H1I serotype whereas the two distinct isolates: ST-10 and ST-1251 were serotyped as O176:H48 and O132:H28, respectively. The three non-transferrable isolates (ST-752) were identified as the O123:H40 serotype.

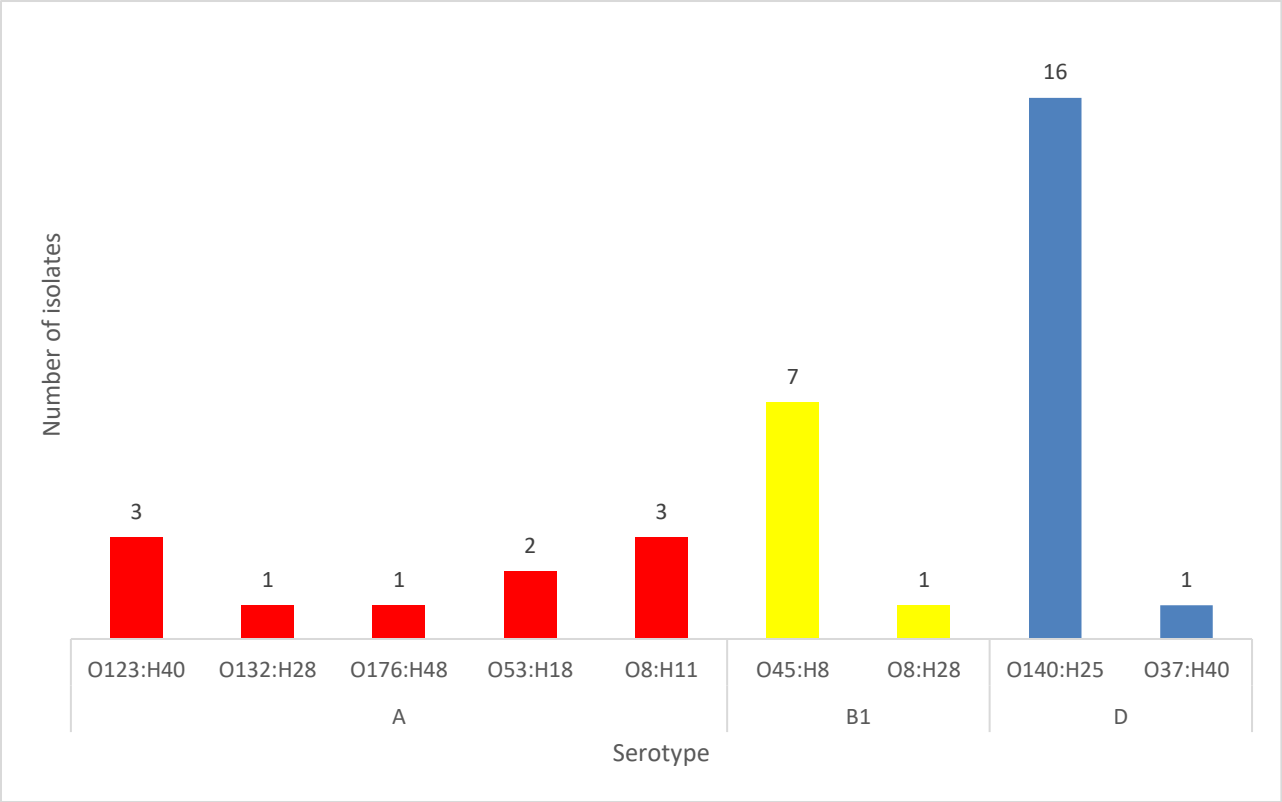


Figure 19. SerotypeFinder results of the 35 *E. coli* isolates. Phylogenetic grouping (A, B1, and D) of the isolates is also shown.

3.6.6 Whole genome plasmid typing

During the BAP analysis, PlasmidFinder tool identified 25 plasmid replicons among the WGS data of the 35 *E. coli* isolates (Table 10). Of these plasmid replicons, IncI1α and IncFIB were the dominant types. Other major Inc groups included IncB/O, IncK, IncZ, and ColpVC. However, only the IncI1α, IncFIB, and IncB/O plasmids were detected in the transconjugants.

The IncI1α replicon was detected in all 35 (100%) isolates followed by IncFIB(AP001918) replicon in 32 (91.4%) of the 35 isolates. However, IncFIB was detected in 41% (13/32) of the transconjugants. Similarly, IncB/O was detected in 11 (31.4%) isolates but was identified in only three transconjugants.

Overall WGS analysis confirmed that the IncI1α plasmids contained the $bla_{\text{CTX-M-1}}$ genes in all 35 isolates, because $bla_{\text{CTX-M-1}}$ together with the IncI1α plasmid were present on the same contig. Thereafter, the $bla_{\text{CTX-M-1}}$ IncI1α plasmids were further subtyped by pMLST.

Table 10. Detection of replicons in the 35 ESBL-producing *E. coli* using PlasmidFinder. Replicons detected in transconjugants by PBRT is also shown.

Plasmid replicon	Number of isolates	Plasmid detected in number of transconjugant
***IncI1α**	**35**	**33**
***IncFIB(AP001918)**	**32**	**13**
ColRNAI	14	N.I
Col(MG828)	13	N.I
IncK	12	0
IncZ	12	0
***Inc B/O**	**12**	**3**
ColpVC	12	N.I
IncFII	8	0
IncFIC(FII)	7	0
IncFIA	7	0
IncHI1B(CIT)	4	0
IncFII(pRSB107)	3	0
p0111	3	N.I
Col(KPHS6)	3	N.I
IncX4	2	0
Col8282	2	N.I
IncI2	2	0
IncFIA(HI1)	1	0
IncFII(29)	1	0
IncFIB(pB171)	1	0
IncFII(pHN7A8)	1	0
IncFII(pCoo)	1	0
Col(MGD2)	1	N.I
Col156	1	N.I

*Plasmids that transferred and were detected in transconjugants via PBRT (marked red). N.I= plasmids excluded from the PBRT kit due to unavailable primers.

3.6.7 pMLST of IncI1α plasmids

pMLST determined sequence types (STs) of the IncI1α plasmids based on the combination of the alleles identified for the genes *(ardA, trbA, pill, sogS, and repI1)* (García-Fernández et al., 2008). Figure 20 depicts the identification of an IncI1α sequence type using the pMLST 1.4 webserver, which in this case was ST-3.

The pMLST analysis assigned the 35 IncI1α plasmids containing the $bla_{\text{CTX-M-1}}$ to three different sequence types (Figure 14 and 21). However, the pMLST profile of five (17%) IncI1α plasmids were unknown. Of the 30 IncI1α plasmids assigned, 26 (74%) belonged ST-3, 3 (9%) to ST-7, and 1 belonged to the ST-42.

pMLST-1.4 Server - Typing Results

Sequence Type: *ST-3*

Locus	% Identity	HSP Length	Allele Length	Gaps	Allele
ardA	100.00	343	343	0	*ardA_1*
trbA	100.00	465	465	0	*trbA_4*
pilL	100.00	254	254	0	*pilL_2*
sogS	100.00	235	235	0	*sogS_1*
repI1	100.00	83	83	0	*repI1_2*

Results as text | Hit in genome sequences | pMLST allele sequences | extended output

pMLST Profile: IncI1

Figure 20. pMLST profile of an IncI1α plasmid, which is IncI1α[ST3]. A perfect match to the allele is marked in green.

