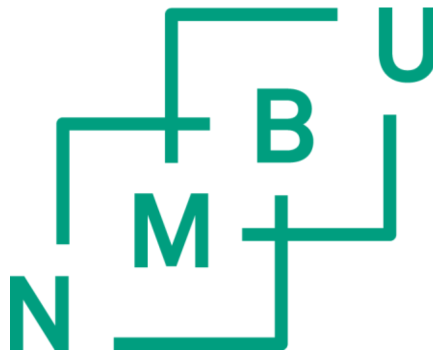
Cephalosporin-resistant *Escherichia coli* in the Norwegian broiler production pyramid

-genetic characterization and determination of risk factors

Philosophiae Doctor (PhD) Thesis

Solveig Sølverød Mo



Department of Production Animal Clinical Sciences

Faculty of Veterinary Medicine and Biosciences

Norwegian University of Life Sciences

Adamstuen 2016

Veterinærinstituttet
Norwegian Veterinary Institute

Thesis number 2016:63

ISSN 1894-6402

ISBN 978-82-575-1975-9

“A scientist in his laboratory is not a mere technician; he is also a child confronting natural phenomena that impress him as though they were fairytales”

Marie Curie (1867-1934)

Table of contents

1. Acknowledgements ... 7
2. List of abbreviations ... 9
3. Summary ... 11
4. Sammendrag (summary in Norwegian) ... 13
5. List of papers ... 15
6.1 The Norwegian broiler production pyramid ... 17
6.2 Antimicrobial resistance ... 20
6.2.1. Monitoring of antimicrobial resistance ... 24
6.2.2. Cephalosporin resistance in *E. coli* ... 25
6.3 Epidemiology of cephalosporin-resistant *E. coli* ... 28
6.3.1. Epidemiology in the broiler production pyramid ... 29
6.3.2. Epidemiology in humans ... 34
6.4 Public health aspects ... 36
6.5 Knowledge gaps ... 39
7. Aims of the study ... 41
8. Summary of materials and methods ... 43
8.1. Materials ... 43
8.1.1. Study population ... 43
8.1.2. Samples collected from parent- and broiler flocks ... 43
8.1.3. Samples collected in previous studies ... 44
8.1.4. Collection of data for identification of risk factors (Paper II) ... 44
8.2. Laboratory methods ... 45
8.2.1. Detection and identification of cephalosporin-resistant *E. coli* ... 45
8.2.2. Antimicrobial susceptibility testing ... 45
8.2.3. Characterization of cephalosporin-resistant *E. coli* ... 46
8.2.4. Characterization of bla_{CMY-2} carrying plasmids ... 46
8.3. Statistical methods ... 47
8.3.1. Study design ... 48
9. Summary of papers ... 49
9.1. Paper I. ... 49
9.2. Paper II. ... 49
9.3. Paper III. ... 49

9.4. Paper IV. 50
10. Discussion 51
10.1. Material and methodological considerations 51
10.1.1. Materials and methods applied in the laboratory 51
10.1.2. Materials and methods used in the epidemiological study 56
10.2 Results and general discussion 61
10.2.1. Occurrence of cephalosporin-resistant *E. coli* in the Norwegian broiler production 61
10.2.2. Risk factors for occurrence of cephalosporin-resistant *E. coli* in Norwegian broilers 62
10.2.3. Characterization of cephalosporin-resistant *E. coli* 64
10.2.4. Persistence of cephalosporin-resistant *E. coli* in the Norwegian broiler production 67
10.2.5. Preventive measures 70
11. Main conclusions 71
12. Future perspectives 73
13. Errata 75
14. References 77
14. Enclosed papers I-IV 99

1. Acknowledgements

The work presented in this thesis was carried out at Section for bacteriology-aquatic and terrestrial and Section for epidemiology at the Norwegian Veterinary Institute (NVI) in Oslo during 2013-2016. The project was funded by the Research Council of Norway through the Research Levy on Agricultural products, “Matfondavtalen” (grant number 225165/E40), with the Norwegian Meat and Poultry Research Centre (Animalia) as an industrial partner. I would like to acknowledge the funding parties for making this project possible. Also, I am grateful to the Norwegian University of Life Sciences (NMBU) for admitting me to the PhD programme.

I wish to express my sincere gratitude to my main supervisor, Marianne Sunde, for her enthusiasm, patience, and confident guidance and support. Thank you for always being available and for sharing your immense knowledge on antimicrobial resistance! Furthermore, I wish to thank my co-supervisor Madelaine Norström for excellent guidance through the world of epidemiology, and for always having a good story lurking. I would also like to thank my co-supervisors Ane Nødtvedt and Even Heir for good advice and feedback along the way.

I want to thank all my wonderful colleagues at the NVI for providing a good working environment and for supporting me through the PhD process. Thank you, Jannice Schau Slettemeås for sharing your knowledge and skills, and for all the laughs we have shared in the “Resistance corner”. A great thank you goes to Camilla Sekse for patiently sharing office with me the last years. I greatly appreciate our work-space and all our scientific and non-scientific discussions. Aina Steihaug Barstad, Bjørg Kvitle and Anette Wold Åsli are acknowledged for providing excellent technical assistance, and Attila Tarpai is acknowledged for making nice maps.

I am very grateful to all producers contributing with samples from their flocks to enable the risk factor study. Also, Atle Løvland is acknowledged for sharing his knowledge on the Norwegian broiler production.

Everyone at department of Food safety and quality at Nofima are acknowledged for welcoming me into their lab and working environment. Special thanks go to Hanna Ilag for excellent cooperation.

Also, I would like to thank all co-authors for their invaluable contributions to the papers presented in this thesis.

The staff at NMBU University library Campus Adamstuen is greatly acknowledged for always being helpful.

Finally, I would like to express my sincere gratitude to my family and friends for all your love, support and encouragement and to my dear Trond Stian for making me so incredibly happy.

Oslo, June 2016

Solveig Sølverød Mo

2. List of abbreviations

AMR: Antimicrobial resistance

BPC: British Poultry Council

DNA: Deoxyribonucleic acid

ECOFF: Epidemiological cut-off value

EFSA: European Food Safety Authority

ESBL: Extended-spectrum beta-lactamase

ESC: Extended-spectrum cephalosporins

EUCAST: European Committee on Antimicrobial Susceptibility Testing

ExPEC: Extra-intestinal pathogenic *Escherichia coli*

Inc: Incompatibility

MALDI-TOF: Matrix-assisted laser desorption/ionization-time of flight

MIC: Minimum inhibitory concentration

MLVA: Multiple-locus variable number tandem repeat analysis

NORM: The Norwegian monitoring programme for antimicrobial resistance in bacteria from humans

NORM-VET: The Norwegian monitoring programme for antimicrobial resistance in bacteria from food, feed and animals

NVI: Norwegian Veterinary Institute

OR: Odds ratio

pAmpC: Plasmid-mediated AmpC

PCR: Polymerase chain reaction

PFGE: Pulsed-field gel electrophoresis

SNP: Single nucleotide polymorphism

ST: Multilocus sequence type

TA: Toxin-antitoxin

T/R: Transconjugant/recipient ratio

TSA: Tryptone soya agar

WGS: Whole genome sequencing

WHO: World Health Organization

3. Summary

Antimicrobial resistance is defined as one of our times' main public health challenges. The prevalence of *Escherichia coli* displaying resistance against extended-spectrum cephalosporins, a class of antimicrobials that are critically important for treatment of human infections, has increased in both human and veterinary medicine during the last decade. In Norway, the use of antimicrobial agents for food producing animals is low, and in the broiler production almost non-existent. Therefore, it was surprising to find a relatively high occurrence of cephalosporin-resistant *E. coli* in broilers following implementation of a selective detection method of such resistance as part of the Norwegian monitoring programme for antimicrobial resistance in bacteria from food, feed and animals (NORM-VET) from 2011. The aim of the present study was to increase knowledge on cephalosporin-resistant *E. coli* in the Norwegian broiler production in order to provide scientifically based advice to the authorities and the broiler industry regarding preventive measures related to introduction, persistence and spread of cephalosporin-resistant *E. coli*.

Data collected in 2011 and 2012 were compiled and analysed. An epidemiological study was performed in order to determine risk factors related to the occurrence of cephalosporin-resistant *E. coli* in Norwegian broiler flocks. Furthermore, thorough genetic characterization of cephalosporin-resistant isolates was performed in order to determine whether successful clones and/or resistance plasmids were present in the broiler production. Further, transfer experiments using relevant recipient strains and two donor strains harbouring well characterized plasmids with cephalosporin-encoding genes were carried out under different conditions.

All cephalosporin-resistant *E. coli* included in the present study displayed an AmpC-phenotype, and the vast majority carried the $bla_{\text{CMY-2}}$ gene. AmpC-producing *E. coli* have been detected at all levels of the Norwegian broiler production pyramid, and are present in all areas of Norway with broiler production. The risk for occurrence of cephalosporin-resistant *E. coli* was associated with the status of the previous flock in the broiler house, number of parent flocks supplying the broiler flock with day-old chickens, routines for disinfection of floor between production cycles, and transport personnel entering the room where the broilers are raised. The results indicated that a high level of biosecurity, including implementation of thorough cleaning and disinfection routines, will aid in minimizing the odds of cephalosporin-resistant *E. coli* occurring in broiler flocks. Characterization of

isolates revealed that cephalosporin-resistance was generally mediated by bla_{CMY-2} on IncI1 or IncK plasmids. The majority of the plasmids were self-transmissible. Furthermore, a large cluster of closely related isolates grouping into multilocus sequence type (ST)38, and a cluster grouping into ST1158 were identified. These isolates harboured bla_{CMY-2} on IncK plasmids. Interestingly, *E. coli* ST38 and IncK and IncI1 plasmids highly similar to plasmids found in Norway have been reported from other European countries, indicating that some successful clones and plasmids are circulating in the European broiler production. In addition, plasmid stability systems were identified in both IncK (pNVI1292/IncK) and IncI1 plasmids (pNVI2798/IncI1). The presence of such systems might partly explain the persistence of cephalosporin-resistant *E. coli* in the Norwegian broiler production despite the lack of antimicrobial selection pressure. Transfer experiments revealed the ability of IncK and IncI1 plasmids to self-transfer to other hosts within biofilm. Furthermore, self-transfer of pNVI1292/IncK into *Serratia* spp. was observed. Bacteria in biofilm have an increased ability to survive cleaning and disinfection. Environmental bacteria can also have intrinsic properties making them more tolerant to some disinfectants. Therefore, the results might indicate that biofilms and environmental bacteria can act as reservoirs for cephalosporin-resistant *E. coli* and plasmids encoding cephalosporin-resistance in the broiler production.

Overall, the results presented in this thesis provide important knowledge regarding the molecular epidemiology of cephalosporin-resistant *E. coli* in the Norwegian broiler production. Furthermore, the identification of risk factors for occurrence of cephalosporin-resistant *E. coli* in broiler flocks can contribute to implementation of preventive measures aimed at minimization of persistence of these bacteria.

4. Sammendrag (summary in Norwegian)

Antibiotikaresistens er definert som en av de største helseutfordringene verden står overfor. Forekomsten av *Escherichia coli* som uttrykker resistens mot bredspektrede cefalosporiner, en gruppe antibiotika som er kritisk viktige for behandling av infeksjoner hos mennesker, har økt i både human- og veterinærmedisinen det siste tiåret. I Norge er bruken av antibiotika til husdyr lav, og forbruket i slaktekyllingproduksjonen er minimalt. Derfor var det overraskende å finne en relativt høy forekomst av cefalosporin-resistente *E. coli* i slaktekylling etter innføring av en selektiv metode for deteksjon av slik resistens i det norske overvåkningsprogrammet for antibiotikaresistens i mat, fôr og dyr (NORM-VET) fra 2011. Målsettingen for denne studien har vært å øke kunnskapen om cefalosporin-resistente *E. coli* i den norske slaktekyllingproduksjonen for å kunne gi kunnskapsbaserte anbefalinger til myndigheter og fjørfenæringen om forebyggende tiltak mot introduksjon, persistens og spredning av cefalosporin-resistente *E. coli*.

Data innsamlet i 2011 og 2012 ble ferdigstilt og analysert. En epidemiologisk studie ble utført for å identifisere risikofaktorer for forekomst av cefalosporin-resistente *E. coli* i norske slaktekyllingflokker. Videre ble cefalosporin-resistente isolater grundig karakterisert for å undersøke om vellykkede kloner og/eller resistensplasmider forekom i slaktekyllingproduksjonen. I tillegg ble det gjort overføringsforsøk med resistensplasmider ved forskjellige forutsetninger. To donorer inneholdende godt karakteriserte plasmider med gener som koder for cefalosporin resistens, og relevante mottakerstammer ble brukt.

Alle cefalosporin-resistente *E. coli* inkludert i denne studien hadde en AmpC-fenotype, og de fleste inneholdt genet bla_{CMY-2}. AmpC-produserende *E. coli* ble påvist på alle nivåer av den norske slaktekyllingproduksjonen og var spredt i alle områder i Norge med slaktekyllingproduksjon. Risiko for tilstedeværelse av cefalosporin-resistente *E. coli* var assosiert med status på foregående flokk i samme kyllinghus, antall foreldreflokker som leverte daggamle kyllinger til slaktekyllingflokken, rutiner for desinfeksjon av gulv mellom innsett, og om transportpersonell gikk inn i dyrerommet i kyllinghuset. Resultatene indikerte at god biosikkerhet, inkludert implementering av grundige rengjørings- og desinfeksjonsrutiner, kan bidra til å minimere risikoen for forekomst av cefalosporin-resistente *E. coli* i slaktekyllingflokker. Karakterisering av isolatene viste at cefalosporin-resistensen generelt ble mediert av IncK eller IncI1 plasmider med bla_{CMY-2}. Majoriteten av plasmidene var overførbare. I tillegg viste studiene tilstedeværelse av en stor gruppe av nært

beslektede isolater som tilhørte multilokus sekvenstype (ST)38, og en gruppe som tilhørte ST1158. Disse isolatene inneholdt IncK plasmider. *E. coli* ST38, samt IncK og IncI1 plasmider som er svært like plasmidene funnet i Norge, har også blitt funnet i andre europeiske land. Dette er en indikasjon på at enkelte vellykkede kloner og plasmider sirkulerer i den europeiske slaktekyllingproduksjonen. I nukleotidsekvensene til ett IncK plasmid (pNVI1292/IncK) og ett IncI1 plasmid (pNVI2798/IncI1) ble det funnet gener som koder for plasmidstabilitets-systemer. Tilstedeværelsen av slike systemer kan være en av grunnene til at cefalosporin-resistente *E. coli* kan persistere i den norske slaktekyllingproduksjonen til tross for mangelen på seleksjonspress fra antibiotikabruk. Overføringsforsøk viste at plasmider med $bla_{\text{CMY-2}}$ kunne overføres til andre bakterieverter i biofilm. I tillegg ble overføring av IncK plasmidet (pNVI1292/IncK) til *Serratia* spp. observert. Bakterier i biofilm har generelt økt evne til å overleve renhold og desinfeksjon. Miljøbakterier kan også ha iboende egenskaper som gjør dem mer tolerante overfor visse typer desinfeksjonsmidler. Resultatene kan derfor tyde på at biofilm og miljøbakterier kan fungere som et reservoar for cefalosporin-resistente *E. coli* og plasmider som koder for cefalosporin-resistens i kyllingproduksjonen.

Resultatene presentert i denne studien gir viktig kunnskap om den molekylære epidemiologien til cefalosporin-resistente *E. coli* i den norske slaktekyllingproduksjonen. I tillegg vil kunnskap om hvilke risikofaktorer som påvirker forekomst av cefalosporin-resistente *E. coli* i slaktekyllingflokker kunne bidra til implementering av forebyggende tiltak for å minimere forekomsten av disse bakteriene.

5. List of papers

Paper I

Mo, S.S., Norström, M., Slettemeås, J.S., Løvland, A., Urdahl, A.M., Sunde, M. 2014. Emergence of AmpC-producing *Escherichia coli* in the broiler production chain in a country with a low antimicrobial usage profile. Veterinary Microbiology 171: 315-320.

Paper II

Mo, S.S., Kristoffersen, A.B., Sunde, M., Nødtvedt, A., Norström, M. 2016. Risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in Norwegian broiler flocks. Preventive Veterinary Medicine 130, 112-118.

Paper III

Mo, S.S., Slettemeås, J.S., Berg, E.S., Norström, M., Sunde, M. 2016. Plasmid and host strain characteristics of *Escherichia coli* resistant to extended-spectrum cephalosporins in the Norwegian broiler production. PLoS ONE 11: e0154019.

Paper IV

Mo, S.S., Sunde, M., Ilag, H.K., Langsrud, S., Heir, E. 2016. Transfer potential of plasmids encoding extended-spectrum cephalosporin resistance in *Escherichia coli* from poultry into different *Enterobacteriaceae* under various conditions. Manuscript in preparation.