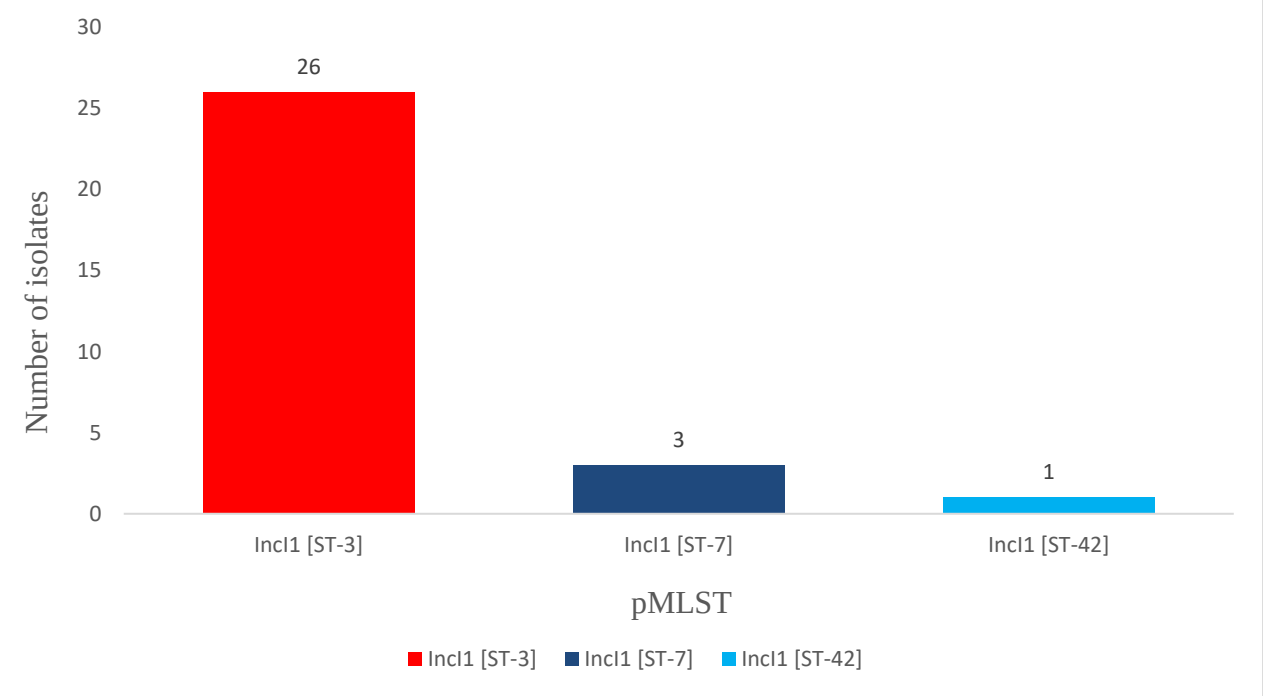


Figure 21. pMLST identification of the 30 Inc1I plasmids.

3.6.8 IncI1α plasmid characterization

An IncI1α/ST3 plasmid from the phylogroup D/ ST-57-O140:H25 isolate (2016-40-14272) was determined and characterized. The *bla*$_{CTX-M-1}$ and all five genes (*ardA*, *trbA*, *pill*, *sogS*, and *repI1*) used in pMLST for the IncI1α plasmids were located on a contig with 94.91 kb in size. Alignment results showed the *bla*$_{CTX-M-1}$ IncI1α plasmid in this study was 99% identical to the three reference plasmids in broilers from Switzerland and France. Of the reference plasmids, IncI1α/ST-3 plasmid in the present study showed more similarity to the Swiss IncI1α plasmids based on the genetic organization (accession no. KM377238 and

KM377239) (Figure 23). Thus, the plasmid with *bla*$_{\text{CTX-M-1}}$ from isolate 2016-40-14272 was constructed using the Swiss plasmid (KM377239).

When compared with the Swiss plasmids, the contig with *bla*$_{\text{CTX-M-1}}$-IncI1α lacked a variable sequence region consisting of the sulphonamide resistance gene (*sul2*), *yac*ABC genes, and mobile protein elements including transposases, integrons, and IS-elements. Further analysis of the isolate's wgs data showed these genes were located on another contig (NODE_49) with 5.9 kB in size (Figure 22). Thus, it was hypothesized the *sul2* contig (5.9 kB) was a missing region of the *bla*$_{\text{CTX-M-1}}$-IncI1α contig.

However, primers designed **(in 2.10)** in attempt to close the gaps yielded a PCR product of approximately 1.5 kb, which was not long enough to overlap the sequence from the two contig ends. Based on this one can say the primers designed were not robust enough to determine the sequences that could close the gaps between the contig with *bla*$_{\text{CTX-M-1}}$-IncI1α and the *sul2* contig. Hence, genetic organization of the *bla*$_{\text{CTX-M-1}}$ IncI1α/ST-3 plasmid and *sul2* contig is depicted in Figure 23 and 24, respectively. In addition, the Swiss plasmid (KM377239) contained the aminoglycoside resistance gene (*aadA5*) and trimethoprim resistance gene (*dfrA17*). A complete structure of the Swiss IncI1α/ST-3 plasmid is shown S1 Figure.

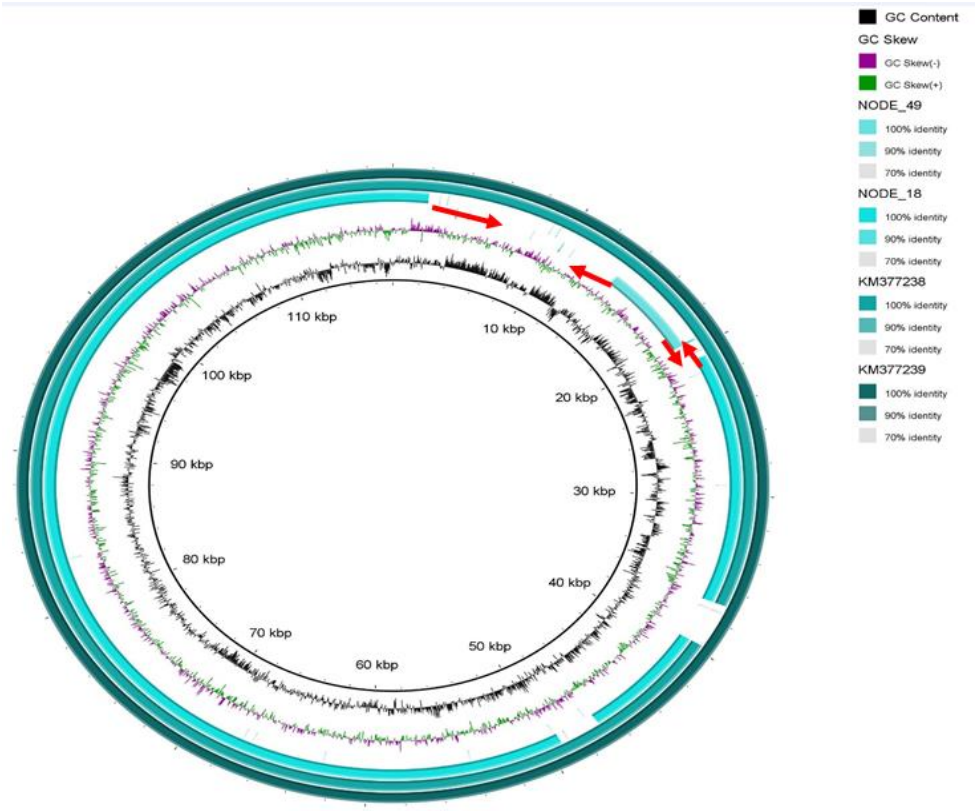


Figure 22. BRIG display of the sequencing alignments of the IncI1α/ST3 plasmid from this study. The two outmost circles (green and light green) represent two IncI1α/ST3 plasmids from Switzerland used as reference.

Red arrows show the direction of primer design to close the gaps between the two contigs: NODE_18 and NODE_49. Figure created by Jannice Schau Slettemås (researcher at NVI).

Majority of the genes present on the contig with IncI1 were identical to the Swiss plasmids. The IncI1 plasmid contig included the plasmid maintenance and stability genes, *tra*/*trb*ABC genes (associated with conjugal transfer; in yellow), *pil* genes (formation of pilus; in blue), and the *repZ* gene, which initiate replication (Figure 23). Similar to the Swiss plasmids, IS*Ecp1*-*bla*$_{\text{CTX-M-1}}$ (in red and blue) were flanked by *rci* downstream and *pilV* upstream. The *tra*ABCD gene cluster was located upstream of the *repZ* gene. In addition, a type IV-secretion system and post-segregational killing protein gene, *hok* were present on this contig.

Figure 23. Partial annotation of the full length 94.9 kB contig with *bla*$_{\text{CTX-M-1}}$-carrying IncII/ST-3 plasmid in *E. coli* from a broiler isolate. ISEcpI-*bla*$_{\text{CTX-M-1}}$ encoding resistance to third-generation cephalosporin is marked in a blue square. Hypothetical proteins are excluded.

Figure 24 depicts the missing 5.9 kB sequence region from the *bla*$_{CTX\text{-}M\text{-}1}$ contig. The *sul2* (in red) gene located on this contig was flanked by a hypothetical protein and the phosphoglucosamine synthase gene, *glmM*. One of the *yac* genes, *yac*B was located upstream of this sequence. Prevent host death protein, Phd (green), which is an antitoxin was located in this contig.