6. Introduction

6.1 The Norwegian broiler production pyramid

The broiler production follows a pyramidal structure with purebred animals at the top, followed by breeding animals in the middle and broilers producing retail chicken meat at the bottom (Figure 1). Until 1994, the broiler production in Norway was self-supplied with breeding animals. However, after allowing import of breeding animals from abroad (EFTA, 2014), maintenance of the breeding stock was too costly (NL, 2014), and the Norwegian breeding animals were outcompeted. Nowadays, the broiler production in Norway depends on import of hatching eggs from grandparent animals in Sweden. However, a single grandparent flock was imported to Norway in 2011 due to an increased demand for chicken meat in the Norwegian market (KOORIMP, 2012). The majority of grandparent animals originate from Scotland, while a minor proportion originate from Germany. Only parent animals and broiler chickens are produced in Norway (Figure 1).

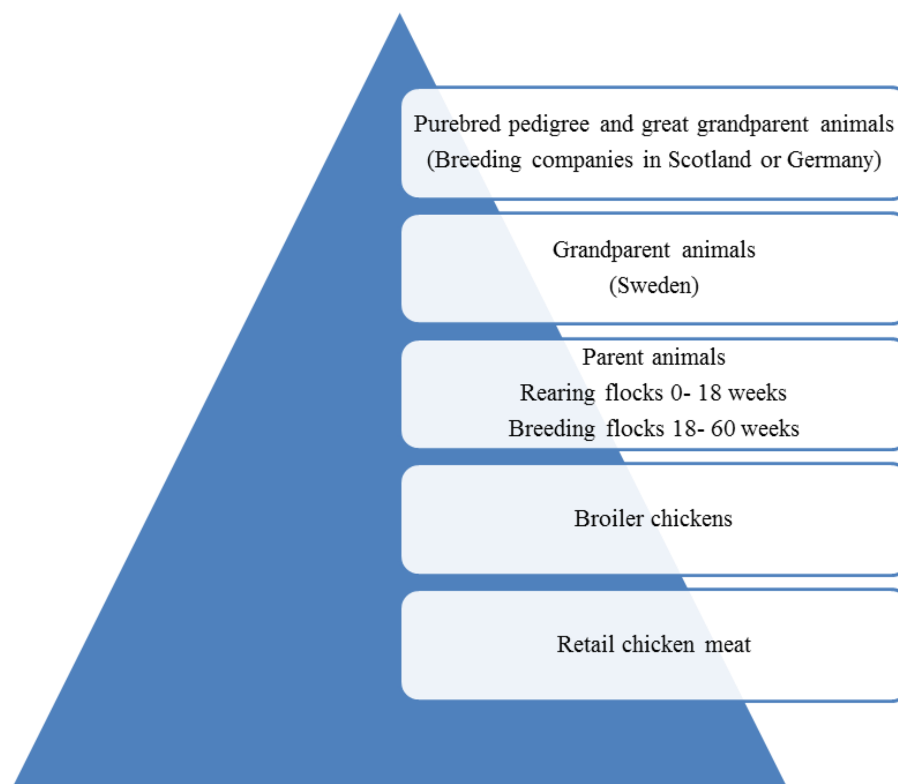


Figure 1: The complete broiler production pyramid. Only parent animals and broiler chickens are reared in Norway. Illustration: S. S. Mo.

Eggs imported from Sweden are hatched to parent animals, and sent to rearing farms where they are held until they are approximately 18 weeks old. Then, the flock is sent to a production farm, where they start laying eggs at around 20 weeks of age. Eggs from breeding parent flocks are sent to a broiler hatchery, and day old chickens are further supplied to broiler producers. Broiler chickens are raised to approximately 28-32 days of age before they are slaughtered (Nortura, 2015). An overview of the Norwegian broiler production is shown in Figure 2.

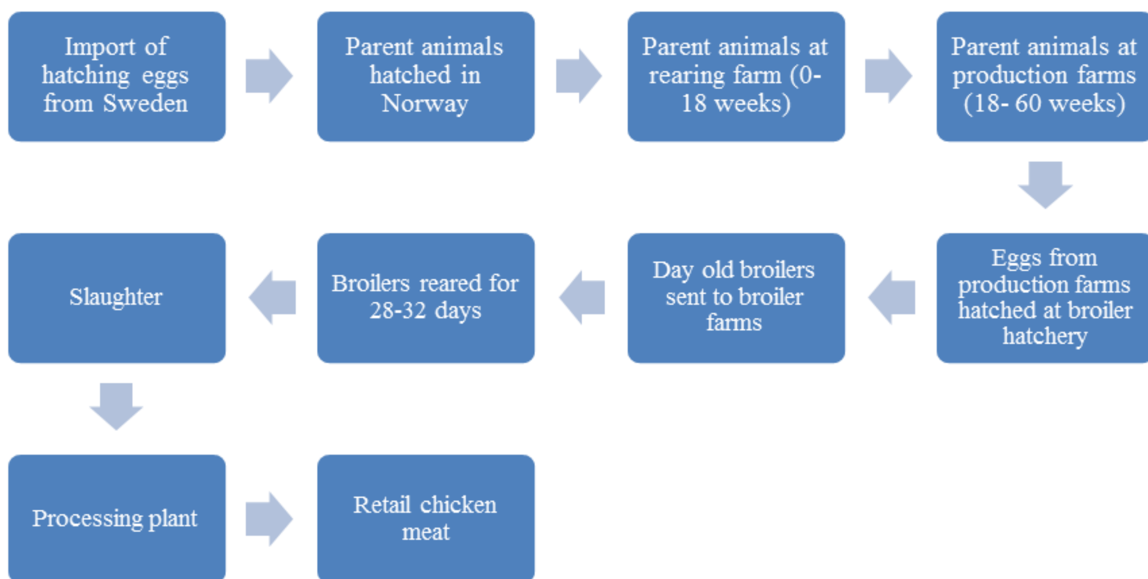


Figure 2: Step- by- step presentation of the Norwegian broiler production. Illustration: S. S. Mo.

In Norway, broiler chickens are raised in free-range houses with a standard animal density of 25 kg/m^2. However, the animal density can be increased to 36 kg/m^2 if the producer is committed to an animal welfare programme and a range of requirements are fulfilled (Lovdata, 2001). Since 2015, the concession limit for each broiler producer is 280 000 animals per year (Lovdata, 2004, 2014). Broiler production is a growing industry in Norway. From 2007 to 2016, the number of broilers reared annually has increased by approximately 36 %. In 2015, more than 63.3 million broilers were reared in Norway, in approximately 650 broiler farms (SN, 2016). The Norwegian broiler production is large enough to supply the Norwegian market. Thus, most commercial chicken meat available at retail in Norway is domestically produced. A limited amount of chicken meat is imported to Norway yearly (NAA, 2016), of which most is halal chicken (Personal communication, Julie Kilde Mjelva, Norwegian Agriculture Agency). The regions with highest production of broiler chickens in Norway are Hedmark, Østfold, Rogaland and Trøndelag (Figure 3).

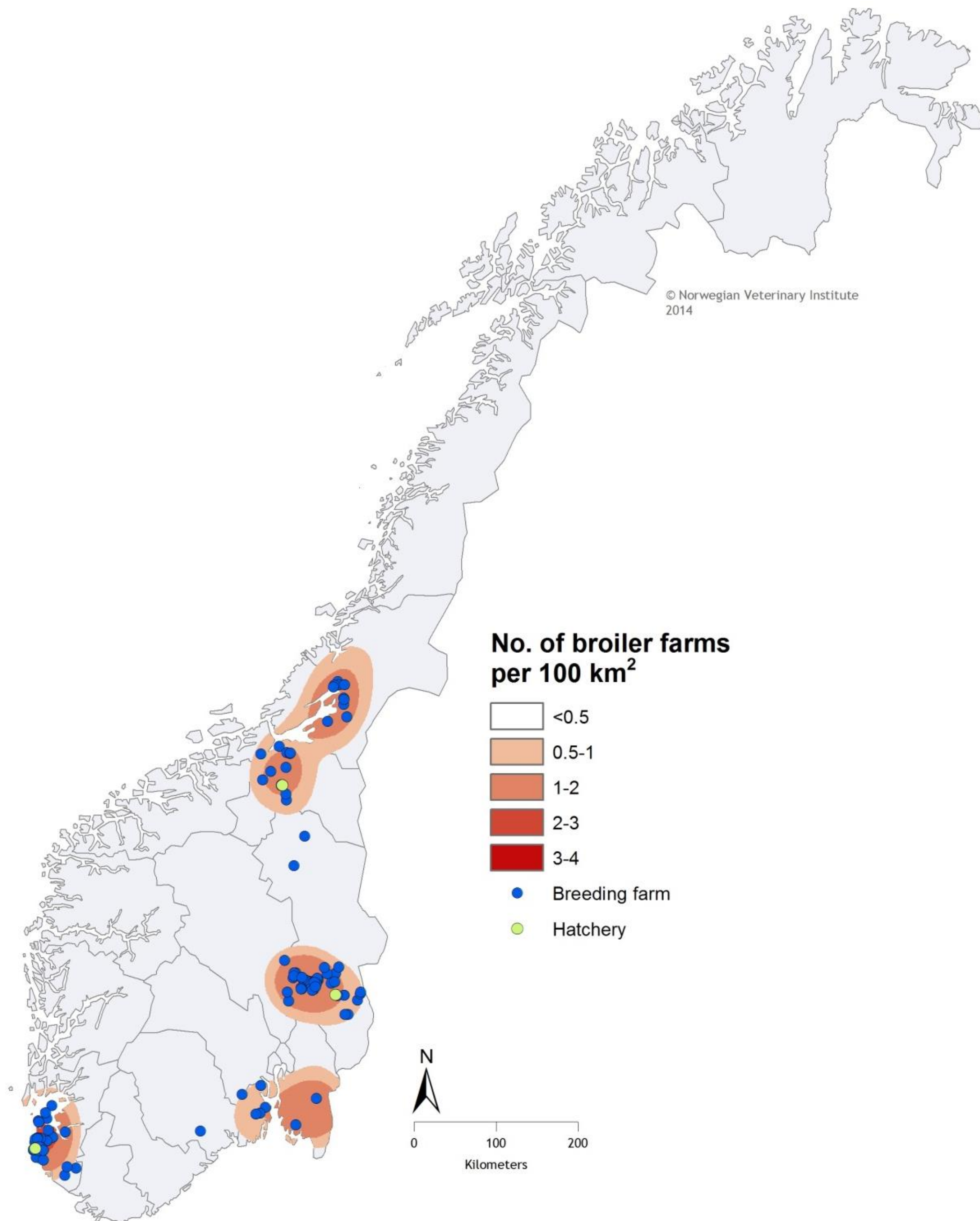


Figure 3. Map showing the density of broiler farms per 100 km^2 in Norway in 2014. Farms with parent flocks are indicated by blue dots and broiler hatcheries are indicated by yellow dots. Illustration: A. Tarpai, NVI.

Antimicrobial usage in the Norwegian broiler production is rare, with only a single broiler flock (0.02 %) treated annually from 2013-2015. Furthermore, only two parent flocks (1.1 %) were treated in 2013 and 2014 (Animalia, 2015; Refsum, 2015; Animalia, 2016). The antimicrobial growth promoter avoparcin was banned in 1995, and the Norwegian broiler industry voluntarily abandoned the use of all other antimicrobial growth promoter the same year (Grave et al., 2004). After the ban of antimicrobial growth promoters, the ionophore coccidiostat narasin was licensed for use as a feed additive for broilers (Grave et al., 2004),

and has been used routinely since (Grave et al., 2004; NORM/NORM-VET, 2015). However, the broiler industry in Norway aims to phase out the use of narasin during 2016 (Nortura, 2014; Hommelsgård, 2016), and, the largest broiler cooperative in Norway has already succeeded (Nortura, 2016).

6.2 Antimicrobial resistance

Antimicrobial resistance (AMR) has been identified as one of the main global health challenges by the World Health Organization (WHO) (Dziekan et al., 2012). Several definitions of AMR exist, but the most commonly used definitions are that an isolate displays AMR if it can grow in the presence of higher antimicrobial concentrations than wild-type isolates (Guardabassi and Courvalin, 2006) or if it can survive treatment with an antimicrobial that would normally kill it or limit its growth (WHO, 2014). The emergence of bacteria displaying AMR is especially provoked by the extensive use of antimicrobials (Schwarz et al., 2001; Guardabassi and Kruse, 2008; Davies and Davies, 2010; WHO, 2014), and a direct correlation between the use of antimicrobial agents and the emergence of strains resistant to these antimicrobials has been reported (Chantziaras et al., 2014). This is due to the selection pressure the antimicrobial exerts on the bacterial population by killing susceptible strains, while resistant strains survive and become the dominant phenotype. The main mechanisms of AMR in bacteria are enzymatic inactivation of the antimicrobial, replacement or modification of the antimicrobial target in the cell, active efflux of the antimicrobial from the bacteria, and reduced uptake of the antimicrobial (Levy and Marshall, 2004; Guardabassi and Courvalin, 2006; Schwarz et al., 2006; Tenover, 2006).

AMR can be intrinsic or acquired. Intrinsic resistance relates to features that are inherently associated to a particular type or group of bacteria, e.g. the outer membrane of Gram-negative bacteria, which is impermeable to several antimicrobials used to treat infections caused by Gram-positive bacteria (Guardabassi and Courvalin, 2006; Tenover, 2006; Guardabassi and Kruse, 2008; Cox and Wright, 2013). Acquired resistance arises due to changes in the bacterial genome. These changes can be due to mutations in the bacterial chromosome, or horizontal acquisition of foreign DNA. Horizontal acquisition of DNA can occur via transformation, transduction or conjugation (Figure 4). Transformation represents the uptake of naked DNA from the surrounding environment; transduction is acquisition of

DNA from bacteriophages, while conjugation is transfer of mobile DNA elements between two bacterial cells (Bennett, 1995; Guardabassi and Courvalin, 2006; Guardabassi and Kruse, 2008; Norman et al., 2009; Sykes, 2010; Tortora et al., 2010).

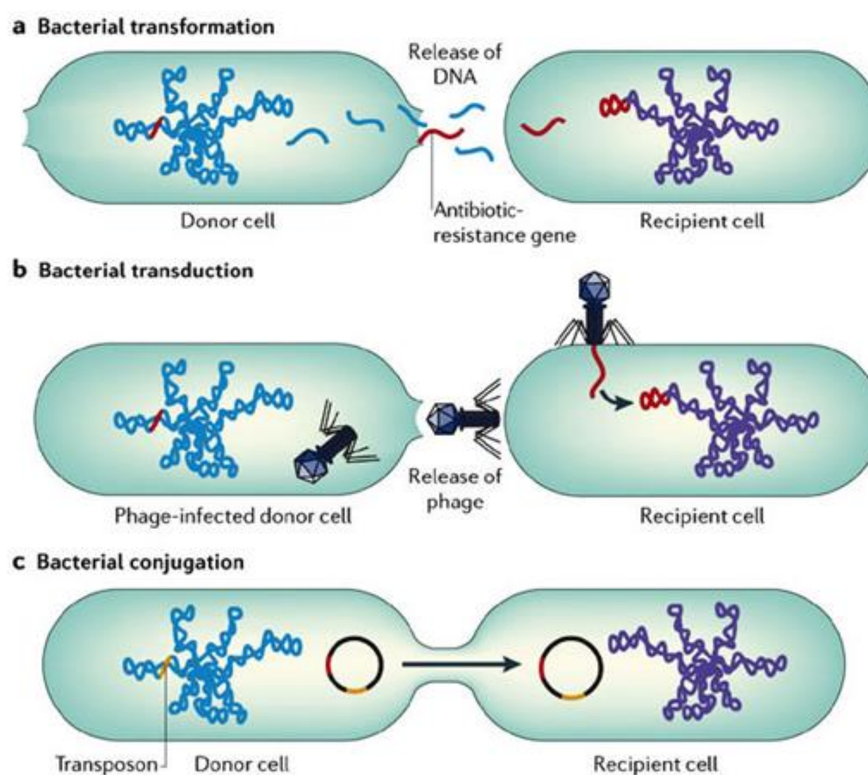


Figure 4. Routes for horizontal acquisition of DNA by bacteria. Transformation (a) represents the uptake of naked DNA from the surroundings; transduction (b) is acquisition of DNA from bacteriophages, while conjugation (c) is direct transfer of DNA between two bacterial cells. Figure reprinted from Furuya and Lowy (2006) with permission from Macmillan Publishers Ltd.

The contribution of transformation and transduction to development of antimicrobial resistance is considered to be limited. Transformation requires homology between the DNA in donor and recipient strains, and can therefore only occur between closely related bacteria (Guardabassi and Courvalin, 2006; Schwarz et al., 2006; Norman et al., 2009). Furthermore, free DNA is degraded quickly in the environment after being released from lysed bacteria (Guardabassi and Courvalin, 2006; Schwarz et al., 2006; Norman et al., 2009). Phages involved in transduction have high host specificity, and thus the range of hosts able to receive AMR genes through transduction is probably restricted (Guardabassi and Courvalin, 2006; Schwarz et al., 2006; Norman et al., 2009). Thus, conjugation is the most important mode of horizontal gene transfer between bacteria *in vivo*. This is both due to the frequent location of AMR genes on conjugative genetic elements, and to the ability of these elements

to transfer DNA between a broad range of different bacterial species (Schwarz et al., 2001; Guardabassi and Courvalin, 2006; Schwarz et al., 2006; Guardabassi and Kruse, 2008; Norman et al., 2009; Sykes, 2010). Conjugative spread of AMR genes is mediated by mobile genetic elements, with dissemination of plasmids being the main contributor (Su et al., 2008; Carattoli, 2009, 2013).

Plasmids are circular, extrachromosomal DNA elements, which are capable of self-replication within a bacterial host (Novick, 1987; Norman et al., 2009; Tortora et al., 2010). Plasmid transfer by conjugation is a stepwise process. First, a conjugation pilus is formed to connect the donor bacteria with the recipient bacteria to ensure cell-to-cell contact. Subsequently, the plasmid is nicked and a relaxosome including single-stranded plasmid DNA is formed. The relaxosome is then transferred into the recipient bacterium (Bennett, 1995; Wilkins, 1995; Norman et al., 2009; Tortora et al., 2010). Finally, the second plasmid DNA strand is synthesized in both the donor and recipient bacteria (Bennett, 1995; Norman et al., 2009; Tortora et al., 2010) (Figure 5).

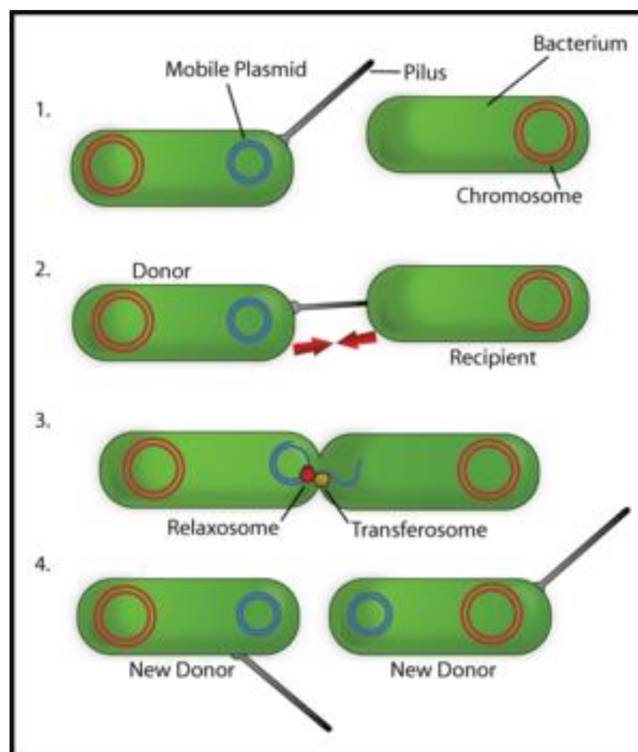


Figure 5. Schematic presentation of the conjugation process. A conjugation pilus is formed (1), and the donor and recipient cells are connected (2). The relaxosome including single stranded plasmid DNA is formed and transferred from the donor to the recipient cell (3). The second strand of plasmid DNA is synthesized, and the mobile plasmid is now present in both the donor and recipient cell (4). Figure adapted from https://en.wikipedia.org/wiki/Bacterial_conjugation.

Normally, the expression of genes encoding pili formation is repressed, probably due to the energy costs (Norman et al., 2009). However, external factors, such as temperature (Alonso et al., 2005), or proximity to plasmid-free cells (Norman et al., 2009) can influence the expression of genes encoding these pili. In addition, moisture, pH, water movement, changes in amount of available nutrients, type of soil and the presence of other microorganisms have been reported to influence the rate of plasmid transfer in natural environments (Sørensen et al., 2005). Furthermore, the environment in which the bacteria are present affect the rates of conjugation, as the pilus morphology determines whether a liquid or solid environment (i.e. biofilm) is optimal for conjugation (Bradley et al., 1980). Factors encoded by several conjugative plasmids have been shown to increase the biofilm-forming ability of *E. coli* and to enable *E. coli* to enter already existing biofilms (Ghigo, 2001; Burmølle et al., 2008). Also, the process of conjugative plasmid transfer itself has been shown to promote biofilm formation in *E. coli* (Reisner et al., 2006). This might enhance the ability of conjugative spread of plasmids harboured by these bacteria, as biofilms are considered to represent hot-spots for horizontal gene transfer (Jefferson, 2004; Sørensen et al., 2005).

Two plasmids sharing the same replication system cannot stably exist within the same host, a phenomenon referred to as incompatibility. Incompatibility is a consequence of the homogeneity of sequences controlling replication. This has been used to divide plasmids into different incompatibility (Inc) groups on the basis of their replicon type (Novick, 1987; Carattoli, 2013). Replicon typing of plasmids is a useful tool in monitoring dissemination of plasmids carrying AMR genes in bacteria from different niches, and to monitor horizontal spread of AMR genes between bacteria (Carattoli et al., 2005). Major plasmid families associated with dissemination of AMR genes in *Enterobacteriaceae* include IncF, IncA/C, Inc L/M, IncI1, IncHI2 and IncN (Carattoli, 2009).

Plasmids may possess several factors contributing to their stable maintenance within a bacterial host, including during cell division (Hayes, 2003; Unterholzner et al., 2013). These factors are known as toxin-antitoxin (TA) modules, post-segregational killing systems, addiction systems, or plasmid stability systems (Hayes, 2003). TA modules consist of two genes encoding a stable toxin and a labile antitoxin, respectively (Hayes, 2003; Norman et al., 2009). The antitoxin binds to the toxin forming a TA complex that neutralizes the toxin (Unterholzner et al., 2013). Daughter cells that do not inherit a copy of the plasmid will still inherit the TA complex (Hayes, 2003). The antitoxin is more rapidly degraded than the

toxin, which will lead to killing or growth restriction of plasmid-free daughter cells (Hayes, 2003; Norman et al., 2009; Van Melderen and Saavedra De Bast, 2009; Unterholzner et al., 2013). In addition, TA modules aid the exclusion of other compatible plasmids from the bacteria (Cooper and Heinemann, 2000). Two plasmids with the same Inc-group cannot stably exist in the same bacterial cell. Exclusion of the plasmid with the TA module will kill or retard the growth of the bacteria, while exclusion of the plasmid without the TA module will leave the bacteria viable. Thus, the presence of TA modules will increase the relative fitness of their host plasmid by eliminating competitive plasmids in the bacterial population (Van Melderen and Saavedra De Bast, 2009). These attributes contribute efficiently to the stable maintenance and persistence of plasmids within the bacterial population (Hayes, 2003; Van Melderen and Saavedra De Bast, 2009; Unterholzner et al., 2013).

When AMR genes target a successful plasmid type, it will have a significant effect on the dissemination of that gene to different bacterial hosts. This is due to the fact that such plasmids are often stably maintained within its hosts, which will contribute to both spread and persistence of resistance genes in the bacterial population (Carattoli, 2013). Therefore, plasmids represent a serious challenge due to their contribution in the dissemination and persistence of relevant AMR genes (Su et al., 2008; Carattoli, 2013).

6.2.1. Monitoring of antimicrobial resistance

Since January 2014, all EU/EEA member states are obliged to monitor the occurrence of AMR in zoonotic and commensal bacteria from food and food-producing animals as implemented by the European Commission (SANCO/11591/2012). To estimate the occurrence of AMR in Gram-negative and Gram-positive bacterial populations, indicator bacteria are monitored for the occurrence of AMR. Indicator bacteria are bacteria with relevance for human medicine that are commonly isolated from the faecal flora of animals. In addition, the most relevant resistance phenotypes can be found in indicator bacteria, and they represent a reservoir of resistance genes that might have the potential to be transferred to other bacteria, including pathogenic strains. Furthermore, as indicator bacteria are a part of the normal flora, they provide a good indication of the selection pressure generated by antimicrobial use on the intestinal flora of food-producing animals. *E. coli* are used as indicator organisms for the Gram-negative bacterial population, while *Enterococcus* spp. are used as indicator organisms for the Gram-positive population (EFSA, 2008). In order to optimize detection of acquired resistance, epidemiological cut-off values (ECOFFs)

determined by the European Committee for Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org) are used to classify isolates as susceptible or resistant (EFSA, 2008). The ECOFFs are normally set at a lower MIC value than clinical breakpoints, which are used to predict the clinical efficacy of antimicrobial treatment (Aarestrup et al., 2007).

In Norway, the monitoring programme for the occurrence of antimicrobial resistance in bacteria from food, feed and animals (NORM-VET) has been running since 2000. A joint report with the monitoring programme for the occurrence of antimicrobial resistance in bacteria from humans (NORM) is published annually (http://www.vetinst.no/eng/Publications/NORM-NORM-VET-Report). The NORM-VET programme is coordinated by the Norwegian Veterinary Institute (NVI) and commissioned by the Norwegian Food Safety Authority.

6.2.2. Cephalosporin resistance in *E. coli*

Cephalosporins are beta-lactam antimicrobials that exert their antimicrobial effect by interfering with cell wall synthesis. The cell wall is thereby weakened, causing disruption and death of the bacteria (Papich and Riviere, 2009). The extended-spectrum cephalosporins (ESC) are defined as critically important by WHO for treatment of human infections (Dziekan et al., 2012), and the emergence of resistance towards these antimicrobials is therefore concerning. Resistance to ESC in *E. coli* is mainly mediated by hydrolysis of the cephalosporin by beta-lactamases (Pfeifer et al., 2010) (Figure 6).

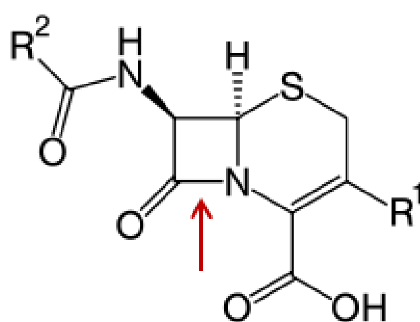


Figure 6. The chemical structure of cephalosporins. Beta-lactamases hydrolyse the beta-lactam ring, indicated by the red arrow, causing inactivation of the antimicrobial. Figure adapted from https://en.wikipedia.org/wiki/Cephalosporin.

A variety of different enzymes with small differences in their spectrums of activity exist (www.lahey.org/studies). Attempts have been made to classify the different beta-lactamases.

Ambler (1980) suggested a classification on the background of the beta-lactamase structure, while Bush et al. (1995) made a classification based on the functional characteristics of the enzymes. In addition, a classification scheme based on the spectrum of beta-lactamase resistance was proposed by Giske et al. (2009) to simplify the understanding of differences between beta-lactamase groups. In this thesis, the terms “ESBL” and “AmpC”, as specified underneath, will be used, while “cephalosporin-resistant” will be used as a collective term for ESC resistance.

Extended-spectrum beta-lactamases

Enzymes classified as extended-spectrum beta-lactamases (ESBLs), also called “classical” ESBLs, are the major cause of cephalosporin resistance in *E. coli* worldwide. They have a broad spectrum of activity, including both 3rd and 4th generation cephalosporins, and monobactams. However, ESBLs do not confer resistance to cephamycins, and they are susceptible to beta-lactamase inhibitors such as clavulanic acid (Bush et al., 1995; Bradford, 2001; Pfeifer et al., 2010). The classical ESBLs belong to Ambler class A, Bush-Jacoby-Medeiros group 2be and Giske group $ESBL_A$ (Ambler, 1980; Bush et al., 1995; Giske et al., 2009). The enzymes can be divided into different groups based on their origin (Bradford, 2001). Hundreds of different ESBLs exist, and the major groups include CTX-M, TEM and SHV (www.lahey.org/studies). Different enzymes in the TEM and SHV groups are generated by amino acid substitutions in the bla_{TEM} and bla_{SHV} genes, which result in small alterations in the resistance spectrum. The CTX-M enzymes can be divided into five major groups, where each group consist of highly similar enzymes resulting from mutations in the $bla_{CTX\text{-}M}$ genes (Pfeifer et al., 2010).

ESBLs are frequently associated with plasmids belonging to Inc groups F, A/C, L/M, I1, HI2 and N in *E. coli* isolated from both humans and animals (Carattoli, 2009). Plasmid borne $bla_{CTX\text{-}M}$ genes have been mobilized from the chromosomal cephalosporinase of *Kluyvera* spp. (Humeniuk et al., 2002; Poirel et al., 2002), while $bla_{SHV\text{-}1}$ is thought to originate from the chromosome of *Klebsiella pneumoniae* (Hæggman et al., 1997). The ancestor of the bla_{TEM} genes is thought to originate from naturally occurring plasmids from where it has translocated onto other resistance plasmids (Heffron et al., 1975).

AmpC beta-lactamases

The AmpC beta-lactamases mediate resistance to 3rd generation cephalosporins, monobactams and cephamycins. In addition, they are resistant to deactivation by clavulanic

acid and other beta-lactamase inhibitors, but susceptible to 4th generation cephalosporins and cloxacillin (Philippon et al., 2002; Jacoby, 2009). The AmpC beta-lactamases are classified into Ambler class C, Bush-Jacoby-Medeiros group 1 and Giske group $ESBL_M$ (Ambler, 1980; Bush et al., 1995; Giske et al., 2009). In *E. coli*, cephalosporin resistance caused by production of AmpC beta-lactamases can be mediated either by an up-regulated expression of the chromosomal *ampC* gene, or by acquisition of a plasmid carrying a bla_{AmpC} gene (Jacoby, 2009; Pfeifer et al., 2010).

Up-regulated chromosomal ampC

E. coli have a chromosomal *ampC* gene which is expressed at a low level due to a defect promoter region (Pfeifer et al., 2010). In contrast to chromosomal *ampC* genes in several *Enterobacteriaceae*, the expression of chromosomal *ampC* in *E. coli* is not inducible by exposure to beta-lactams (Jacoby, 2009) due to the lack of an *ampR* regulator (Pfeifer et al., 2010). The expression of chromosomal *ampC* in *E. coli* is regulated by promoter and attenuator regions (Jacoby, 2009). Mutations and insertions in certain areas of the promoter region can enable up-regulation in chromosomal *ampC* expression, resulting in overproduction of ampC enzyme and expression of cephalosporin resistance (Caroff et al., 2000; Siu et al., 2003; Pfeifer et al., 2010).

Plasmid-mediated AmpC (pAmpC)

Genes encoding AmpC beta-lactamases located on plasmids (pAmpC) have been mobilized from chromosomal AmpC-encoding genes found in *Enterobacteriaceae* and other Gram-negative bacteria (Walther-Rasmussen and Høiby, 2002; Su et al., 2008). The pAmpC beta-lactamases are grouped based on their origin, including CMY, MOX, FOX, DHA, and ACT (Walther-Rasmussen and Høiby, 2002; Jacoby, 2009; Pfeifer et al., 2010). The most commonly occurring pAmpC beta-lactamase is CMY-2, which is globally distributed (Jacoby, 2009; Ewers et al., 2012). The gene encoding CMY-2, bla_{CMY-2}, originates from the chromosome of *Citrobacter freundii* (Wu et al., 1999) and has been frequently reported on IncF, IncA/C and IncI1 plasmids (Carattoli, 2009). In addition, an association between bla_{CMY-2} and IncK plasmids has been reported in recent years (Dierikx et al., 2010; Börjesson et al., 2013b; Börjesson et al., 2013c; Voets et al., 2013; Agersø et al., 2014; Egervärn et al., 2014).

6.3 Epidemiology of cephalosporin-resistant *E. coli*

The epidemiology of AMR is highly complex. In addition to clonal spread of AMR strains, plasmids carrying AMR-encoding genes can spread horizontally in the bacterial population, increasing the complexity of transmission pathways (Coque et al., 2008a; Brolund, 2014). AMR can be attributed to the «One Health» concept, describing the complex interplay between humans and animals, and their respective social and environmental contexts (Zinsstag et al., 2011). An increasing occurrence of resistant bacteria in one compartment may cause emergence of resistant strains in other compartments as well (EFSA, 2011). In Figure 7, a schematic overview of possible ways by which AMR bacteria, including cephalosporin-resistant *E. coli*, can spread between different populations is illustrated (Davies and Davies, 2010).