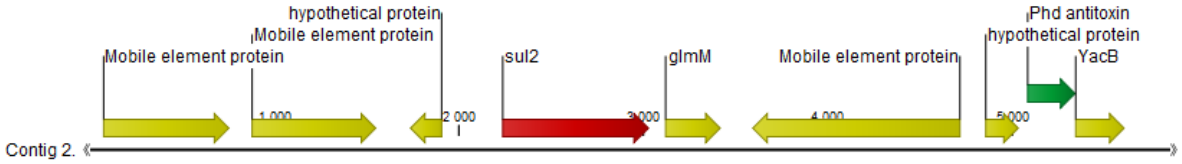


Figure 24. RAST annotation of the 5.6 kB contig sequence with the sulphonamide resistance gene (*sul2*), *yac*B, and mobile element proteins.

3.6.9 Isolates with non-transferrable plasmids

Based on a previous study from (Kim et al., 1993), *traBC* are essential for the conjugal transfer of resistance plasmids. Hence, whole genome analysis of the three non-transferrable isolates by conjugation revealed a missing *tra*C gen. Figure 25 depicts a 9.0 kb sequence region in the contig with *bla*$_{CTX\text{-}M\text{-}1}$ IncI1α lacking a *traC* gene in one of the non-transferrable isolates: 2016-40-14263, 2016-40-20703, and 2016-40-23575.

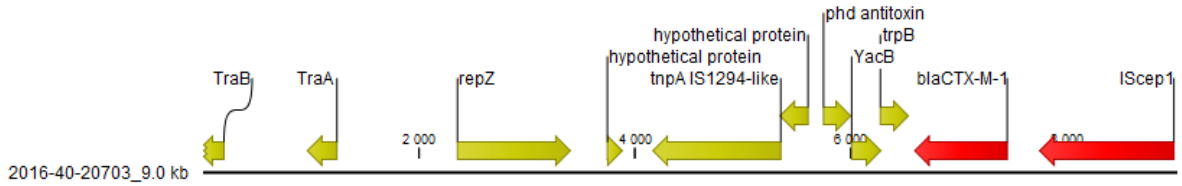


Figure 25. Genetic organization of a 13.5 kb contig with *bla*$_{CTX\text{-}M\text{-}1}$ IncI1α plasmid in one non-transferrable isolate (2016-40-20703). As depicted, *traC* gene is missing.

3.6.10 MDR isolates

The seven antimicrobial resistance genes detected in the MDR isolates were distributed among two contigs. Four resistance genes: *aaAd1*, *bla*$_{TEM\text{-}1B}$, *sul1*, and *drfA1* were present on one contig whereas the second contig contained *sul2*, *bla*$_{CTX\text{-}M\text{-}1}$, and *tet(A)* (Figure 26). A class 1 integron (*int*) was found in contig 1 (6.9 kb) containing a *drfA1-aadA1* gene cassette that encodes resistance to trimethoprim and streptomycin.

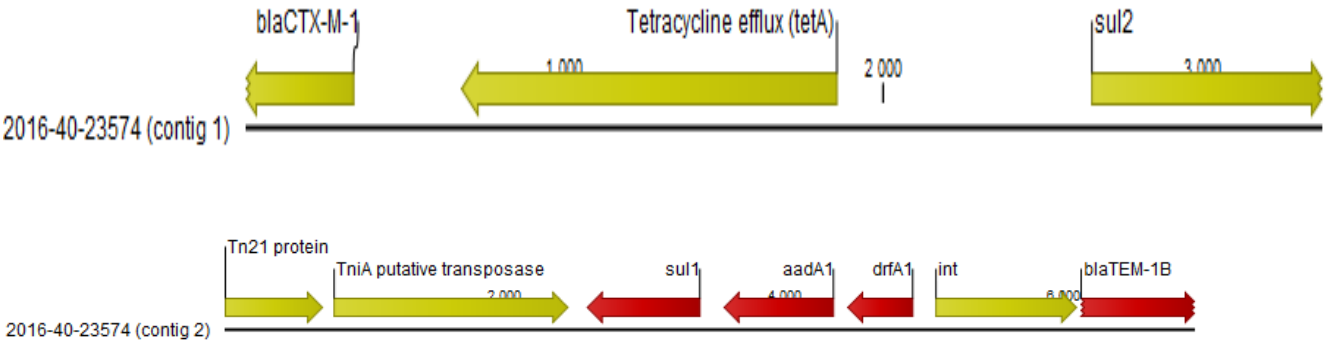


Figure 26. Schematic representation of the acquired antimicrobial resistance genes located on a 3.4 kb sequence and 6.9 kb sequence region from the two different contigs. Contig 1(3.4 kb): *bla*$_{\text{CTX-M-1}}$, *tetA*, and *sul2* and contig 2 (6.9 kb): *sul1*, *aadA1*, *drfA1*, and *bla*$_{\text{TEM-1B}}$ (red)

4. Discussion

This study investigated the emergence of an ESBL-producing *E. coli* with *bla*$_{\text{CTX-M-1}}$ in the Norwegian broiler production pyramid. In general, the use of third-generation cephalosporins select these ESBL-producing bacteria where they in turn, disseminate their resistance via clonal transmission or horizontal gene transfer. However, since these antimicrobial agents are never used in the broiler production in Norway, their occurrence raised several questions regarding if the occurrence resulted from a clonal spread of one particular *E. coli* sequence type or the horizontal transfer of mobile genetic elements such as plasmids. Characterization of the isolates was performed using phenotypic tests, molecular typing, and whole genome sequencing data.

4.1 Phylotyping, PFGE, and MLST, serotyping of isolates, SNP-analysis

Phylotyping, PFGE, MLST, serotyping and SNP-analysis determined the genetic relatedness between the isolates. Overall, the genotypic methods, i.e. phylotyping and PFGE agreed with the SNP- phylogeny regarding clonal analysis of the isolates. In phylogenetic grouping of the isolates, none of the isolates belonged to phylogroup B2 whereas the phylogroup D was dominant among the 35 *E. coli* isolates, particularly from broilers. Similar findings were reported in Mo et al., (2016) and Agersø et al., (2014) where phylogroup D mostly represented *E. coli* from broilers and broiler meat. Nonetheless, there is no indication of a phylogenetic group being dominant at a level in the broiler production pyramid. Despite the parent flock isolates showing little variation in their phylogenetic grouping, they displayed a higher genetic diversity than the broiler isolates based on the PFGE and WGS data analysis.

Three phylogroup A isolates characterized as ST-752/O123:H40 were highly similar and grouped into one PFGE cluster. *E. coli* ST-752 was discovered in two parent isolates and one broiler isolate, suggesting a possible vertical transmission of this sequence type and horizontal transfer of the *bla*$_{\text{CTX-M-1}}$-IncI1α plasmid between the three isolates. Additionally, *E. coli* ST-752 is known to occur in a variety of hosts and was identified in a healthy Italian poultry flock with ESBL genotype (*bla*$_{\text{SHV-12}}$) (Bortolaia et al., 2011) and a Swiss broiler with *bla*$_{\text{CTX-M-1}}$ (Zurfluh et al., 2014).

Three more STs/serotypes (ST-1251/O176:H48, ST-10/O132:H28, and ST-1638/O53:H18) were identified among the phylogroup A isolates. Of the three, *E. coli* ST-10 in addition to broiler and broiler meat, have been shown to associate with human ExPEC disease such as urinary tract infections in North America and the Netherland (Manges, 2016). *E. coli* ST-1638 appears uncommon in poultry or other reservoirs. However, *E. coli* ST-1638 with a different ESBL genotype (*bla*$_{\text{TEM-52}}$) was discovered from a rock faecal sample in Czech Republic (Jamborova et al., 2015). Similarly, there are few reports about *E. coli* ST-1251 in the broiler production pyramid but this *E. coli* sequence type was identified in chicken meat from Switzerland (Zurfluh et al., 2017). In contrast to *bla*$_{\text{CTX-M-1}}$-IncI1α, the isolate from Switzerland harboured *mcr-1* plasmid conferring resistance to colistin. To the best of my knowledge, this study could be among few where both CTX-M-1 producing *E. coli* ST-1251 and ST-1638 exist in the broiler production pyramid.

The majority of the isolates showed a clonal relationship with the most frequent clone being ST-57 (among phylogroup D) and ST-297 (among phylogroup B1), respectively. In addition, the two isolates from Iceland were characterized as ST-57. The phylogroup D isolates characterized as ST-57 in this study agrees with a previous study from Tunisia where a phylogroup D *E. coli* isolate from broilers belonged to the ST-57 (Sallem et al., 2014). As a result, it is possible one *E. coli* ST-57 acquired the *bla*$_{\text{CTX-M-1}}$-plasmid and further spread third-generation cephalosporin resistance among the broiler flocks in the production pyramid**.** Moreover**,** in Norway, an *E. coli* ST-57 isolate was found in a human urine sample after a comparative study of ESBL-producing *E. coli* from wastewater and recreational water (Jørgensen et al., 2017). Hence, this may points to a clonal transmission of the ST-57 ESBL-producing bacteria from the environment/animals to humans and vice versa. The ST-297 *E. coli* isolates found in this study have also been identified in chickens and other livestock from China (Chan et al., 2014). Regarding the whole genome SNP analysis of the isolates, few SNP differences were seen between the majority of isolates from broilers and parent flocks that were clonally related. Hence, isolates with identical MLST profiles, serotypes, and few SNP

variations highlighted a clonal dissemination of ESBL-producing *E. coli* isolates in the Norwegian broiler production.

4.2 Plasmid typing: conjugation, PBRT, PlasmidFinder and pMLST.

Identifying and characterizing *bla*$_{\text{CTX-M-1}}$-carrying plasmids was one of the main objectives in this study. This was achieved through a combination of conjugation experiments, PBRT, and whole genome- plasmid typing. IncI1α was the dominant plasmid replicon found in the ESBL-producing *E. coli* of the different MLST types. Most of the IncI1α plasmids identified were conjugative and readily transferred the *bla*$_{\text{CTX-M-1}}$ gene to the *E. coli* DH5α recipient strain. As a result, this showed an evidence of horizontal transfer of resistance plasmids between bacteria (Mo et al., 2016). Further subtyping of the IncI1α plasmids revealed most belong to the ST-3 lineage.

The IncI1α/ST-3 plasmid is known to disseminate *bla*$_{\text{CTX-M-1}}$ gene in *E. coli* isolated from the broiler production pyramid and retail chicken meat (Leverstein-van Hall et al., 2011, Zurfluh et al., 2014, Wang et al., 2013, Madec et al., 2015). Furthermore, IncI1α/ST-3 plasmids have been prevalent in ESBL-producing *E. coli* isolated from a recreational water, wastewater and human urine in Norway (Jørgensen et al., 2017). Similar to the study from Jørgensen et al., 2017, a French study discovered highly similar IncI1α/ST-3 plasmids in a ST-48 *E. coli* isolated from drinking water, urinal samples from human, and animals (Madec et al., 2016). In the same manner, studies from Zurfluh et al., 2014 and de Been et al., 2014 discovered highly similar IncI1α/ST-3 plasmids in *E. coli* from the broiler production pyramid. Thus, these reports together with the findings in this study indicate the higher occurrence of *bla*$_{\text{CTX-M-1}}$-IncI1α/ST3 in different reservoirs. On the contrary to plasmid transferability, three IncI1α plasmids were non-conjugative in this study and belonged to the ST-7 lineage.

Similar to IncI1α/ST3, IncI1α/ST7 plasmids have also been detected in two different ESBL-producing *E. coli* MLSTs isolated from recreational water (ST-131) and a urine sample (ST-88) in Norway (Jørgensen et al., 2017). Identical IncI1α/ST-7 plasmids were discovered in cephalosporin resistant *E. coli* isolates from human, poultry, and pig in the Netherlands (de Been et al., 2014). Hence, this suggests that these plasmids can disseminate cephalosporin resistance genes such as *bla*$_{\text{CTX-M-1}}$ in both humans and food-producing animals. In this study, one IncI1α plasmid belonged to ST-42, which to the best of my knowledge is uncommon in *E. coli* from broiler production pyramid or other reservoirs. Nonetheless, an IncI1α/ST-42

plasmid was discovered in a *bla*$_{\text{CTX-M-1}}$-producing *E. coli* ST-359 from a human urine sample in Norway (Silje B. Jørgensen, personal communication, May 3, 2018) (Jørgensen et al., 2017). Last but not least, one major plasmid associated with the *bla*$_{\text{CTX-M-1}}$ gene involves the IncN group, which has been identified in different hosts and reservoirs. A Danish study discovered highly similar *bla*$_{\text{CTX-M-1}}$-carrying IncN plasmids in *E. coli* from pigs and farm workers, suggesting a transmission of IncN plasmids (Moodley and Guardabassi, 2009). In addition to the conjugal transfer genes and *pil* genes, the IncI1α plasmid constructed carried a type IV secretion system and post-segregational killing protein gene, *hok* on the same contig. Bacteria use type IV secretion system to invade and adhere to host gut whereas *hok* is the toxic component of the toxin/antitoxin system, *hok/sok* that maintains resistance plasmids in the bacterial population (UniProt, 2018). Thus, persistence of *bla*$_{\text{CTX-M-1}}$-IncI1α plasmids in the Norwegian broiler production, despite no selection pressure from antimicrobials could have resulted from the *hok* gene

4.3 Phenotypic resistance testing and acquired resistance genes (ResFinder):

The 35 ESBL-producing *E. coli* isolates showed phenotypic resistance to more than one antimicrobial class. Of concern involves the isolates that showed resistance to the highly important antimicrobial agents applied in human medicine such as sulfamethoxazole, tetracycline, trimethoprim, and sulfamethoxazole/trimethoprim (WHO, 2017). Genes coding resistance to these antimicrobial agents, mostly *tetA* (tetracycline resistance) and *sul2* (sulphonamide resistance) were detected on the same transferable *bla*$_{\text{CTX-M-1}}$ IncI1α/ST-3 plasmids and will therefore be co-transferred. Similar to this result, a sequenced *bla*$_{\text{CTX-M-1}}$ IncI1α/ST-3 plasmid (Accession number: SAMN07197432) from a French broiler carried the *tetA* and *sul2* resistance genes (Touzain et al., 2018).

Moreover, three isolates were sensitive to ceftazdime, which is another third-generation cephalosporin. The isolates displaying sensitivity to ceftazidime contradicts with the fact that ESBL-producing bacteria resist third-generation cephalosporins. However, this finding agrees with a report from EFSA (2011) on the notion that some ESBL-producing bacteria are “off and on” resistant to ceftazdime because the CTX-M enzymes have a higher hydrolytic activity against cefotaxime better (Poirel et al., 2002). Thus, in general, cefotaxime is recommended as the ideal substrate to detect ESBL-positive bacteria with *bla*$_{\text{CTX-M}}$ genes. Less hydrolytic activity of the CTX-M enzymes against ceftazidime was observed in the transconjugants. During conjugation, *bla*$_{\text{CTX-M-1}}$ transferred but did not express ceftazidime resistance in almost all the transconjugants. In addition, significant differences regarding

susceptibility testing of ceftazidime might be due to the fact the results were interpreted using epidemiological cut off (ECOFF) values from EUCAST for the donor strains and clinical breakpoint for the transconjugants.

4.4 Virulence genes associated with the different *E. coli* MLSTs/phylogenetic groups:

In the two isolates (2016-40-17381 and 2016-40-23575), the *iroN* and *iss* virulence genes were located on the same contig as $bla_{\text{CTX-M-1}}$ IncI1α plasmids. These virulence in addition to *tsh, iroN,* and *iss* genes are strongly associated with avian pathogenic *E. coli* (APEC) and normally located on ColV plasmids as described in (Maluta et al., 2014, Johnson et al., 2006). Hence, highly transmissible plasmids such as IncI1α can transfer antimicrobial resistance and pathogenicity traits to other bacteria in the same population. It was expected the phylogroup D isolates (ST-57 and ST-641) would detect virulence genes associated with ExPEC infections than the commensal phylogroup A and B1 isolates. However, this was not the case since majority of the *E. coli* isolates regardless of their phylogenetic group contained the *tsh*, *iss* and *iroN* ExPEC virulence genes. Moreover, three phylogroup A isolates detected 10 more virulence genes than the phylogroup D isolates. Thus, to confirm the connection between *E. coli* pathogenicity and the different phylogenetic groupings needs more investigation.

4.5 Isolates with non-transferrable plasmids

The IncI1α plasmids in the three *E. coli* isolates characterized as phylogroup A/ST-752-O123:H40 were unable to transfer $bla_{\text{CTX-M-1}}$ by conjugation. Failure of these isolates to transfer cephalosporin resistance was probably the result of a missing *traC* gene, which is essential for the conjugation process (Kim et al., 1993). Another study also discovered the inability of an IncI1α plasmid to transfer by conjugation resulted from insertion mutations in the *traP* region (Hansen et al., 2016). However, the isolate in Hansen et al., (2016) was an AmpC-producing producing *E. coli* from a healthy parent flock. In cases where resistance plasmids are non-transferrable by conjugation, several studies apply a transformation technique to transfer plasmids into an electro-competent plasmid free (F^-) recipient strains (Bielak et al., 2011, Hammerum et al., 2014).