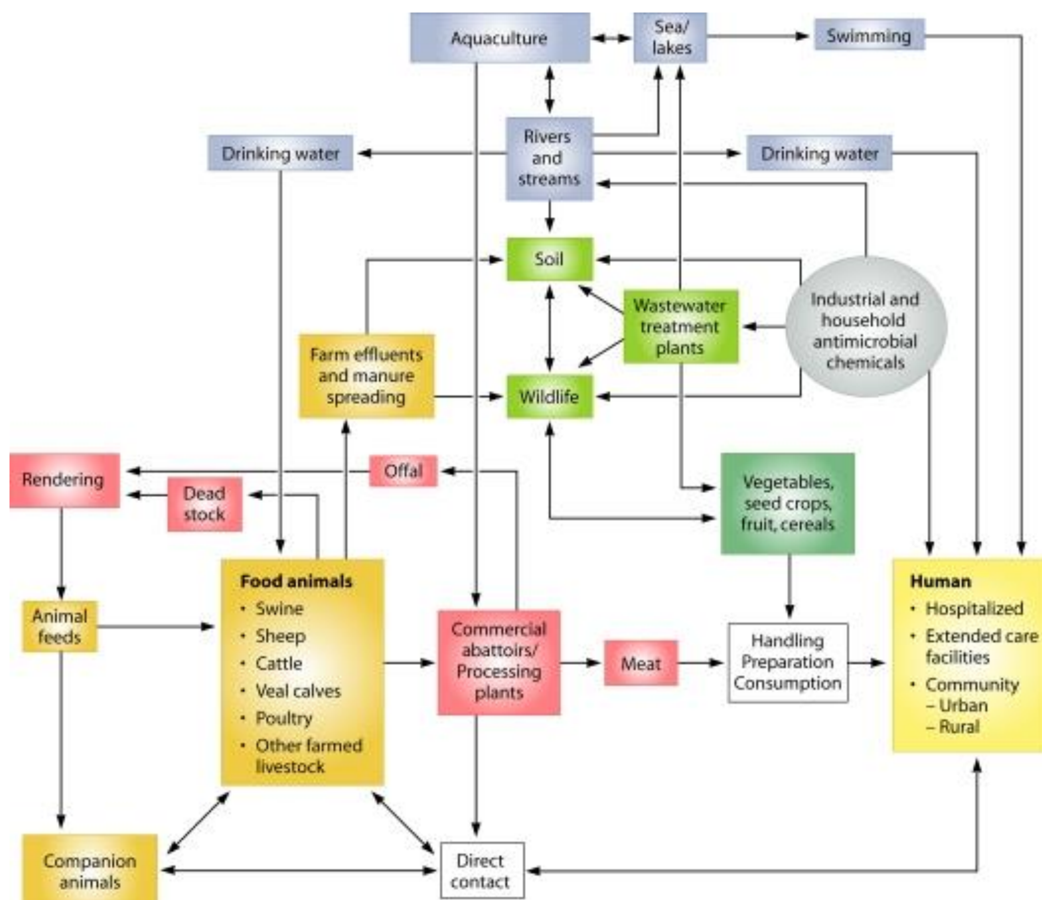


Figure 7. Possible routes by which bacteria, displaying antimicrobial resistance can move between different environmental compartments. Figure reprinted from Davies and Davies (2010) with permission from the American Society for Microbiology, John Wiley and Sons, and BMJ Publishing Group Ltd.

The occurrence of cephalosporin-resistant *E. coli* has increased substantially in both human and veterinary medicine during the course of the last decade (Cantón et al., 2008; Carattoli,

2008; Coque et al., 2008a; Pitout and Laupland, 2008; Overdevest et al., 2011; Ewers et al., 2012; ECDC, 2015; EFSA and ECDC, 2016). This increased occurrence is likely attributed to both expansion of pandemic and successful cephalosporin-resistant clones (Coque et al., 2008a; Mathers et al., 2015b; Schaufler et al., 2016), and by horizontal transfer of ESBL/AmpC-encoding plasmids within and between *E. coli* clones (Coque et al., 2008a; Woerther et al., 2013; de Been et al., 2014). The epidemiology of cephalosporin-resistant bacteria in humans, and to some extent broilers, is complicated by the occurrence of similar plasmids carrying identical ESBL/AmpC-encoding genes in different bacterial species, including *E. coli*, *Klebsiella* spp. and *Salmonella* spp. (Coque et al., 2008a; Sunde et al., 2009; Dierikx et al., 2010; Woerther et al., 2013).

In humans, a global pandemic of ESBL-producing *E. coli* of multilocus sequence type (ST)131 has been documented (Coque et al., 2008a). Although this clone has also been isolated from companion animals (Pomba et al., 2009; Ewers et al., 2010), wild birds (Rogers et al., 2011; Schaufler et al., 2016), rodents, food-producing animals and retail meat (Rogers et al., 2011), it appears that *E. coli* ST131 is well adapted to human hosts (Mathers et al., 2015b, a). Other successful *E. coli* clones displaying cephalosporin resistance may be better adapted to several hosts. Examples include ST410 isolated from humans, wild birds (Schaufler et al., 2016), companion animals (Huber et al., 2013) and retail meat (López-Cerero et al., 2011), or ST648, isolated from humans, companion animals and food-producing animals (Wieler et al., 2011; Ewers et al., 2014).

In this section, a brief summary on the epidemiology of cephalosporin-resistant *E. coli* in the broiler production pyramid and humans in Norway and Europe will be presented.

6.3.1. Epidemiology in the broiler production pyramid

In veterinary medicine, the highest frequencies of cephalosporin-resistant *E. coli* are found in poultry (Smet et al., 2008; Ewers et al., 2012; Dierikx et al., 2013a; MARAN, 2015). The first description dates back to 2000-2001, when *E. coli* harbouring ESBL- and AmpC-encoding genes were detected in broilers in Spain (Briñas et al., 2003). Since then, numerous studies on the occurrence of cephalosporin-resistant *E. coli* in broilers and their products have shown that the distribution is global (Carattoli, 2008; Smet et al., 2008; Dierikx et al., 2010; Doi et al., 2010; Ewers et al., 2012; Hiroi et al., 2012b).

The situation in Norway

The first *E. coli* resistant to cephalosporins detected in the veterinary sector in Norway was isolated from a faecal sample from healthy broilers collected within the frame of the NORM-VET programme in 2006 (NORM/NORM-VET, 2007). Genetic characterization of the isolate revealed a $bla_{\text{TEM-20}}$ gene on an IncI1 plasmid as the cause of the cephalosporin resistant phenotype. The IncI1 plasmid was closely related to plasmids originating from broilers in the Netherlands, and poultry and humans in Belgium and France (Sunde et al., 2009). This finding was considered an "early warning", indicating that the broiler production could be a reservoir of cephalosporin-resistant bacteria in Norway (NORM/NORM-VET, 2012), and suggested that international trade of breeding animals for broiler production also contributed to the international spread of cephalosporin-resistant *Enterobacteriaceae* (NORM/NORM-VET, 2007; Sunde et al., 2009).

In 2011, a selective method for detection of cephalosporin-resistant *E. coli* was implemented in the NORM-VET programme (NORM/NORM-VET, 2012). Samples were plated directly out on MacConkey agar supplemented with 1 mg/L cefotaxime and MacConkey agar supplemented with 2 mg/L ceftazidime. In parallel, the original non-selective method with plating out on MacConkey agar without supplement followed by isolation and susceptibility testing of one indicator *E. coli* was applied. Using the selective method, the occurrence of cephalosporin-resistant *E. coli* was surprisingly high in faecal samples from broilers (35.7-42.9 %) (NORM/NORM-VET, 2012, 2015) and retail chicken meat (28.9-32.2 %) (NORM/NORM-VET, 2013, 2015). However, a very low occurrence was identified when the non-selective method was used, as cephalosporin-resistant *E. coli* was detected in only 1.0-1.5 % of broiler flocks (2011 and 2014), and 0.5 % of retail chicken meat samples (2012) (NORM/NORM-VET, 2012, 2013, 2015).

Since the introduction of a selective detection method in 2011, all cephalosporin-resistant *E. coli* isolated from the broiler production pyramid in the NORM-VET programme have displayed an AmpC-phenotype. All isolates with an acquired AmpC-encoding gene have been found to carry the $bla_{\text{CMY-2}}$ gene (NORM/NORM-VET, 2012, 2013, 2015).

A semi-quantitative method was applied to detect the proportion of cephalosporin-resistant *E. coli* of total *E. coli* in broiler caecal samples during 2014 (NORM/NORM-VET, 2015). The results indicated that in more than 78.3 % of the samples, 0.1 % or less of the total *E. coli* population were cephalosporin-resistant. Also, quantification of cephalosporin-resistant

E. coli in retail chicken meat showed that in 87.9 % of the samples, the occurrence was ≤ 0.2 cfu/g (NORM/NORM-VET, 2015). Together, these findings indicated that the proportion of cephalosporin-resistant *E. coli* in positive samples from Norwegian broilers and retail meat was low.

In 2014, the Norwegian poultry industry initiated an action plan against cephalosporin-resistant *E. coli* in the broiler production pyramid (Animalia, 2013). Samples were collected from each batch of imported parent animals after hatching and from parent flocks in production. Cephalosporin-resistant *E. coli* were detected in 5 of 87 (5.7 %) batches of newly hatched parent animals. In parent flocks, 23 of 85 (27.1 %) flocks sampled were positive (Animalia, 2015). In 2015, cephalosporin-resistant *E. coli* were detected in 2 of 84 imports (2.4 %), and in 13 of 60 (21.7 %) parent flocks in production (Animalia, 2016). Samples collected during this action plan were analysed using a pre-enrichment step in MacConkey broth supplemented with 1 mg/L cefotaxime, before plating out on MacConkey agar supplemented with 1 mg/L cefotaxime. Thus, it is assumed that this method is more sensitive than the method used in the NORM-VET programme (2011, 2012 and 3014), and the results cannot be directly compared.

The situation in Europe

In Denmark and Sweden, the occurrence of cephalosporin-resistant *E. coli* in the broiler production pyramid is comparable to Norwegian conditions. The pAmpC-beta-lactamase encoded by the $bla_{\text{CMY-2}}$ gene dominates, with only sporadic findings of ESBLs encoded by $bla_{\text{CTX-M}}$, bla_{SHV} and bla_{TEM} genes. However, direct comparison of the occurrences is problematic due to small differences in the detection methods used (DANMAP, 2011; SVARM, 2011; Agersø et al., 2012; DANMAP, 2012; SVARM, 2012; Börjesson et al., 2013a; Börjesson et al., 2013c; DANMAP, 2013; SWEDRES/SVARM, 2013; Agersø et al., 2014; DANMAP, 2014; SWEDRES/SVARM, 2014; DANMAP, 2015; SWEDRES/SVARM, 2015; Börjesson et al., 2016). In Iceland, a very limited number of AmpC-producing isolates have been detected. However, only a limited screening has been performed (MAST, 2015). Cephalosporins have not been used for treatment of flocks in the broiler production in any of these Nordic countries, and the general consumption of antimicrobial agents for treatment of disease is low. Thus, the selection pressure from antimicrobial use is virtually non-existent (NORM/NORM-VET, 2007; Sunde et al., 2009; SVARM, 2011; SWEDRES/SVARM, 2013; Agersø et al., 2014; MAST, 2015;

NORM/NORM-VET, 2015). Therefore, import of breeding animals or hatching eggs has been identified as the likely source of introduction of cephalosporin-resistant *E. coli* to the broiler production in the Nordic countries (Sunde et al., 2009; NORM/NORM-VET, 2012; Agersø et al., 2014; Nilsson et al., 2014). In Sweden, pAmpC-producing *E. coli* have been detected in samples taken from day old grandparent animals imported from Scotland (SVARM, 2011; Nilsson et al., 2014). Furthermore, clonally related pAmpC-producing *E. coli* were detected through all levels of the Swedish broiler production, suggesting vertical transmission through the pyramid (Nilsson et al., 2014).

In several other European countries, a higher occurrence of cephalosporin-resistant *E. coli* compared to the Nordic countries is seen. Prevalence's of cephalosporin resistance in indicator *E. coli* among European broilers ranging from 0.0-32.2 % were reported in 2014, using non-selective methods for detection (Figure 8) (EFSA and ECDC, 2016).

Figure 8. Occurrence of cephalosporin resistance in indicator *E. coli* isolated from broilers in European countries in 2014. Reprinted from "The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2014", www.ecdc.europa.eu (EFSA and ECDC, 2016).

Furthermore, numerous studies investigating the occurrence of cephalosporin-resistant *E. coli* in broilers and retail chicken meat in European countries using selective methods have detected high occurrences. In broilers, occurrences have ranged from 54.5-100.0 % (Smet et al., 2008; Wasyl et al., 2012; Dierikx et al., 2013a; Reich et al., 2013; Huijbers et al., 2014), while in meat samples, occurrences of 34.0-100.0 % have been reported (Overdevest et al., 2011; Agersø et al., 2012; Cohen Stuart et al., 2012; Egea et al., 2012; Belmar Campos et al., 2014; Egervärn et al., 2014; Vogt et al., 2014). Furthermore, a high within-flock occurrence of cephalosporin-resistant *E. coli* has been identified in the Netherlands and Germany (Dierikx et al., 2013a; Reich et al., 2013).

In comparison to Norway and the other Nordic countries, the genetic background encoding cephalosporin resistance in *E. coli* isolated from the broiler production pyramid in European countries is heterogeneous. While $bla_{\mathrm{CMY\text{-}2}}$ dominates largely in the Nordic countries (Agersø et al., 2012; NORM/NORM-VET, 2012; Börjesson et al., 2013b; Börjesson et al., 2013c; NORM/NORM-VET, 2013; Agersø et al., 2014; NORM/NORM-VET, 2015; Börjesson et al., 2016), genes encoding enzymes of the CTX-M, SHV and TEM type beta-lactamases are also frequently detected in other European countries (Smet et al., 2008; Bortolaia et al., 2010; Cortés et al., 2010; Dierikx et al., 2010; Overdevest et al., 2011; Egea et al., 2012; Ewers et al., 2012; Dierikx et al., 2013a; Belmar Campos et al., 2014; Huijbers et al., 2014; Vogt et al., 2014; Day et al., 2016).

The high occurrence of cephalosporin-resistant *E. coli* detected in some European countries may be partly explained by the extensive use of antimicrobial agents (EMA, 2013; MARAN, 2015), as antimicrobial use has been identified as a risk factor for occurrence of cephalosporin-resistant *E. coli* in broilers (Persoons et al., 2011). Although cephalosporins are not approved for use in broilers in Europe, off-label use for prophylactic treatment of newly hatched chickens in broiler hatcheries has been practiced (Liebana et al., 2013). In January 2012, the British Poultry Council (BPC) voluntarily abandoned the use of cephalosporins in the broiler production (WorldPoultry, 2011). However, it has been hypothesized that use of cephalosporins in animals at the top of the pyramid prior to this ban might have provoked the emergence of cephalosporin-resistant *E. coli*, which have then been exported with breeding animals to Sweden (Agersø et al., 2014) and to other European countries (Dierikx et al., 2013b). Following the ban of off-label use of cephalosporins for prophylactic treatment of day-old chickens in Dutch hatcheries, the occurrence of

cephalosporin-resistant *E. coli* isolated from broilers decreased drastically within a short time period (MARAN, 2015; EFSA and ECDC, 2016).

6.3.2. Epidemiology in humans

A variety of ESBL/AmpC-enzymes have been reported in *E. coli* from humans (Hawkey and Jones, 2009). However, a global epidemic of ESBL-producing *E. coli* associated with the clonal spread of the highly virulent strain *E. coli* O25:H4-ST131-CTX-M-15 has been identified (Coque et al., 2008a; Woodford et al., 2011; Mathers et al., 2015b, a). This strain is often associated with urinary tract infections (Nicolas-Chanoine et al., 2008). In Europe, CTX-M-15 is the most commonly occurring beta-lactamase (Hawkey and Jones, 2009; Woerther et al., 2013) and is often associated with IncF plasmids (Coque et al., 2008b; Carattoli, 2009; Brolund, 2014; Mathers et al., 2015b). The success of the CTX-M-15 enzyme relies both on the endemic spread of *E. coli* ST131 and horizontal spread of plasmids carrying $bla_{\text{CTX-M-15}}$ (Peirano and Pitout, 2010). Recently, CTX-M-15 has also been associated with the emerging successful clones ST410 (Schaufler et al., 2016) and ST648 (van der Bij et al., 2011; Wieler et al., 2011). Globalization and intercontinental travel have also been found to play major roles in the worldwide spread of resistant bacteria (Woerther et al., 2013; Prescott, 2014; Hawkey, 2015).

The situation in Norway

In the NORM programme, *E. coli* isolated from clinical cases of sepsis and urinary tract infections are susceptibility tested. In 2014, the occurrence of cephalosporin resistance was 5.8 % and 3.8 % in isolates from sepsis and urinary tract infections, respectively. The majority of the isolates harboured $bla_{\text{CTX-M-1}}$ or $bla_{\text{CTX-M-9}}$, while a few were carriers of bla_{SHV} or bla_{CMY} genes (NORM/NORM-VET, 2015). An increasing trend in the occurrence of cephalosporin-resistant *E. coli* in clinical samples has been evident in recent years (ECDC, 2015; NORM/NORM-VET, 2015). A nosocomial outbreak of *E. coli* carrying $bla_{\text{CTX-M-15}}$ has been reported (Naseer et al., 2007), and molecular characterization of *E. coli* isolates with $bla_{\text{CTX-M-15}}$ isolated from diagnostic laboratories in Norway revealed that the majority of these strains could be assigned to the global multidrug-resistant clone O25:H4-ST131-CTX-M-15 (Naseer et al., 2009). Isolates displaying AmpC-phenotypes seem to occur only sporadically in human patients, with $bla_{\text{CMY-2}}$ being the most frequently detected pAmpC-encoding gene (Naseer et al., 2010). Currently, a research project is investigating the occurrence of resistant bacteria in the faecal flora of healthy humans in Norway,

including cephalosporin-resistant *E. coli* (personal communication, Marianne Sunde, NVI and Norwegian Institute of Public Health).