4.6 Multi-resistance isolates

Certain isolates, mostly phylogroup A, were MDR (n= 9). Of importance were the isolates that showed six resistance phenotypes and detected six acquired resistance genes to different antimicrobial classes. Tetracycline resistance and sulphonamide resistance were

encoded on the *bla*$_{\text{CTX-M-1}}$ plasmid. As a result, a bacterium can resist multiple antimicrobial agents if it receives such resistance plasmids in the population. Another concern involves the co-selection of resistance genes by using an antimicrobial agent. For instance, exposing a bacteria population to third-generation cephalosporin might select *bla*$_{\text{CTX-M-1}}$, which is located on plasmids with multiple antimicrobials resistance genes. Co-selection of resistance genes was demonstrated by (Vien et al., 2012) where beta-lactams used to treat an acute respiratory tract infection in children, selected the plasmid-mediated quinolones resistance genes (PMQR):*qnr*

4.7 Non-typeable isolates PFGE

In this study, the PFGE method was unable to characterize the phylogroup A/ST-1638-O53:H18 isolates. Similar situations have been described in several studies where occasionally, PFGE is ineffective in typing certain bacterial isolates. The study from (Bens et al., 2006) demonstrated that DNA methylation in the genome results in PFGE failure due to the presence of a "5 methylcytosine blocking the restriction enzyme's activity, which involves DNA digestion. Hence, a methylated region in the two isolates' genome could have prevented the PFGE analysis.

4.8 Limitations of study

In general, a limitation of this study was the use of whole genome data to construct large plasmids. Using this approach is time consuming, ineffective and laborious. For instance, primers designed was challenging but failed to determine the full sequences between the two contigs after PCR and sequencing. Compared to other studies, IncI1α plasmid characterization have been based on actual plasmid sequences or the transconjugants sequences (Wang et al., 2014, Zurfluh et al., 2014). Hence, the drawback of using WGS-based data to construct plasmids is that some genes including the mobile genetic elements might not be included in the plasmid's genetic organization. In addition, the use of short sequence reads to reconstruct large plasmids can generates errors in the plasmids' variable regions (Knudsen et al., 2018). Hence, other sequencing platforms such as the Oxford Nanopore or Pacific Biosciences (PacBio) that generate longer reads (40 kb) are recommended for proper plasmid characterization (Knudsen et al., 2018).

Despite Oxford Nanopore and PacBio platforms generating longer reads, it often inserts wrong bases into the nucleotide sequences. Thus, reads from Illumina and long reads from either Oxford Nanopore or PacBio are combined to determine sequences missing from large plasmids (personal communication, Marianne Sunde, NVI, 2018). Finally, the

BioNumerics software version 7.6 used to analyse the PFGE fingerprints failed to include the new fingerprints into the original dendogram.

5 Conclusion

In conclusion, the recent emergence and dissemination of ESBL-producing *E. coli* in the Norwegian broiler production pyramid resulted from both clonal transfer and horizontal transfer of *bla*$_{\text{CTX-M-1}}$-IncI1α/ST3 plasmids. High similarity of the IncI1α plasmids from broilers in this study and both France and Switzerland suggest that the *bla*$_{\text{CTX-M-1}}$-carrying IncI1α plasmids is circulating in the European broiler production. Thus, third-generation cephalosporin use in the broiler production should be restricted to avoid further risk of spreading certain ESBL-producing bacteria clones. In addition, import of breeding animals into Norway should be heavily monitored to avoid introducing ESBL-producing bacteria into Norwegian livestock.

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7. Supplementary

S1 Table. Results from conjugation experiments carried out in broth matings. Plasmids able to transfer the $bla_{CTX\text{-}M\text{-}1}$ to *E. coli* DH5α is indicated with **"+"**, whereas **"-"** indicates negative transfer of $bla_{CTX\text{-}M.\text{-}1}$ carrying plasmids.

	Time		
Mating pair (donor→ recipient)	4 h	6 h	24 h
***2016-40-14263 → *E. coli* DH5α**	– –	– –	– –
2016-40-14272 → *E. coli* DH5α	+		+
2016-40-14497 → *E. coli* DH5α	+		+
2016-40-16262 → *E. coli* DH5α	+		+
2016-40-16344 → *E. coli* DH5α	+		+
2016-40-16990 → *E. coli* DH5α	+		+
2016-40-17074 → *E. coli* DH5α	+		+
2016-40-17091 → *E. coli* DH5α	+		+
2016-40-17093 → *E. coli* DH5α	+		+
2016-40-17200 → *E. coli* DH5α	+		+
2016-40-17381 → *E. coli* DH5α	+		+
2016-40-17437 → *E. coli* DH5α	+		+
2016-40-17495 → *E. coli* DH5α	+		+
2016-40-19016 → *E. coli* DH5α	+		+
2016-40-19138 → *E. coli* DH5α	+		+
2016-40-19148 → *E. coli* DH5α	+		+
2016-40-19583 → *E. coli* DH5α	+		+
2016-40-19738 → *E. coli* DH5α	+		+
2016-40-19970 → *E. coli* DH5α	+		+
2016-40-20426 → *E. coli* DH5α	+		+
2016-40-20481 → *E. coli* DH5α	+		+
***2016-40-20703 → *E. coli* DH5α**	– –	– –	– –
2016-40-21210 → *E. coli* DH5α	+		+
2016-40-21249 → *E. coli* DH5α	+		+
2016-40-21254 → *E. coli* DH5α	+		+
2016-40-21270 → *E. coli* DH5α	+		+

2016-40-22440 → *E. coli* DH5α	+	[redacted]	+
2016-40-22638 → *E. coli* DH5α	+	[redacted]	+
2016-40-23572 → *E. coli* DH5α	+	[redacted]	+
2016-40-23574 → *E. coli* DH5α	+	[redacted]	+
***2016-40-23575 → *E. coli* DH5α**	– –	– –	– –
2016-40-24003 → *E. coli* DH5α	+	[redacted]	+
2016-40-24053 → *E. coli* DH5α	+	[redacted]	+
2016-40-ISL-4500 → *E. coli* DH5α	+	[redacted]	+
2016-40-ISL-5109 → *E. coli* DH5α	+	[redacted]	+

*Plasmids-carrying $bla_{CTX\text{-}M\text{-}1}$ were non-transferable in the *E. coli* isolates 2016-40-14263, 2016-40-20703, and 2016-40-2375 after 4 h and 24 h broth matings. To be certain, conjugation experiments were repeated for 4 h, 6h, and 24 h broth matings. Plasmids were still non-transferable.

S2 Table. Minimal Inhibitory Concentration (mg/L) resistance testing

			MIC (mg/L)													
Isolate ID	**Source**	**Genotype after PCR and WGS**	**SMX (64 mg/L)**	**TEM (2 mg/L)**	**CIP (0.064 mg/L)**	**TET (8 mg/L)**	**MER (0.125 mg/L)**	**AZI (N.A)**	**NAL (16 mg/L)**	**FOT (0.25 mg/L)**	**CHL (16 mg/L)**	**TGC (0.5 mg/L)**	**TAZ (0.5 mg/L)**	**COL (2 mg/L)**	**AMP (8 mg/L)**	**GEN (2 mg/L)**
2016-40-14263	broiler	CTX-M-1	<=8	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	1	<=1	>64	<=0.5
2016-40-14272	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-14497	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-16262	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-16344	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	1	<=1	>64	<=0.5
2016-40-16990	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	1	<=1	>64	1
2016-40-17074	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-17091	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-17093	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	1	<=1	>64	<=0.5
2016-40-17200	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-17381	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-17437	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-17495	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-19016	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	1	<=1	>64	1
2016-40-19138	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-19148	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	1	<=1	>64	<=0.5
2016-40-19583	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-19738	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-19970	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	1	<=1	>64	<=0.5
2016-40-20426	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	<=0.5	<=1	>64	<=0.5
2016-40-20481	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-20703	broiler	CTX-M-1	<=8	<=0.25	<=0.015	<=2	<=0.03	8	<=4	>4	<=8	<=0.25	1	<=1	>64	<=0.5