The situation in Europe

In Europe, the population-weighted mean occurrence of cephalosporin-resistant *E. coli* from human clinical isolates increased significantly between 2011 and 2014 from 9.6 % to 12.0 % (ECDC, 2015). The occurrence varied between countries with a range from 3.3 % in Iceland to 40.4 % in Bulgaria. The lowest occurrences were found in the Nordic countries and the Netherlands, while the highest occurrences were reported from countries in the south- and south-eastern parts of Europe (ECDC, 2015). The occurrences of cephalosporin-resistant *E. coli* in different European countries in 2009 and 2014 are illustrated in Figures 9 and 10, respectively. The change in colour from green and yellow towards orange and red indicates an increase in the occurrence during this period.

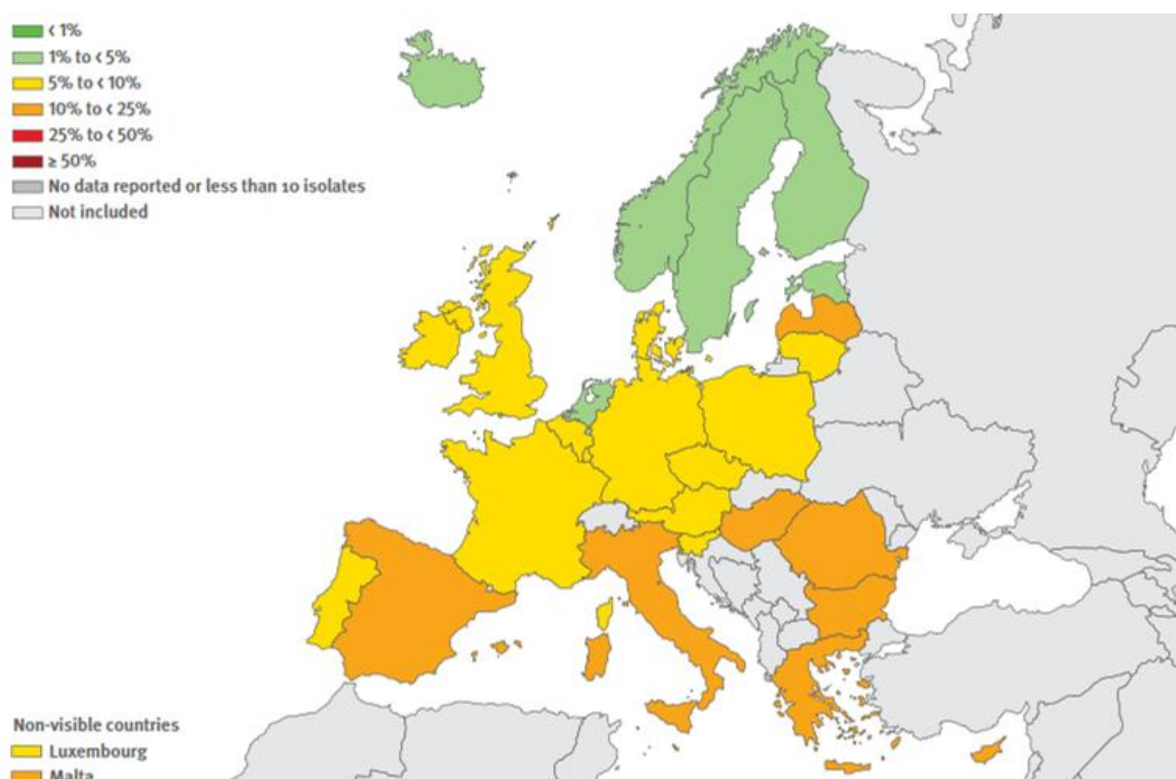


Figure 9. Occurrence of cephalosporin resistance in invasive *E. coli* isolated from human patients per country in 2009. Reprinted from "Antimicrobial resistance surveillance in Europe 2009, www.ecdc.europa.eu (ECDC, 2010).

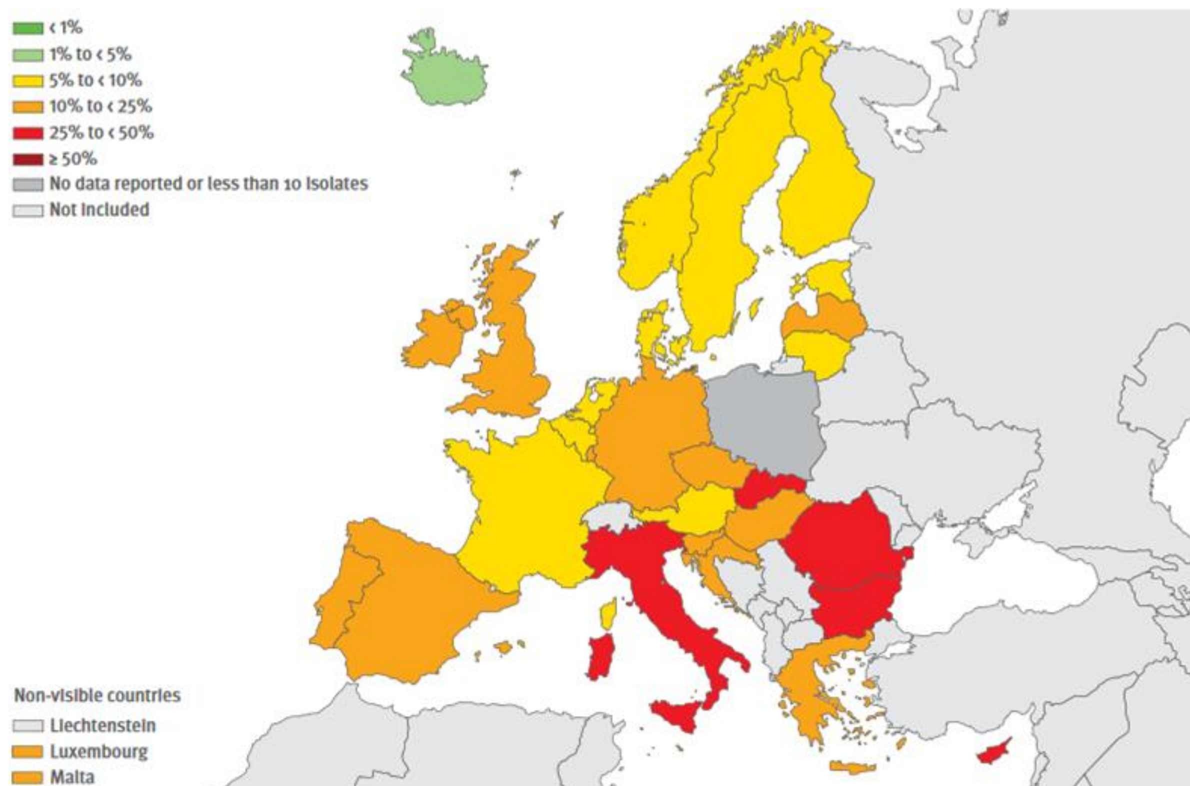


Figure 10. Occurrence of cephalosporin resistance in invasive *E. coli* isolated from human patients per country in 2014. Reprinted from "Antimicrobial resistance surveillance in Europe 2014, www.ecdc.europa.eu (ECDC, 2015).

A review of data available from healthy humans carrying cephalosporin-resistant *Enterobacteriaceae* showed that the occurrence in Europe was generally low to moderate, ranging from 0.0-11.6 %. This level is low compared to other regions in the world, such as Southeast Asia (up to 69.3 %). Based on modelling of data from studies on intestinal carriage, it has been estimated that approximately 34 million people in Europe are healthy carriers of cephalosporin-resistant *Enterobacteriaceae* (Woerther et al., 2013). The most prevalent enzyme by far is CTX-M-15 (Woerther et al., 2013), which is also associated with the global spread of the highly virulent *E. coli* variants O25:H4-ST131-CTX-M-15 (Coque et al., 2008a; Naseer and Sundsfjord, 2011; Mathers et al., 2015b, a), ST405 (Coque et al., 2008b; Naseer and Sundsfjord, 2011), ST410 (López-Cerero et al., 2011; Schaufler et al., 2016) and ST648 (van der Bij et al., 2011).

6.4 Public health aspects

The increasing incidence of infections caused by cephalosporin-resistant bacteria observed in Europe (ECDC, 2015) is considered a serious threat to public health due to a limitation in

the number of antimicrobial agents available for treatment of such infections (Pitout and Laupland, 2008; ECDC and EMEA, 2009; Dziekan et al., 2012; Mathers et al., 2015a). Furthermore, infections caused by resistant bacteria result in increased morbidity, mortality, and increased costs. In 2007, it was estimated that infections caused by AMR bacteria were the direct cause of 25,000 deaths annually in Europe, and represented a cost of approximately 1.5 billion Euros. Furthermore, these infections lead to approximately 2.5 million additional hospital days (ECDC and EMEA, 2009). A recent report has estimated that AMR infections alone will cause 10 million deaths yearly by 2050, and that the annual cost will be 100 trillion US dollars (O'Neill, 2016). However, it has been suggested that the true cost of AMR is even higher, as current estimates do not take the whole healthcare system into consideration (Smith and Coast, 2013).

It has been hypothesized that food-producing animals and their products can constitute a reservoir of cephalosporin-resistant *E. coli*, which may be transferred to humans (Winokur et al., 2001; Coque et al., 2008a; Doi et al., 2010; EFSA, 2011; Leverstein-van Hall et al., 2011; Overdevest et al., 2011; Ewers et al., 2012; Kluytmans et al., 2013; Voets et al., 2013). Poultry has been suggested as the most likely source of cephalosporin-resistant extraintestinal pathogenic *E. coli* (ExPEC) acquired from the food chain (Lazarus et al., 2015). Also, studies have proposed retail chicken meat as a source of ExPEC, causing urinary tract infections in humans (Jakobsen et al., 2010; Vincent et al., 2010; Manges, 2016).

Studies from the Netherlands have identified an increased risk of colonization with cephalosporin-resistant *E. coli* for people working or living on broiler farms compared to the general population, indicating direct transmission of resistant strains between broilers and humans (Dierikx et al., 2013a; Huijbers et al., 2014). However, living in close proximity of a broiler farm did not increase the risk of being colonized with cephalosporin-resistant *E. coli* (Huijbers et al., 2013), suggesting that direct contact with animals is important for transmission of cephalosporin-resistant strains. Detection of identical *E. coli* STs, resistance genes, and plasmids belonging to the same Inc groups in broilers and humans also supports the hypothesis that such transmission occurs (Winokur et al., 2001; Leverstein-van Hall et al., 2011; Overdevest et al., 2011; Kluytmans et al., 2013; Lazarus et al., 2015). Furthermore, *in situ* experiments have shown the ability of ESBL-producing *E. coli* of avian origin to establish and persist in a model mimicking the human intestine (Smet et al., 2011). Also, conjugal transfer of ESBL-encoding plasmids from avian to human *E.*

coli has been demonstrated (Smet et al., 2011). In addition, resistant *E. coli* from humans have been shown to have a higher genetic similarity to *E. coli* isolated from poultry than to susceptible *E. coli* from humans (Johnson et al., 2007).

A small study in Sweden showed no close relationship between pAmpC-producing *E. coli* isolated from broilers and humans. However, IncK plasmids carrying $bla_{\text{CMY-2}}$ were detected in both populations. Further characterization of the IncK plasmids was not performed, and transmission of the resistance plasmids between broiler and human strains could therefore not be ruled out (Börjesson et al., 2013c). Conjugal transfer of plasmids carrying ESBL/AmpC-encoding genes from *E. coli* originating from broilers or retail chicken meat to human strains has also been suggested by others (Winokur et al., 2001; Carattoli, 2008; Leverstein-van Hall et al., 2011; Kluytmans et al., 2013; Voets et al., 2013; Wu et al., 2013; de Been et al., 2014; Day et al., 2016). Another study from Sweden concluded that the contribution of cephalosporin-resistant *E. coli* from food products to the occurrence of cephalosporin-resistant *E. coli* in healthy humans in Sweden was very limited (Börjesson et al., 2016). A scientific report from Norway concluded that the possibility of transmission of cephalosporin-resistant *E. coli* from broilers and retail chicken meat to humans is non-negligible, but that more knowledge is required to determine the probability of such transmission (VKM, 2015). Both Sweden and Norway have a low occurrence of cephalosporin-resistant *E. coli* in the broiler production compared to other European countries. As both the frequency of occurrence and quantity of cephalosporin-resistant *E. coli* in broilers and retail chicken meat will influence the likelihood of zoonotic transmission of resistant strains and/or resistance plasmids (Liebana et al., 2013), transmission may be more likely in countries where the occurrence is higher than in the Nordic countries.

In a study from the Netherlands, the genetic relatedness between cephalosporin-resistant *E. coli* from broilers, retail chicken meat and humans was investigated by the use of whole genome sequencing (WGS) (de Been et al., 2014). WGS has a higher discriminatory power than traditional typing methods (den Bakker et al., 2014; Joensen et al., 2014). The study showed that highly related plasmids carrying ESBL/AmpC-encoding genes were present in isolates from humans, animals and meat. However, there was no indication of recent clonal dissemination of cephalosporin-resistant *E. coli* strains between the reservoirs. Notably, only a limited number of isolates from each reservoir was investigated (de Been et al., 2014). On the contrary, a small study in Norway also utilizing WGS, found a limited number of human clinical *E. coli* isolates with a high degree of genetic relatedness to

pAmpC-producing *E. coli* from chicken. In addition, the plasmids found in human and broiler isolates were highly similar, both in closely and more distantly related *E. coli* isolates (Berg et al., 2015). Highly similar plasmids harbouring *bla*$_{CMY-2}$ were also detected in *E. coli* of different STs isolated from humans, parent- and broiler flocks, and retail meat in Denmark (Hansen et al., 2016).

Transmission of cephalosporin-resistant *E. coli* from retail meat might occur through handling or consumption of meat contaminated with resistant bacteria (Leverstein-van Hall et al., 2011; Voets et al., 2013), or via contaminated kitchen supplies such as cutting boards (Tschudin-Sutter et al., 2014). The overall goal should therefore be to aspire to keep the occurrence of cephalosporin-resistant *E. coli* in the broiler production pyramid and retail chicken meat at the lowest possible level. This would minimize the probability of zoonotic transfer of resistant strains and/or resistance plasmids via the food chain (Liebana et al., 2013).

6.5 Knowledge gaps

Before the start of this PhD project in 2013, knowledge relating to cephalosporin-resistant *E. coli* in the Norwegian broiler production pyramid was limited. In the NORM-VET programme, cephalosporin-resistant *E. coli* had been found at several levels of the broiler production pyramid, but there was lack of knowledge of the epidemiology of these bacteria. Also, there was a need to establish knowledge about how the resistance situation developed, and to identify and estimate possible risk factors for the presence of cephalosporin-resistant *E. coli* in Norwegian broiler flocks. In order to identify successful clones and/or resistance plasmids, and possibly determine their origin, studies on the genetic relationships between resistant isolates and resistance plasmids were needed. This would provide information regarding the ability of cephalosporin-resistant *E. coli* to persist in a population without selection pressure from antimicrobial use.

7. Aims of the study

The aim of the study was to provide knowledge about cephalosporin-resistant *E. coli* in the Norwegian broiler production pyramid, in order to give scientifically based advice to the authorities and the broiler industry regarding preventive measures for introduction, persistence and spread of cephalosporin-resistant *E. coli*.

The aim was accomplished through the following objectives:

- Determination of the occurrence of cephalosporin-resistant *E. coli* in the Norwegian broiler production pyramid (Papers I and II).
- Creation of a model for identification and estimation of risk factors for occurrence of cephalosporin-resistant *E. coli* in the Norwegian broiler production (Paper II).
- Characterization of cephalosporin-resistant *E. coli* and their associated resistance plasmids (Papers I, III and IV).
- Determination of the ability of cephalosporin-resistant *E. coli* and their plasmids to disseminate and persist in the Norwegian broiler production pyramid (Papers I-IV).

8. Summary of materials and methods

This section gives a brief summary of the sample materials and methods used in the study. Methodological considerations will be addressed in the discussion. A detailed description of materials and methods is provided in Papers I-IV.