2016-40-21210	broiler	CTX-M-1	>1024	<=0.25	0.03	<=2	<=0.03	8	<=4	>4	<=8	<=0.25	1	<=1	>64	1
2016-40-21249	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	8	<=4	>4	<=8	<=0.25	1	<=1	>64	<=0.5
2016-40-21254	broiler	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	8	<=4	>4	<=8	<=0.25	2	<=1	>64	1
2016-40-21270	broiler	CTX-M-1	>1024	<=0.25	0.03	<=2	<=0.03	8	<=4	>4	<=8	<=0.25	1	<=1	>64	1
2016-40-22440	parent	CTX-M-1	>1024	<=0.25	0.015	64	<=0.03	8	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-22638	parent	CTX-M-1, TEM-1B	>1024	>36	<=0.015	>64	<=0.03	8	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-23572	parent	CTX-M-1	>1024	<=0.25	<=0.015	64	<=0.03	8	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-23574	parent	CTX-M-1, TEM-1B	>1024	>36	<=0.015	>64	<=0.03	8	<=4	>4	<=8	<=0.25	2	<=1	>64	1
2016-40-23575	parent	CTX-M-1	<=8	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	1	<=1	>64	<=0.5
2016-40-24003	parent	CTX-M-1, TEM-1B	>1024	>36	<=0.015	>64	<=0.03	8	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-24053	parent	CTX-M-1	>1024	<=0.25	0.03	64	<=0.03	8	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
E- coli-ISL-4500	poultry	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	2	<=1	>64	<=0.5
2016-40-ISL-5109	Poultry	CTX-M-1	>1024	<=0.25	<=0.015	<=2	<=0.03	4	<=4	>4	<=8	<=0.25	1	<=1	>64	<=0.5

S3 Table. Overview of the inhibition zone diameters (mm) of the 16 tested antimicrobials against *E. coli* isolates

Isolate ID	**AMP S ≥14 R<14**	**AMC S≥19 R<19**	**SXT S≥14 R<11**	**TE S≥19 S<19**	**CL S≥14 R<14**	**CIP S≥26 R<24**	**N S≥13 R<12**	**CN S≥17 S<14**	**F S≥11 R<11**	**PB S≥10 R<10**	**W S≥18 R<15**	**NA S≥16 R<16**	**CTX S≥20 R<17**	**E**	***P**
2016-40-14263	0	19	28	20	15	38	18	20	18	14	34	22	10	2	0
2016-40-14272	0	19	20	18	10	26	17	20	19	14	27	20	0	0	0
2016-40-14497	0	20	25	25	15	30	18	22	20	16	31	26	0	2	0
2016-40-16262	0	23	24	25	15	32	19	20	21	16	30	26	0	2	0
2016-40-16344	0	20	24	25	15	32	19	22	20	16	29	26	0	2	0
2016-40-16990	0	18	23	25	25	38	20	22	23	17	28	30	0	2	0
2016-40-17074	0	20	24	26	10	35	19	20	21	16	30	30	0	4	0
2016-40-17091	0	20	24	24	10	32	18	21	21	16	28	26	0	0	0
2016-40-17093	0	19	24	24	11	34	19	20	21	16	30	28	0	0	0
2016-40-17200	0	20	25	24	11	26	20	21	22	16	31	26	0	2	0
2016-40-17381	0	20	23	22	9	32	19	21	21	15	31	26	0	4	0
2016-40-17437	0	20	24	24	17	40	19	23	21	17	33	28	0	4	0
2016-40-17495	0	19	26	24	12	34	19	21	20	16	30	28	0	0	0
2016-40-19016	0	21	23	26	14	28	18	21	22	16	30	30	8	6	0
2016-40-19138	0	22	30	26	20	36	19	22	20	16	28	30	0	0	0
2016-40-19148	0	19	21	20	7	36	18	21	22	16	26	30	0	0	0
2016-40-19583	0	20	26	26	15	36	18	21	20	16	30	30	0	0	0
2016-40-19738	0	20	25	26	15	34	19	22	23	16	31	28	0	0	0
2016-40-19970	0	20	19	21	7	36	19	20	21	16	25	28	0	0	0
2016-40-20426	0	19	25	26	18	40	20	20	20	16	31	30	9	0	0

2016-40-20481	0	19	24	24	15	26	19	22	19	15	29	26	0	0	0
2016-40-20703	0	20	34	28	17	40	19	22	20	15	36	30	11	2	0
2016-40-21210	0	20	30	26	20	32	18	20	20	16	28	26	0	0	0
2016-40-21249	0	18	24	26	12	36	20	22	20	19	29	26	0	0	0
2016-40-21254	0	20	30	26	16	38	19	22	22	16	34	30	0	0	0
2016-40-21270	0	20	22	24	15	34	19	20	20	16	29	28	0	0	0
2016-40-22440	0	19	22	0	15	34	19	20	18	15	29	26	0	0	0
2016-40-22638	0	18	0	0	15	36	19	20	22	16	0	26	0	0	0
2016-40-23572	0	20	23	0	15	34	19	21	18	15	29	26	0	0	0
2016-40-23574	0	18	0	0	15	36	18	21	22	16	0	28	0	0	0
2016-40-23575	0	20	32	24	14	40	20	21	21	16	34	30	1	0	0
2016-40-24003	0	17	0	0	16	38	18	20	24	15	0	26	1	0	0
2016-40-24053	0	20	24	0	18	36	20	22	20	16	30	26	0	0	0
2016-40-ISL-4500	0	21	22	24	16	36	19	24	22	15	29	28	0	0	0
2016-40-ISL-5109	0	10	30	26	16	36	19	21	18	15	26	26	0	0	0

AMPC: ampicillin (10 µg) , AMC: amoxicillin/clavulanic acid (30 µg), CL: cefalexin (30 µg), SXT: sulfa/trimethoprim (23.75+1.25 µg), TE: tetracycline (30 µg), PB: polymyxin/colisitin (300 units), NA: nalidixic acid (30 µg), CIP: ciprofloxacin (5 µg), DA: clindamycin (2 µg), CTX: cefotaxime (5 µg), N: neomycin (30 µg), CN: gentamicin (10 µg), F: nitrofurantoin (100 µg), W: trimethoprim (5 µg), E: erythromycin (15 µg), P: penicillin (1 unit).

S5 Table. Assembly data from the 35 whole genome sequenced ESBL-producing *E. coli* isolates

Isolate ID	*Coverage	Number of contigs*	Total sequence length*
ISL_2016-5109	163,4	161	5068709
ISL_2016-4500	163,2	136	5062830
2016-40-24053	159,3	229	4941838
2016-40-24003	156,3	203	4848992
2016-40-23575	172,7	372	5357211
2016-40-23574	157,9	192	4897678
2016-40-23572	158,5	236	4917170
2016-40-22638	159,8	228	4956195
2016-40-22440	158,6	242	4919974
2016-40-21270	169,2	282	5247686
2016-40-21254	164,4	223	5101418
2016-40-21249	159,6	155	4951700
2016-40-21210	169,2	186	5248159
2016-40-20703	171,2	408	5310703
2016-40-20481	164,8	144	5110878
2016-40-20426	165,9	135	5145427
2016-40-19970	170,1	256	5277938
2016-40-19738	164,9	144	5114564
2016-40-19583	162,1	119	5028709
2016-40-19148	169,6	185	5260847

2016-40-19138	169,8	205	5267189
2016-40-19016	169,6	214	5262472
2016-40-17495	167,1	178	5183638
2016-40-17437	162,2	120	5030816
2016-40-17381	171,1	218	5306861
2016-40-17200	164,8	161	5111777
2016-40-17093	163,2	109	5062617
2016-40-17091	163,2	107	5062128
2016-40-17074	163,4	118	5069557
2016-40-16990	163,2	106	5063166
2016-40-16344	162,1	134	5027832
2016-40-16262	166,7	175	5172342
2016-40-14497	162,2	128	5030954
2016-40-14272	162,1	115	5029777
2016-40-14263	170,9	352	5302266

*Contigs ≥150 bp: total number of bases in each contig greater or equal to 150.