8.1. Materials

8.1.1. Study population

In Paper II, 62 randomly selected commercial broiler producers affiliated to a single broiler production company and hatchery, namely Nortura Samvirkekylling were considered for inclusion. This affiliation was chosen because Nortura SA was partner in the project, and it enabled us to obtain information regarding sampled flocks, including ancestry, flock size, slaughter age and slaughter date. Furthermore, we were able to sample the parent flocks most likely to supply the broiler flocks with day-old chickens. This provided us with the opportunity to detect a possible association between occurrence of cephalosporin-resistant *E. coli* in supplying parent flocks and in receiving broiler flocks. In order to be included in the study, broiler farmers had to fulfil the following inclusion criteria; active commercial broiler producer, farm located in Hedmark county, affiliation to Nortura Samvirkekylling only, respond to questionnaire, and provide samples from a minimum of two broiler flocks housed at the farm during the study period. The final study group consisted of 27 broiler producers with a total of 34 broiler houses.

8.1.2. Samples collected from parent- and broiler flocks

Boot swabs (Sodibox, Nevez, France) were collected from selected parent flocks every four weeks from November 2013 to October 2014. Furthermore, boot swabs were collected from broiler flocks at selected farms from February 2014 to January 2015. All samples were collected by the farmers and sent per mail to the NVI in Oslo for analysis. The sampling was performed in order to estimate the occurrence of cephalosporin-resistant *E. coli* in Norwegian parent and broiler flocks. Furthermore, the results were used to identify and estimate risk factors for occurrence of cephalosporin-resistant *E. coli* in broiler flocks (Paper II).

8.1.3. Samples collected in previous studies

AmpC-producing isolates originating from healthy broiler chickens (Papers I and III), parent animals, grandparent animals (Paper I), and retail chicken meat (Papers I and III) were available from the in-house strain collection at the NVI. The isolates were collected within the frame of the NORM-VET programme in 2011 (broilers), 2012 (retail meat and parent animals) and 2014 (retail meat) (NORM/NORM-VET, 2012, 2013, 2015), a survey conducted on an imported grandparent flock in 2011, and a small study initiated by the largest poultry cooperative in Norway (Nortura SA) in 2011 (grandparent- and parent animals in production). Samples from the imported grandparent flock (day-old) were collected from paper linings and meconium. Broiler-, parent- and grandparent flocks in production were sampled using boot swabs. Retail meat samples were collected at retail following a proportionate stratified sampling scheme representing production units from all areas of Norway with broiler production.

Serratia spp. included in Paper IV were selected from the in-house strain collection at Nofima and originated from retail chicken meat and food processing units in Norway.

8.1.4. Collection of data for identification of risk factors (Paper II)

Online questionnaire

Randomly selected broiler producers affiliated to Nortura Samvirkekylling (n=62) were requested to respond to an online questionnaire distributed per e-mail. The questions referred to antimicrobial use at the farm, the size of the farm and the production, management factors at the farm, and the area surrounding the farm and broiler house(s). Also, farmers were asked to describe the washing and disinfection routines applied in the broiler house between production cycles.

Data provided by Nortura SA

Nortura SA provided data on the status of grandparent flocks (i.e. cephalosporin-resistant *E. coli* present or absent) supplying the included parent flocks, and the ancestry of parent and broiler flocks sampled. In addition, flock size, slaughter age and slaughter date for each sampled broiler flock was provided.

Data on status of parent- and broiler flocks

Data on the status of parent and broiler flocks (i.e. cephalosporin-resistant *E. coli* present or absent) were obtained from the samples collected as described in section 8.1.2. Nine parent flocks supplying day-old chickens to the sampled broiler flocks were not sampled in Paper II. However, information regarding their status was available from the action plan against cephalosporin-resistant *E. coli* initiated by the poultry industry (Animalia, 2015).

8.2. Laboratory methods

8.2.1. Detection and identification of cephalosporin-resistant *E. coli*

Boot swabs (Paper I) were dissolved in sterile water and plated directly onto MacConkey agar supplemented with 1 mg/L cefotaxime and MacConkey agar supplemented with 2 mg/L ceftazidime. For other samples, overnight pre-enrichment in MacConkey broth (retail meat samples, Paper I), peptone water (meconium and paper linings, Paper I) or in MacConkey broth supplemented with 1 mg/L cefotaxime (boot swabs, Paper II) was done before plating out as described above. Agar plates were incubated for 24-48 hours at 37-38 ^{o}C. Colonies displaying typical morphology were selected and confirmed as *E. coli* using API 20 E (bioMérieux, Marcy L'Etoile, Rhone, France) or PCR (Heininger et al., 1999) (Paper I), or by the use of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF, Bruker Daltonics GmbH, Bremen, Germany) (Paper II). All isolates included in Paper III were collected in the NORM-VET programme, and had previously been confirmed as *E. coli* (NORM/NORM-VET, 2012, 2013, 2015).

8.2.2. Antimicrobial susceptibility testing

Isolates with suspected ESBL/AmpC-production were subjected to disk-diffusion as recommended by EUCAST (www.eucast.org) to determine the cephalosporin resistance phenotype (Papers I and II). Furthermore, isolates included in Paper I were subjected to broth microdilution to determine the minimum inhibitory concentrations (MICs) to a panel of different antimicrobials (VET-MIC^{TM} GN-mo, National Veterinary Institute, Uppsala, Sweden). Recipient- and transconjugant isolates included in Paper IV were also subjected to MIC determination by the use of the broth microdilution method in order to determine the beta-lactam resistance pattern before and after acquisition of a plasmid encoding cephalosporin resistance (EUVSEC2, Sensititre®, TREK Diagnostic LTD, Thermo Scientific, Grinstead, UK).

8.2.3. Characterization of cephalosporin-resistant *E. coli*

All *E. coli* isolates included in the study displayed cephalosporin resistance with an AmpC-phenotype. In Paper I, all isolates were subjected to PCR (Pérez-Pérez and Hanson, 2002) and sequencing to determine the gene responsible for resistance. Isolates with a negative PCR result were further investigated for mutations in the promoter region of the chromosomal *ampC* gene (Agersø et al., 2012). Isolates collected in Paper II were subjected to real-time PCR with previously published primers and probe (Schmidt et al., 2015) for detection of bla_{CMY-2}. Isolates included in Paper III had previously been characterized with respect to AmpC-encoding genes. Transconjugants obtained from conjugation experiments (Paper III and IV) were subjected to real-time PCR as described above to confirm transfer of plasmids carrying bla_{CMY-2}.

Isolates included in Paper III were subjected to thorough characterization. To obtain an overall classification of all isolates, they were subjected to phylotyping (Doumith et al., 2012). For more discriminatory typing, pulsed-field gel electrophoresis (PFGE) with the *Xba*I restriction enzyme was performed (Ribot et al., 2006; Sunde et al., 2015). Isolates originating from retail meat that were non-typeable by PFGE (with *Xba*I) were subjected to multiple-locus variable number tandem repeat analysis (MLVA) including 10 loci (Løbersli et al., 2012). Finally, WGS was performed on 15 isolates using Illumina technology. WGS was conducted by BGI (BGI Tech solutions Co. Ltd., Hong Kong). Sequences were used to perform *in silico* typing using tools available online from Centre for Genomic Epidemiology, Technical University Denmark (www.genomicepidemiology.org). Furthermore, single nucleotide polymorphism (SNP) analysis was performed using CSI Phylogeny 1.1 (Kaas et al., 2014).

8.2.4. Characterization of bla_{CMY-2} carrying plasmids

To determine whether plasmids carrying bla_{CMY-2} were conjugative, all isolates included in Paper III were subjected to conjugation experiments in broth. *E. coli* DH5α (nalidixic acid resistant) was used as recipient strain for isolates that were quinolone susceptible, while *E. coli* One Shot^{TM} with pCRTM II vector encoding kanamycin resistance was used as recipient strain for isolates that were quinolone resistant. Transconjugants obtained from the conjugation experiments in Paper III were subjected to PCR- based replicon typing using the commercial PBRT-kit (Diatheva, Fano, Italy) to determine the replicon type of plasmids transferred to the recipient strain.

The nucleotide sequence of an IncK plasmid carrying *bla*$_{CMY-2}$ (pNVI1292/IncK) was reconstructed from a WGS sequence (Paper III). Genes encoded on the plasmid sequence were annotated using a combination of RAST v. 4.0 (Aziz et al., 2008) and manual annotation in CLC Main Workbench (CLC bio, Qiagen, Aarhus, Denmark).

In Paper IV, further investigations of the transferability of the IncK (pNVI1292/IncK) and IncI1 (pNVI2798/IncI1) plasmids with *bla*$_{CMY-2}$ were performed. Selected *E. coli* and *Serratia* spp. were included as recipient strains. Conjugation experiments were performed by colony mating on agar plates, in broth, and in biofilm. For colony mating, one fresh colony of the donor strain was mixed with one fresh colony of the recipient strain on a tryptone soya agar (TSA) plate. Broth matings were prepared as described earlier (Sunde and Sørum, 2001). For the biofilm experiments, a biofilm of the recipient strain was prepared on steel coupons as previously described (Nesse et al., 2014). Thereafter, the steel coupons were washed and transferred to tubes containing overnight cultures of the donor strain (Król et al., 2011). All experiments in Paper IV were performed under various conditions mimicking those relevant for food processing units.

8.3. Statistical methods

Data management and statistical analysis of significance was performed in SAS Enterprise guide version 4.3 for Windows (Paper I) or version 6.1 (Paper II) (SAS Institute Inc., Cary, NC, USA). Multivariable logistic regression analysis was performed to create a model predicting risk factors (Paper II). The model was built in R i386 version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria) using the glm command in the lme4 library to model generalized linear models. The study unit was a broiler flock and the outcome variable was the status of the flock (i.e. cephalosporin-resistant *E. coli* present or absent). All potential risk factors were first tested for association to flock status (i.e. cephalosporin-resistant *E. coli* present or absent) by univariable analysis. All independent variables with $p \leq 0.20$ were available for inclusion in the multivariable analysis. Association between variables available for inclusion in the multivariable model was investigated using the Pearson Chi-square test. The presence of a possible nested random effect of house within farm was also investigated. The final model was built using manual stepwise backward selection.

8.3.1. Study design

The risk factor study (Paper II) was designed as a longitudinal study with broiler flock as the study unit.

9. Summary of papers

9.1. Paper I. Emergence of AmpC-producing *Escherichia coli* in the broiler production chain in a country with a low antimicrobial usage profile

Samples were collected from all levels of the Norwegian broiler production pyramid, including retail chicken meat (n=649), and analysed using a selective method for detection. Cephalosporin-resistant *E. coli* were detected at all levels of the pyramid, with the occurrence ranging from 8-43 %. The beta-lactam resistance profile displayed by all cephalosporin-resistant isolates was equivalent to an AmpC-phenotype. The majority of the isolates were found to harbour the $bla_{\text{CMY-2}}$ gene, while a few isolates had up-regulated chromosomal *ampC* production. The use of antimicrobial agents in the Norwegian broiler production is minimal, and cephalosporins have never been used. Despite the lack of selection pressure from antimicrobial use, AmpC-producing *E. coli* were disseminated throughout the Norwegian broiler production pyramid.

9.2. Paper II. Risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in Norwegian broiler flocks

A longitudinal study of broiler flocks affiliated to the largest broiler cooperative in Norway was performed to determine risk factors for occurrence of cephalosporin-resistant *E. coli* in Norwegian broiler flocks. Four risk factors were identified; positive status (i.e. cephalosporin-resistant *E. coli* present) of the previous broiler flock in the same house, three or more parent flocks supplying the broiler flock with day-old chickens, transport personnel occasionally entering the room where the broilers are raised, and irregular disinfection of floors in the broiler house between production cycles. The results indicated that implementation of a high level of biosecurity, including rigorous cleaning and disinfection routines, would be favourable in order to minimize the occurrence of cephalosporin-resistant *E. coli* in Norwegian broiler flocks.

9.3. Paper III. Plasmid and host strain characteristics of *Escherichia coli* resistant to extended-spectrum cephalosporins in the Norwegian broiler production

E. coli isolates with pAmpC-production (n=232) were characterized and compared to investigate the possibility of successful clones and/or plasmids being present. All isolates were subjected to phylotyping, PFGE and conjugation experiments. PCR-based replicon typing was performed on transconjugants. Subsets of isolates were subjected to MLVA and

WGS, respectively. The nucleotide sequence of an IncK plasmid harbouring *bla*$_{CMY-2}$ was determined. The genetic diversity was higher in isolates originating from broiler intestines than among isolates originating from retail chicken meat. Two clusters of isolates belonging to ST38 and ST1158, respectively, were detected. Isolates in both clusters grouped into phylotype D and harboured IncK plasmids with *bla*$_{CMY-2}$. Genes encoding plasmid stability systems were present within the IncK nucleotide sequence. SNP analysis revealed few differences between isolates within the same ST. Also, highly similar IncK plasmids were present in both ST38 and ST1158 isolates. Both clonal expansion and horizontal transfer of plasmids have contributed to the dissemination of pAmpC-producing *E. coli* in the Norwegian broiler production. Also, high homology was detected between the IncK plasmid originating from Norwegian retail chicken meat and an IncK plasmid originating from Dutch retail meat, indicating that a this plasmid could be widespread within the European broiler production.

9.4. Paper IV. Transfer potential of plasmids encoding extended-spectrum cephalosporin resistance in *Escherichia coli* from poultry into different *Enterobacteriaceae* under various conditions

Two *E. coli* donors (isolated from retail chicken meat) harbouring an IncK (pNVI1292/IncK) and an IncI1 (pNVI2798/IncI1) plasmid, respectively, were subjected to conjugation experiments with *E. coli* and *Serratia* spp. recipients. The recipients originated from retail chicken meat, broiler intestines or food processing units. Colony mating on agar, broth mating and conjugation experiments in biofilm was performed at different temperatures to investigate the ability of the plasmids to self-transfer under different conditions. Plasmid transfer was observed using all three methods. Both the pNVI1292/IncK and the pNVI2798/IncI1 plasmids were able to self-transfer to the *E. coli* recipient. Furthermore, pNVI1292/IncK transferred to *Serratia marcescens* recipients. Interestingly, *S. marcescens* could act as a secondary donor of pNVI1292/IncK, and it was further transferred to *E. coli* and *S. proteamaculans* recipients. Transfer of plasmids was observed in conditions sub-optimal for bacterial growth, indicating a large potential for horizontal transfer of IncK and IncI1 plasmids. Both *Serratia* spp. and bacteria in biofilms have been shown to have an increased ability to survive disinfection. The ability of the plasmids to self-transfer to *Serratia* spp. and to hosts with good biofilm forming ability, together with the potential of transconjugants to act as secondary donors, could contribute to the maintenance of these plasmids throughout the food chain.

10. Discussion

10.1. Material and methodological considerations

10.1.1. Materials and methods applied in the laboratory

Bacterial isolates

All *E. coli* isolates included in Papers I, III and IV originated from the in-house strain collection at the NVI and were collected from grandparent animals, parent animals, broiler chickens and retail chicken meat between 2011 and 2014. The majority of isolates originated from the NORM-VET programme. In NORM-VET, samples are collected using defined sampling schemes and are thus considered as a representative selection of the actual population of *E. coli* at the different levels of the Norwegian broiler production pyramid. Some isolates included in Paper I (parent flocks and one grandparent flock sampled in 2011) originated from a small study performed by the largest broiler cooperative in Norway (Nortura SA) and a small study on imported breeding animals performed at the NVI. Recent results from the broiler industry's action plan against cephalosporin-resistant bacteria have shown that the occurrence varies between the different broiler cooperatives (Animalia, 2015, 2016). Therefore, over-representation of samples from the company with the lowest occurrence in Paper I and Paper II may have biased the results to some degree.

Detection of cephalosporin-resistant E. coli

All cephalosporin-resistant *E. coli* included in the present study were detected using a selective method as described in section 8.2.1. Some isolates were detected using a single selection step, while others were detected using double selection. Double selection is recommended by the European Food Safety Authority (EFSA) in order to achieve optimal sensitivity for detection of cephalosporin-resistant *E. coli* (EFSA, 2011). It is assumed that the sensitivity for the detection method using double selection is higher than 95% (Wasyl et al., 2010). For isolates collected in Paper II, both methods were performed in parallel (unpublished data). Following use of a single selection step, the occurrence of pAmpC-producing *E. coli* was lower in both parent and broiler flocks than when double selection was used (8.6 % vs 13.8 % and 13.9 % vs 22.5 %, respectively) (Paper II and unpublished data). It is important to bear in mind that the use of different methods for detection means that direct comparison of occurrence of cephalosporin-resistant *E. coli* in the various studies is not possible.

Characterization of cephalosporin-resistant E. coli

Phylotyping was performed to roughly classify isolates included in Paper III. The protocol suggested by Doumith et al. (2012) was used, although an updated protocol including more phylotypes (A, B1, B2, C, D, E, F and clade I-V) (Clermont et al., 2013) than the one used (A, B1, B2 and D) has been published. All isolates were subjected to further typing by PFGE, and a subset subjected to MLVA and WGS. Therefore, the additional discrimination provided by the updated phylotyping protocol provided by Clermont et al. (2013) would have been of limited value. Also, use of the already established protocol enabled comparison with reports from other countries using the same method.