S6 Table. SNP variations among the phylogroup D isolates

	2016-40-14272	2016-40-14497	206-40-16262	2016-40-16344	2016-40-16990	2016-40-17074	2016-40-17091	2016-40-17093	2016-40-17200	2016-40-17437	2016-40-17495	2016-40-19583	2016-40-19738	2016-40-20426	2016-40-20481	2016-ISL-4500	2016-ISL-5109
2016-40-14272	0	31	43	31	33	31	25	33	38	38	30	53		**34264**	50	52	54
2016-40-14497	31	0	54	38	36	28	30	30	47	27	43	56	38	**34259**	55	59	55
2016-40-16262	43	54	0	52	42	42	48	42	28	55	45	56	40	**34263**	44	46	69
2016-40-16344	31	38	52	0	42	26	34	47	49	33	41	40	38	**34259**	44	55	51
2016-40-16990	33	36	42	42	0	30	26	47	41	47	51	40	36	**34259**	35	39	59
2016-40-17074	31	28	42	26	30	0	24	16	39	33	33	30	36	**34253**	47	45	45
2016-40-17091	25	30	48	34	26	24	0	24	51	33	41	30	32	**34257**	41	45	51
2016-40-17093	33	30	42	30	34	16	24	0	43	31	35	34	36	**34267**	47	47	47
2016-40-17200	38	47	28	49	41	39	51	43	0	54	40	53	39	**34267**	50	46	68
2016-40-17437	38	27	55	33	47	33	33	31	54	0	46	29	41	**34262**	58	58	56
2016-40-17495	30	43	45	41	51	33	41	35	40	46	0	43	37	**34260**	64	60	52
2016-40-19583	33	16	56	40	40	30	30	34	53	29	43	0	40	**34267**	55	59	57
2016-40-19738	23	38	40	38	40	36	32	36	39	41	37	40	0	**34269**	53	59	59
2016-40-20426	**34264**	**34259**	**34263**	**34259**	**34259**	**34253**	**34257**	**34252**	**34267**	**34262**	**34260**	**34267**	**34269**	**0**	**34262**	**34260**	**34265**
2016-40-20481	52	55	44	55	35	47	41	47	50	58	64	55	53	**34262**	0	44	74
ISL_2016-4500	52	59	46	55	39	45	45	47	46	58	60	59	59	**34260**	44	0	44
ISL_2016-5109	54	55	69	51	59	45	51	47	68	56	52	57	59	**34265**	74	44	0

S7 Table. SNP variations among the phylogroup B1 isolates

	2016-40-17381	2016-40-19016	2016-40-19138	2016-40-19148	2016-40-19970	2016-40-21210	**2016-40-21249**	2016-40-21270
2016-40-17381	0	46	46	36	39	51	**13464**	74
2016-40-19016	46	0	44	44	51	54	**13473**	84
2016-40-19138	34	44	0	30	23	59	**13462**	58
2016-40-19148	36	44	30	0	31	55	**13466**	60
2016-40-19970	39	51	23	31	0	93	**13461**	57
2016-40-21210	51	54	59	55	60	0	**13477**	93
2016-40-21249	**13464**	**13473**	**13462**	**13466**	**13461**	**60**	**0**	**13486**
2016-40-21270	74	84	58	60	57	93	**13486**	0

S8 Table. SNP variations among the phylogroup A isolates

	2016-40-14263	2016-40-20703	2016-40-21254	2016-40-22440	2016-40-22638	2016-40-23572	2016-40-23574	2016-40-23575	2016-40-24003	2016-40-24053
2016-40-14263	0	**24**	4682	10548	12460	10553	12461	**34**	1058	10556
2016-40-20703	**24**	0	4680	10546	12460	10551	12461	**32**	12058	10554
2016-40-21254	4682	4680	0	10128	12224	10135	12225	4684	11487	10138
2016-40-22440	10548	10546	10128	0	13375	**21**	13379	10548	8994	**20**
2016-40-22638	12460	12460	12224	13375	0	13380	**31**	12454	13824	13379
2016-40-23572	10553	10551	10135	**21**	13380	0	13384	10545	9003	**35**
2016-40-23574	12461	12461	12225	13379	**31**	13384	0	12457	13824	13385
2016-40-23575	34	**32**	4684	10548	12454	10545	12457	0	12054	10552
2016-40-24003	12058	12058	11487	8994	13824	9003	13829	12054	0	9002
2016-40-24053	10556	10554	10138	**20**	13379	**35**	13385	10552	9002	0

S9 Table. Overview of whole genome characterization of the 35 ESBL-producing *E. coli* isolates. MLST, acquired resistance genes, virulence genes, serotype, plasmids, and pMLST of each isolate is shown.

Isolate ID	MLST	Serotype	ResFinder	VirulenceFinder	Plasmid replicon	Unschemed plasmid	pMLST
2016-40-14263	ST-752	O123/O186:H40	*blaCTX-M-1,strA,strB*	*astA,cif,eae,espA,espB,gad,iroN,iss,mchF,nleB,tccP,tir,tsh*	IncFII,IncI1α,IncFIB(AP001918)	ColRNAI	IncI1α [ST-7]
2016-40-14272	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFII,IncI1α,IncFIB(AP001918)		IncI1α [ST-3]
2016-40-14497	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFII(pRSB107),IncI1α,IncFIB(AP001918),IncHI1B(CIT)	p0111,IncX4,ColRNAI	IncI1α [ST-3]
2016-40-16262	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncHI1B(CIT),IncFII,IncI1α,IncFIB(AP001918)	Col8282,p0111,Col(MG828),ColpVC,ColRNAI	IncI1α [Unknown ST]
2016-40-16344	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFIB(AP001918),IncI1α,IncFIC(FII),IncFII(pCoo)	IncI2,Col(KPHS6)	IncI1α [ST-3]
2016-	ST-	O140:H	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFII,IncI1α,IncFIB(AP0019	ColpVC	IncI1α

40-16990	57	25			18)		[ST-3]
2016-40-17074	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFII(pRSB107),IncI1α,IncFIB(AP001918),IncHI1B(CIT)	p0111, ColRNAI	IncI1α [ST-3]
2016-40-17091	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFII,IncI1α,IncFIB(AP001918)	ColpVC	IncI1α [ST-3]
2016-40-17093	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFII(pRSB107),IncI1α,IncFIB(AP001918),IncHI1B(CIT)	p0111,ColRNAI	IncI1α [ST-3]
2016-40-17200	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFIC(FII),IncFIA,IncI1α,IncFIB(AP001918)	IncB/O/K/Z,Col(MG828),ColRNAI	IncI1α [ST-3]
2016-40-17381	ST-297	O45:H8	*blaCTX-M-1,sul2*	*gad,iha,iroN,iss,lpfA,mchB,mchC,mchF,tsh*	IncI1α		IncI1α [Unknown ST]
2016-40-17437	ST-57	O140:H25	*blaCTX-M-1,sul2*	*gad,iroN,iss*	IncFII,IncI1α,IncFIB(AP001918)	IncX1,ColpVC,Col(MG828)	IncI1α [ST-3]
2016-40-17495	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFIC(FII),IncFIA,IncI1α,IncFIB(AP001918)	Col(MG828),ColRNAI, IncB/O/K/Z	IncI1α [ST-3]
2016-40-19016	ST-297	O45:H8	*blaCTX-M-1,sul2*	*gad,iha,iroN,iss,lpfA,mchB,mchC,mchF,tsh*	IncFII,IncI1α,IncFIB(AP001918)	Col(KPHS6)	IncI1α [Unknown ST]
2016-40-19138	ST-297	O45:H8	*blaCTX-M-1,sul2*	*gad,iha,iroN,iss,lpfA,mchB,mchC,mchF,tsh*	IncI1α,IncFIB(AP001918)	IncB/O/K/Z,ColpVC,ColRNAI	IncI1α [ST-3]
2016-40-19148	ST-297	O45:H8	*blaCTX-M-1,sul2*	*gad,iha,iroN,iss,lpfA,mchB,mchC,mchF,tsh*	IncFIA(HI1),IncFII(29),IncI1α,IncFIB(pB171), IncFII(pHN7A8)	Col(MGD2),ColRNAI, ColpVC,IncX4,Col(MG828)	IncI1α [ST-3]
2016-40-19583	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFIB(AP001918),IncFIA,IncI1α,IncFIC(FII)	IncB/O/K/Z,ColRNAI, Col(MG828)	IncI1α [ST-3]
2016-40-19738	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncI1α,IncFIB(AP001918)	ColpVC,IncB/O/K/Z	IncI1α [ST-3]
2016-40-19970	ST-297	O45:H8	*blaCTX-M-1,sul2*	*gad,iha,iroN,iss,lpfA,mchB,mchC,mchF,tsh*	IncI1α,IncFIB(AP001918)	ColpVC	IncI1α [Unknown ST]
2016-40-20426	ST-57	O37:H40	*blaCTX-M-1,sul2*	*gad,lpfA*	IncFIB(AP001918),IncFIA,IncI1α,IncFIC(FII)	IncB/O/K/Z,ColRNAI, Col(MG828)	IncI1α [ST-3]
2016-40-20481	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFIB(AP001918),IncFIA,IncI1α,IncFIC(FII)	IncB/O/K/Z,ColRNAI, Col(MG828)	IncI1α [ST-42]
2016-40-20703	ST-752	O123:H40	*blaCTX-M-1, strA,strB*	*astA,cif,eae,espA,espB,gad,iroN,iss,mchF,nleB,tccP,tir,tsh*	IncFIB(AP001918), IncFIA,IncI1,IncFIC(FII)	Col(MG828),ColRNAI, IncB/O/K/Z	IncI1α [ST-7]
2016-40-21210	ST-297	O45:H8	*blaCTX-M-1, sul2*	*gad,iha,iroN,iss,lpfA,mchB,mchC,mchF,tsh*	IncFII, IncI1α, IncFIB(AP001918)	ColpVC,IncB/O/K/Z	IncI1α [Unknown ST]
2016-40-21249	ST-162	O8:H28	*blaCTX-M-1,sul2*	*gad,iroN,iss,lpfA,mchF*	IncI1α, IncFIB(AP001918)	ColpVC	IncI1α [ST-3]
2016-40-21254	ST-10	O132:H28	*blaCTX-M-1,sul2*	*astA,gad,iss*	IncFIB(AP001918), IncFIA,IncI1,IncFIC(FII)	ColRNAI,IncX1,IncB/O/K/Z,Col156,Col(MG828)	IncI1α [ST-3]
2016-40-21270	ST-297	O45:H8	*blaCTX-M-1,sul2*	*gad,iha,iroN,iss,lpfA,mchB,mchC,mchF,tsh*	IncI1α, IncFIB(AP001918)	ColpVC,IncB/O/K/Z	IncI1α [ST-3]
2016-40-22440	ST-48	O8:H11	*blaCTX-M-1,sul2,tet(A)*	*cma,gad,iroN,iss*	IncI1α, IncFIB(AP001918)		IncI1α [ST-3]
2016-40-22638	ST-1638	O53:H18	*aadA1,blaCTX-M-1,blaTEM-1B-like,dfrA1,sul1,sul2,tet(A)*	*cma,gad,iha,iroN,iss,mchB,mchC,mchF*	IncI1α, IncFIB(AP001918)	Col(MG828)	IncI1α [ST-3]
2016-40-	ST-48	O8:H11	*blaCTX-M-1,sul2,tet(A)*	*cma,gad,iroN,iss*	IncI1α, IncFIB(AP001918)	ColRNAI	IncI1α [ST-3]