PFGE is considered the "golden standard" molecular typing method for several bacterial species, and is known to have good reproducibility and discriminatory power (Tenover et al., 1997; Olive and Bean, 1999). However, the banding pattern of DNA fragments produced after cutting with a restriction enzyme does not provide information of the genetic content of each band. Thus, it is not possible to conclude that isolates are genetically identical (Tenover et al., 1997), only that their banding patterns are indistinguishable or highly similar, and that the isolates are probably closely related (Tenover et al., 1995; Tenover et al., 1997). A single genetic event can cause up to three differences in banding patterns between two isolates (Olive and Bean, 1999), which must be taken into consideration when interpreting the results. A set of criteria for interpretation of PFGE banding patterns of epidemiologically linked isolates have been proposed. However, these criteria should be applied with caution if the isolates investigated are not epidemiologically related (Tenover et al., 1995). Although PFGE does not provide the same level of discrimination as WGS, it is known to be highly associated with the epidemiological relatedness between strains (CDC, 2016). Also, the banding patterns are relatively easy to interpret using software such as BioNumerics (Applies Maths, Sint-Martens-Latem, Belgium). The PFGE method for *E. coli* was already established in our laboratory, and the software for interpretation of banding patterns was available. Therefore, PFGE was chosen as the main method for comparison of cephalosporin-resistant *E. coli* in Paper III.

Some isolates were non-typeable by PFGE. This could be due to degradation of DNA in the agarose plugs. Such degradation can be prevented by the use of thiourea in the electrophoresis buffer (Silbert et al., 2003). It has also been suggested that methylation of sites targeted by the restriction enzyme prevents enzymatic cutting of the DNA strand (Sales

et al., 2007), resulting in discrepancies in PFGE banding patterns. It is, however, considered unlikely that such methylation would have occurred at all restriction sites on the genome. A possible solution to work around the problem of methylation could be to use another restriction enzyme targeting different restriction sites. Instead of performing modified PFGE methods using the highly mutagenic thiourea, or another restriction enzyme than *Xba*I, MLVA was performed on a selection of PFGE non-typeable isolates in order to identify whether they were closely related or genetically diverse. This was done because the method using thiourea in the PFGE electrophoresis buffer or another restriction enzyme was not established in our laboratory, while MLVA was well established.

MLVA has been proposed as a typing method which can be a fast and easy alternative to PFGE, and that might replace PFGE as the method of choice for genotyping of *E. coli* (Løbersli et al., 2012). By using MLVA, isolates that were non-typeable by PFGE could be genotyped. This has also been previously reported, although PFGE was shown to be somewhat more discriminatory (Christiansson et al., 2011).

WGS has been proposed as a good alternative for surveillance and typing of *E. coli* (Zankari et al., 2013; Joensen et al., 2014). Data provided by WGS and standardized phenotypic methods for surveillance of antimicrobial resistance (Zankari et al., 2012) as well as typing and outbreak detection of *E. coli* (Joensen et al., 2014) have a high compliance. Furthermore, WGS provides a rapid and cost-effective typing method compared to the many phenotypic methods that are often needed in order to achieve sufficient discrimination between strains (Joensen et al., 2014). WGS data would have provided a substantial amount of information regarding each strain, and thus a more reliable assumption regarding the genetic similarity between included strains would be available. In retrospect, WGS would most likely have been used extensively in Paper III if the study started at a later time point. Recently, several online tools for *in silico* typing of WGS data have been made available by the Centre for Genomic Epidemiology at the Technical University of Denmark (www.genomicepidemiology.org) and other institutions. For *in silico* detection of antimicrobial resistance genes, several online databases exist (Zankari et al., 2012; McArthur et al., 2013; Gupta et al., 2014). The availability of these online tools simplifies the typing of sequenced isolates. At the time the study started, the knowledge and skills on handling and assembling raw WGS data were limited at the NVI. Also, the price of WGS was higher and the quality of the sequences was not as good as they are today, possibly

complicating assembly and subsequent sequence analysis. Therefore, only a limited number of strains were subjected to WGS in Paper III (n=15).

Conjugation experiments

Conjugation experiments in broth were performed in order to evaluate the self-transmissibility of *bla*$_{CMY-2}$ carrying plasmids. Two different recipient strains were used, depending on the resistance phenotype of the donor isolate. Sampling of the matings was done after four, six and/or 24 hours (Paper III). The majority of the *bla*$_{CMY-2}$ carrying plasmids were self-transmissible. However, some isolates grouping in the main cluster after PFGE analysis, seemed to carry non-transferrable plasmids. This could be erroneous, as isolates grouping closely together in the PFGE analysis are considered to be closely related, and thus are expected to contain the same plasmids. The possible false classification of some of these plasmids as non-transmissible might be due to a low transfer frequency, causing the number of transconjugants to be under the detection limit for the method used. It is also possible that the method is not optimal, and that the time of mating should be longer, or the ratio between donor and recipient strain in the initial mating should be different. Another possible explanation could be that non-transferable plasmids had acquired insertions or other modifications in the nucleotide sequence encoding important proteins involved in the transfer process. This could lead to a non-functional transfer region, leaving the plasmids unable to self-transfer by conjugation into novel hosts (Hansen et al., 2016).

Further conjugation experiments were performed in biofilm. Briefly, a biofilm of the recipient strain was established on a coupon of stainless steel (Nesse et al., 2014). Subsequently, the steel coupon was transferred to a tube with a culture of the donor strain. The method used was derived from that described by Król et al. (2011). However, on the basis of this method, transmission of the plasmid in the broth in which the steel coupon was submerged could not be excluded. Possibly, recipient cells from the biofilm could have detached, entered the broth, received the plasmid from donor cells in the broth and subsequently re-attached to the biofilm. Thus, we cannot be sure that the transmission of the plasmid actually occurred in the biofilm. Use of antimicrobial supplement in the broth could have minimized the probability of this occurring, as the recipient strain would have been killed if entering the broth (Solheim et al., 2013). Broth- and colony matings were run in parallel with the biofilm experiments. Transconjugants were generally detected earlier in broth matings. This could indicate that the conjugation actually occurred in the biofilm, as it

is expected to occur at an earlier time-point in broth. However, it is also possible that the time lag is due to the need for recipients to detach from the biofilm and enter the broth before conjugation can occur, and then re-attaching to the biofilm before they can be detected. On the other hand, it has been suggested that biofilms are hot-spots for conjugative transfer of plasmids (Jefferson, 2004; Sørensen et al., 2005), and conjugative transfer of plasmids in biofilm has been reported previously (Król et al., 2011; Król et al., 2013). Therefore, it is tempting to conclude that the conjugative transfer of plasmids in biofilm observed in Paper IV is genuine.

No plasmid transfer was observed at 12°C in our experiments. This could have been due to a low growth rate of the bacteria or a low number of bacteria in the experiments performed at this temperature. However, self-transfer of plasmids at temperatures down to 8°C have been observed previously (Fernandez-Astorga et al., 1992). Thus, it is possible that horizontal transfer might have occurred in our experiments as well, but at a rate too low to be detected by the methods used. Therefore, we cannot exclude the possibility that pNVI1292/IncK and pNVI2798/IncI1 can self-transfer at low temperatures relevant for food processing.

Sampling the biofilms to investigate whether conjugation had occurred included harvesting the whole biofilm. The steel coupon was rinsed with peptone water to remove unattached cells. In some cases, this led to loosening of large parts of the biofilm. Possibly, this might have led to errors when counting the number of donors, recipients and transconjugants in the samples.

To estimate the transfer frequencies of plasmids from donor to recipients in the biofilm, the transconjugant/recipient (T/R) ratio was calculated based on plating on different selective agar plates, and counting the number of donors, recipients and transconjugants in the sample. A drawback of this method is that we are not able to determine if all transconjugants had received the plasmid from a donor, if a few transconjugants expanded in the biofilm (Sørensen et al., 2005), or if transconjugant strains acted as plasmid donors themselves. Therefore, we cannot be certain that the transfer frequencies calculated are accurate.

10.1.2. Materials and methods used in the epidemiological study

Study design

In Paper II, a longitudinal study was performed in order to identify possible risk factors for occurrence of cephalosporin-resistant *E. coli* in broiler flocks. This study design was chosen because the status of the flocks was unknown, and we wanted to include flocks with a variety of possible exposures of interest for the analysis. The study population was selected from a defined geographic area. This can minimize the risk of unmeasured confounding, as the study population will have numerous characteristics in common, e.g. climatic factors (Dohoo et al., 2010a). A multivariable logistic regression analysis was performed in order to determine the association between the status of a broiler flock (i.e. cephalosporin-resistant *E. coli* present or absent) and a variety of possible risk factors. The favourable situation regarding occurrence of cephalosporin-resistant *E. coli* in the Norwegian broiler production enabled us to include both positive and negative flocks, which facilitated the identification of risk factors.

Selection bias

The target population was broiler producers in Norway and the source population was broiler producers in Hedmark county affiliated to a single hatchery, namely Nortura Samvirkekylling. From the source population, 62 broiler producers were randomly selected and invited to participate in the study. The final study group consisted of 29 broiler producers.

The source population was selected due to cooperation with Nortura SA in the current project. This cooperation enabled acquisition of data regarding ancestry of parent- and broiler flocks included in the study, status (i.e. presence or absence of cephalosporin-resistant *E. coli*) of grandparent flocks supplying parent flocks included in the study, slaughter age, slaughter date, and flock size of sampled broiler flocks.

Only a single broiler hybrid, namely Ross 308, was represented at the farms included in the study. As both hybrid and hatchery of origin have been identified as risk factors for occurrence of cephalosporin-resistant *E. coli* in a previous study (Persoons et al., 2011), it cannot be excluded that these factors might be of importance in Norway as well.

Non-response bias

The response rate of included broiler producers in Paper II was somewhat low (46.8 %) in spite of repeated reminders. It is possible that producers with a higher interest in AMR and higher levels of caring for their animals were more prone to respond than producers with a lower interest for participating in the study. It has been suggested that non-response can be an indication of management differences relating to the outcome (Dohoo et al., 2010b). This should be kept in mind when interpreting the results from Paper II, as it might have led to an under-estimation of the effect of infrequent disinfection of floors and transport personnel entering the house. Furthermore, not all flocks housed at individual farms included in the study were sampled. This, together with the low response rate led to the sample size being smaller than expected. The small sample size might have increased the relative impact of the random error (Dohoo, 2014).

Information bias

An online questionnaire distributed per e-mail to selected broiler producers was the main data source in Paper II. The questionnaire was based on one previously used to collect information regarding possible risk factors for campylobacteriosis in Norwegian broiler farms (Lyngstad et al., 2008), and was prepared in close collaboration with a representative from Nortura SA. Furthermore, it was pilot tested by consultants working with Norwegian broiler farmers to minimize the risk of information bias. However, the possibility of producers misinterpreting questions and/or ticking wrong boxes when answering the questionnaire cannot be ruled out. The included producers replied to the questionnaire prior to the start of sample collection, and samples from their respective broiler flocks were collected during the course of a year. Therefore, it is possible that some of the answers given in the questionnaire regarding e.g. some management factors might have changed during the course of the study. It is difficult to predict the direction of any possible bias resulting from this. Furthermore, it is possible that factors not considered for inclusion in the questionnaire can represent true risk factors that were impossible to detect in this study.

Some samples received in the laboratory were not marked with a sampling date or age of the flock at sampling. Thus, information bias regarding flock age at sampling can be present. This was handled by assigning the mean age at sampling for all included flocks to the flocks where the information was lacking.

Information regarding ancestry for parent and broiler flocks, slaughter age and slaughter date for broiler flocks, and status of grandparent flocks supplying the included parent flocks was provided by Nortura SA. Errors in their databases cannot be excluded, but are considered highly unlikely.

Semi-quantitative studies done on caecal content from Norwegian broilers have shown that only a small fraction of *E. coli* present in the caeca displays cephalosporin resistance (NORM/NORM-VET, 2015). Thus, a detection method with high sensitivity is required. EFSA recommends the use of double selection, i.e. selective enrichment in broth supplemented with cephalosporins before plating out on agar supplemented with cephalosporins (EFSA, 2011). It is assumed that the method used in Paper II has 100 % specificity, and a conservative estimate for the sensitivity is 95 % (Wasyl et al., 2010). Therefore, the possibility of some flocks being misclassified as negative although cephalosporin-resistant *E. coli* are in fact present (false negatives) cannot be excluded. It is possible that this could have influenced the results in the multivariable logistic regression model. However, if the true prevalence is calculated based on the assumption of 95 % sensitivity, a minimal change is observed from 13.8 % to 14.5 % in parent flocks and 22.5 % to 23.7 % in broiler flocks. It is therefore assumed that this possible misclassification of outcome would not have significant influence on the output of the model presented in Paper II.

Another factor that could possibly influence the probability of correct classification is the sampling method. Sampling was performed by each producer, and they were instructed to follow the same sampling method as applied in the Norwegian *Salmonella* spp. control programme (http://www.vetinst.no/eng/Surveillance-programmes/Salmonella). It is not possible to know how thoroughly the sampling was performed. However, both parent- and broiler producers are used to collecting samples from their flocks for the monitoring programme on *Salmonella* spp. The sampling method using boot swabs may have influenced the probability of detecting cephalosporin-resistant *E. coli* if they were in fact present in the flock. Possibly, other methods, such as direct sampling of a defined number of animals in the flock, sampling of caeca at slaughter, or using more than one pair of boot swabs per sampling might be more sensitive. The use of boot swabs is cheap and easy to perform, and was therefore chosen as the method for sample collection in Paper II.

Confounding

A confounding factor is associated with both a variable of interest and the outcome, and might therefore influence the association between these two (Vittinghoff et al., 2012a, b). In the analysis performed in Paper II, confounders were controlled for by offering all non-significant variables one-by-one in the final model. If a confounding factor was present, a large change in the model estimates would be expected (Vittinghoff et al., 2012a). The possible presence of confounding factors associated with the farm and broiler house were controlled for by including a nested random effect of house within farm in the analysis. Confounding factors explained by such random effects could include the managerial ability of the farmer or similar factors that are difficult to measure (Dohoo, 2014). The nested random effect of house within farm was not significant. Although no confounding factors were detected, the presence of confounders not adjusted for cannot be excluded.

External validity

The main focus of this study was to determine the occurrence of and characterize cephalosporin-resistant *E. coli* in the Norwegian broiler production pyramid. Also, risk factors for occurrence of cephalosporin-resistant *E. coli* were identified. The use of antimicrobial agents to food-producing animals is generally higher in other European countries compared to Norway (Grave et al., 2014). Furthermore, the size of the broiler production industry is larger in many other European countries (Marquer et al., 2015). Therefore, the results presented in this thesis are probably not directly valid for countries where a different situation regarding antimicrobial use and occurrence of cephalosporin-resistant *E. coli* is seen.

For countries such as Iceland, Sweden and Denmark, where a situation similar to that in Norway has been reported (DANMAP, 2011; SVARM, 2011; Agersø et al., 2012; DANMAP, 2012; SVARM, 2012; Börjesson et al., 2013b; Börjesson et al., 2013c; SWEDRES/SVARM, 2013; Agersø et al., 2014; DANMAP, 2014; SWEDRES/SVARM, 2014; MAST, 2015), the results from this study might be partly valid.

Recent investigations have shown that the situation regarding the occurrence of cephalosporin-resistant *E. coli* in the broiler production pyramid differs between different hatcheries and geographical locations in Norway (Animalia, 2015, 2016). Therefore, it is possible that the risk factors identified in the longitudinal study presented in Paper II are not

directly applicable to the entire Norwegian broiler production. However, the Norwegian broiler production is quite uniform, and it is assumed that the same risk factors may apply for the whole country, only with different estimates of the effect. Also, other additional risk factors for occurrence of cephalosporin-resistant *E. coli* in broiler flocks might be present in other parts of Norway. The risk factors identified in Paper II might also be extrapolated to populations in other countries that are similar to Norway and with similar exposure to antimicrobials. Furthermore, high levels of biosecurity, including rigorous cleaning and disinfection routines will probably generally be advantageous when attempting to combat cephalosporin-resistant *E. coli* in the broiler production.