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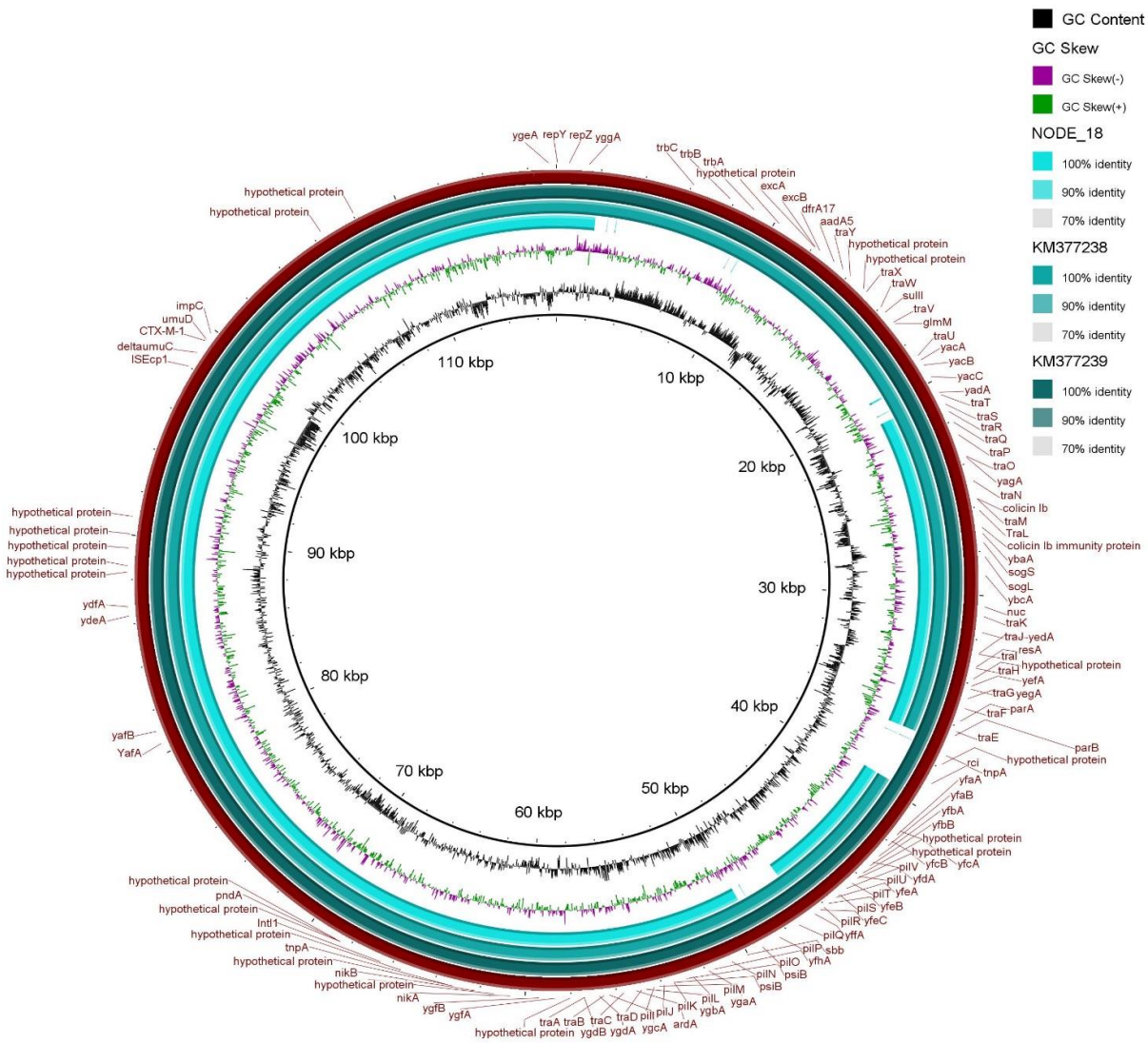
2016-40-23574	ST-1638	O53:H18	*aadA1,blaCTX-M-1,blaTEM-1B,dfrA1,sul1,sul2,tet(A)*	*cma,gad,iha,iroN,iss,mchB,mchC,mchF*	IncI1α, IncFIB(AP001918)		IncI1α [ST-3]
2016-40-23575	ST-752	O123:H40	*blaCTX-M-1,strA,strB*	*astA,cif,eae,espA,espB,gad,iroN,iss,mchF,nleB,tccP,tir,tsh*	IncI1α, IncFIB(AP001918)	ColpVC	IncI1α [ST-7]
2016-40-24003	ST-1251	O176:H48	*aadA1,blaCTX-M-1,blaTEM-1B,dfrA1,sul1,sul2,tet(A)*	*cma,gad,iroN,iss*	IncI1α, IncFIB(AP001918)	IncI2, ColpVC, IncB/O/K/Z	IncI1α [ST-3]
2016-40-24053	ST-48	O8:H11	*blaCTX-M-1,sul2,tet(A)*	*cma,gad,iroN,iss*	IncI1α, IncFIB(AP001918)	ColpVC,Col(MG828)	IncI1α [ST-3]
ISL_2016-4500	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncI1α, IncFIB(AP001918)	Col(MG828)	IncI1α [ST-3]
ISL_2016-5109	ST-57	O140:H25	*blaCTX-M-1,sul2*	*cma,gad,iroN,iss*	IncFIB(AP001918), IncI1α, IncFIC(FII),IncFII(pCoo)	Col(KPHS6)	IncI1α [ST-3]



S1 Figure. Full annotation of the Swiss reference IncI1α/ST3 plasmids:KM377238 and KM377239 (in red) from broilers used for the IncI1α plasmid construction in this study (NODE_18). Figure constructed by Jannice Schau Slettemås. Figure includes alignment of the 94.9 kB contig with *bla*$_{\text{CTX-M-1}}$-carrying IncII/ST-3 plasmid.

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