The genetic characteristics of pAmpC-producing *E. coli* reported in Paper III might to some degree be representative for the corresponding bacterial population in Sweden and Denmark, but a more heterogeneous population has been reported from other countries (Smet et al., 2008; Bortolaia et al., 2010; Cortés et al., 2010; Dierikx et al., 2010; Overdevest et al., 2011; Egea et al., 2012; Ewers et al., 2012; Belmar Campos et al., 2014; Huijbers et al., 2014; Vogt et al., 2014; Day et al., 2016). However, *E. coli* with $bla_{\text{CMY-2}}$ on IncK or IncI1 plasmids with $bla_{\text{CMY-2}}$ are widespread in the European broiler production (Dierikx et al., 2010; Börjesson et al., 2013b; Börjesson et al., 2013c; Voets et al., 2013; Agersø et al., 2014; Egervärn et al., 2014; Börjesson et al., 2016). Also, ST38, which was a common ST for pAmpC-producing *E. coli* in Norwegian broilers and retail chicken meat (Paper III), has also been reported from other countries (Börjesson et al., 2013b; Dierikx et al., 2013a; Voets et al., 2013; Agersø et al., 2014). Furthermore, a high similarity of both IncK and IncI1 plasmids originating from the Netherlands (Voets et al., 2013; de Been et al., 2014; Smith et al., 2015) with IncK and IncI1 plasmids originating from *E. coli* in Norwegian retail chicken meat has been found (Papers III-IV and unpublished data). Thus, the results from the characterization of host strains and plasmids might, in part, be extrapolated to other European counties. It is important to keep in mind that chicken meat samples described in this study were collected from fresh fillets at retail. Knowledge on how packaging in modified atmosphere and the subsequent storage at low temperatures influence the *E. coli* flora on the meat is scarce. Possibly, the dominating *E. coli* ST38 and ST1158 with their IncK plasmids (Paper III) can have an advantage in this environment, but this remains to be determined. If samples from chicken meat had been collected in a different manner, for example from whole carcases at abattoirs, the composition of the *E. coli* flora could possibly be different from that described in this study.

10.2 Results and general discussion

10.2.1. Occurrence of cephalosporin-resistant *E. coli* in the Norwegian broiler production

By the use of a selective detection method, cephalosporin-resistant *E. coli* were identified at all levels of the Norwegian broiler production pyramid in Paper I. All isolates displayed an AmpC-phenotype. The occurrence ranged from 8-43 % with the highest levels found in broiler flocks, closely followed by retail chicken meat. In Paper II, the occurrence of pAmpC-producing *E. coli* in selected parent- and broiler flocks affiliated to Nortura Samvirkekylling was estimated. The occurrence was found to be 13.8 % and 22.2 % in parent- and broiler flocks, respectively. However, a more sensitive method using double selection was used for analysis of these samples. In parallel, the method with only a single selection step was also applied (direct plating on MacConkey agar supplemented with cephalosporin). When a single selection step was used, the occurrence of pAmpC-producing *E. coli* was 8.6 % in parent flocks and 13.9 % in broiler flocks (unpublished data). These results show that the occurrence of pAmpC-producing *E. coli* in parent flocks decreased significantly from 2011 to 2012 ($p < 0.05$), and has remained at a stable relatively low level until 2014 (Paper I and unpublished data). For broilers, a significant decrease was seen from 43 % in 2011 (Paper I) to 13.9 % in 2014-2015 ($p < 0.05$) (unpublished data). For retail chicken meat, a relatively stable occurrence was seen from 32 % in 2012 (Paper I) to 28.9 % in 2014 (NORM/NORM-VET, 2015). The same method for sampling and detection of cephalosporin-resistant *E. coli* was applied for broilers and retail chicken meat, respectively, sampled in different years, enabling direct comparison of the results.

In the NORM-VET programme, the selective method for detection of cephalosporin-resistant *E. coli* was performed in parallel to a non-selective method for detection of indicator *E. coli* from the same samples (NORM/NORM-VET, 2012, 2013). When the non-selective method was applied, only 0.5-1.0 % of the indicator *E. coli* isolates displayed cephalosporin-resistance (NORM/NORM-VET, 2012, 2013). In 2014, a semi-quantitative method was also applied (NORM/NORM-VET, 2015). The results showed that in the majority of broiler flocks tested, a very low level of the total *E. coli* population in the caeca displayed cephalosporin resistance. In addition, less than 0.2 cfu/g cephalosporin-resistant *E. coli* were detected in the vast majority of positive retail meat samples (NORM/NORM-VET, 2015). This indicated that in general, the quantity of cephalosporin-resistant *E. coli* relative to the total number of *E. coli* in positive broiler flocks and meat samples was low.

Also, the difference in occurrences revealed by the use of the two different selective detection methods (single- and double selection step, respectively), support this theory. The within-flock occurrence of cephalosporin-resistant *E. coli* in Norwegian broiler flocks has so far not been assessed.

Interestingly, all cephalosporin-resistant *E. coli* included and collected in this study had a beta-lactam resistance profile with an AmpC-phenotype, displaying cefoxitin resistance and cloxacillin synergy. A limited number of isolates were found to have mutations in the promoter region of the chromosomal *ampC* gene, resulting in increased expression (Paper I). The remaining isolates displayed pAmpC-production, mediated by the $bla_{\text{CMY-2}}$ gene (Papers I-III), which has also been frequently associated with the broiler production in Europe (Ewers et al., 2012; Börjesson et al., 2013b; Börjesson et al., 2013c; Dierikx et al., 2013a; Dierikx et al., 2013b; Agersø et al., 2014). However, a much more heterogeneous combination of cephalosporin resistance genes, also including genes from the CTX-M, SHV and TEM classes of beta-lactamases, are found in the broiler production in several European countries (Smet et al., 2008; Bortolaia et al., 2010; Cortés et al., 2010; Dierikx et al., 2010; Overdevest et al., 2011; Egea et al., 2012; Ewers et al., 2012; Agersø et al., 2014; Belmar Campos et al., 2014; Huijbers et al., 2014; Vogt et al., 2014; Day et al., 2016).

10.2.2. Risk factors for occurrence of cephalosporin-resistant *E. coli* in Norwegian broilers

In order to give scientifically based advice regarding preventive measures against cephalosporin-resistant *E. coli* in Norwegian broiler flocks, a study aimed at identifying risk factors for occurrence was performed (Paper II). Several risk factors had already been reported from a Belgian study (Persoons et al., 2011), but the results could not be directly extrapolated to the Norwegian situation. This was due to considerable differences in the Norwegian and Belgian broiler production with regard to antimicrobial use, size of the farms, and the size of the flocks and the production (Persoons et al., 2012; Dewulf et al., 2013).

Four risk factors were identified. At the flock level, a positive status (i.e. cephalosporin-resistant *E. coli* present) of the previous broiler flock in the same house and three or more parent flocks supplying day-old chickens to the broiler flock were associated with an increased risk of occurrence of cephalosporin-resistant *E. coli*. At the house level, not disinfecting the floors between each production cycle, and transport personnel occasionally

entering the broiler house was associated with increased risk of a positive broiler flock. A positive status of the previous flock was identified as the most important risk factor (odds ratio [OR]=12.7). This was interpreted as an indication that recirculation of cephalosporin-resistant *E. coli* can occur between consecutive flocks in the same house. Also, the fact that houses in which disinfection of floors was performed between each production cycle had a lower occurrence of cephalosporin-resistant *E. coli* (OR=0.1), supported this theory. Survival and recirculation of cephalosporin-resistant *E. coli* in broiler houses between production cycles has also been suggested by others (Hiroi et al., 2012a; Dierikx et al., 2013a; Laube et al., 2013; Agersø et al., 2014; Nilsson et al., 2014). Furthermore, transport personnel occasionally entering the room where the broilers are raised represented a significant increase in the odds of a flock being positive (OR=9.3). This variable was closely associated with the variable describing number of people visiting the broiler house during a production cycle. Thus, it seems that this variable explains an increased risk of cross-contamination from surrounding areas, hatcheries and/or other broiler holdings when the number of people entering the broiler house increases. Although no statistical association between the status of the supplying parent flocks and the status of the broiler flocks receiving day-old chickens from these parent flocks was identified, a significant increase in the odds of being positive was detected by increasing number of parent flocks (three or more) supplying the broiler flock (OR=6.3). It cannot be excluded that some of the parent flocks might have been positive even though no cephalosporin-resistant *E. coli* were detected by the method used. Therefore, it is possible that when several parent flocks with a undetectable positive status supply a broiler flock with day-old chickens, the burden of cephalosporin-resistant *E. coli* accumulates, resulting in a positive broiler flock.

Overall, it seems that the first introduction of cephalosporin-resistant *E. coli* into the Norwegian broiler production pyramid was through import of breeding animals (NORM/NORM-VET, 2007; Sunde et al., 2009). However, as the import of breeding animals positive for cephalosporin-resistant *E. coli* is currently minimal (Animalia, 2015, 2016) local recirculation on farms seems to play a more significant role at present (Paper II). Also, the results indicate that a high level of biosecurity including rigorous cleaning and disinfection routines between production cycles will contribute to preventing occurrence of cephalosporin-resistant *E. coli* in Norwegian broiler flocks.

10.2.3. Characterization of cephalosporin-resistant *E. coli*

Characterization of pAmpC-producing E. coli

In Paper III, all included isolates (n=232) were characterized by phylotyping and PFGE. Furthermore, isolates originating from meat that were non-typeable by PFGE (n=20) were subjected to MLVA. Also, 15 isolates were thoroughly characterized by the use of WGS. The isolates characterized in Paper III included all pAmpC-producing *E. coli* detected in samples from broilers in 2011 and retail meat in 2012 and 2014 collected within the frame of the NORM-VET programme, and are considered to represent an estimate of the true situation in Norway.

Isolates originating from broilers were more heterogeneous with respect to both phylotype and PFGE banding patterns. A large PFGE cluster consisting of 69 isolates (≥ 80 % similarity of banding patterns), including 20 isolates from broilers and 49 isolates from meat was identified. Within this cluster, 25 isolates with identical PFGE profiles (≥ 97 % similarity of banding patterns) were detected. Isolates from both broilers (n=4) and meat (n=21) were included. Of the isolates subjected to MLVA, 15 isolates from 2014 displayed highly similar MLVA-profiles, indicating a close genetic relationship. Of the 15 isolates subjected to WGS, five grouped in the main PFGE cluster, 5 grouped right outside the main PFGE cluster, and five isolates were non-typeable by PFGE, but displayed highly similar MLVA profiles. Analysis of the WGS data *in silico* (www.genomicepidemiology.org) revealed that the ten isolates sharing similar PFGE banding patterns belonged to ST38 and serotype O7:H18. Isolates with highly similar MLVA profiles belonged to ST1158 and serotype O17/77:H34. When analysing the number of SNPs between isolates subjected to WGS (Kaas et al., 2014), few SNP differences were identified between isolates with highly similar PFGE banding patterns and between isolates with highly similar MLVA profiles. Also, isolates originating from different sources (i.e. broiler intestine and retail meat) showed few SNP differences, as did isolates collected in different years. Together, these results suggest that clonal dissemination of ST38 and ST1158 has occurred in the Norwegian broiler production. *E. coli* ST38 with pAmpC production has also been reported from broilers and retail chicken meat in other European countries (Börjesson et al., 2013b; Dierikx et al., 2013a; Voets et al., 2013; Agersø et al., 2014; Vogt et al., 2014; Hansen et al., 2016). Therefore, it is tempting to suggest that clonal dissemination of a successful *E. coli* ST38 clone with pAmpC-production has occurred across the European broiler production.

Characterization of plasmids harbouring bla_{CMY-2}

Conjugation experiments and replicon typing was performed on all pAmpC-producing isolates included in Paper III. In addition, the nucleotide sequence of an IncK plasmid associated with *E. coli* ST38 was determined.

The vast majority of plasmids associated with $bla_{\text{CMY-2}}$ were conjugative. In isolates originating from broilers, an equal distribution of IncK and IncI1 plasmids was seen. In isolates originating from retail chicken meat, however, IncK plasmids were largely predominant. Determination of the nucleotide sequence of an IncK plasmid, pNVI1292/IncK (accession number KU312044) and annotation of its encoded genes, revealed the presence of two plasmid stability systems, namely *relBE/stbDE* and *pndAC* (Paper III). These systems were also identified in an IncI1 plasmid (pNVI2798/IncI1) originating from retail chicken meat (unpublished data). Plasmid stability systems provide a fitness advantage for bacteria carrying the plasmid. This is due to post-segregational killing of plasmid-free daughter cells, and also by elimination of incompatible plasmids from the bacteria during replication (Unterholzner et al., 2013). The presence of these plasmid stability systems might provide some part of the explanation of how pAmpC-producing *E. coli* are maintained in the Norwegian broiler production despite the minimal use of antimicrobials.

Analysis of IncK plasmids using CSI Phylogeny (Kaas et al., 2014) with the nucleotide sequence of pNVI1292/IncK as a reference, revealed the presence of highly similar plasmids in all *E. coli* isolates subjected to WGS, including both ST38 and ST1158 isolates. Also, close homology to an IncK plasmid isolated from retail chicken meat in the Netherlands in 2010 (Paper III), and to IncK plasmids isolated from parent animals and broilers in Denmark, and retail meat both produced in and imported to Denmark during 2009-2011 (Hansen et al., 2016) was observed. SNP analyses using the nucleotide sequence of two different IncI1 plasmids, originating from poultry sampled in the Netherlands in 2007 and 2008 (Smith et al., 2015) as reference, showed close homology between IncI1 plasmids from the Netherlands and pNVI2798/IncI1 from Norway (Paper IV and unpublished data). Thus, there are indications that several successful, possibly endemic, plasmids carrying $bla_{\text{CMY-2}}$ are disseminated across the European broiler production. In addition, the findings suggest that highly similar IncI1 (isolated from 2007-2012) and IncK

plasmids (isolated from 2009-2014) associated with *bla*$_{CMY-2}$ have circulated within the European broiler production for several years (Figure 11).

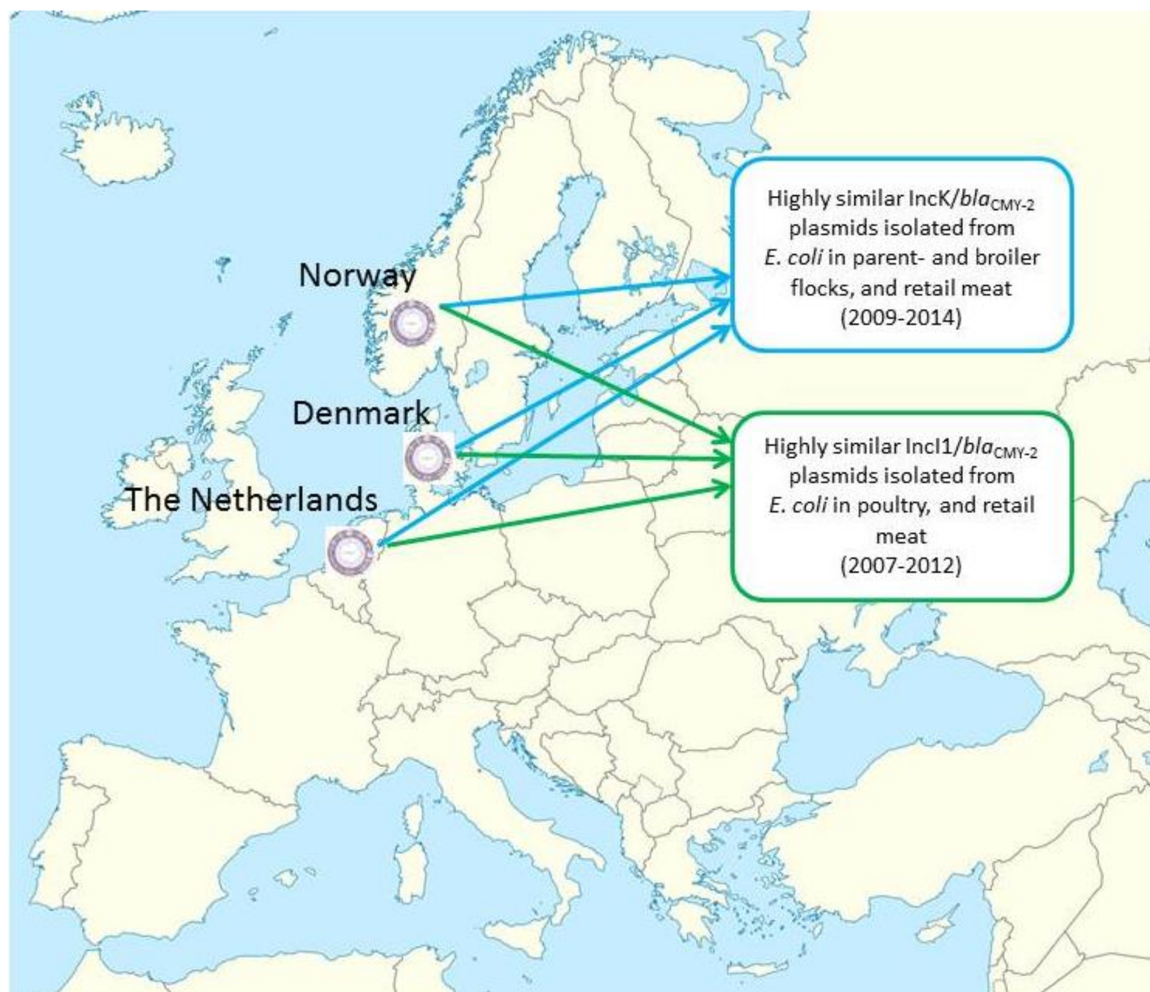


Figure 11. Map showing European countries where highly similar IncK and/or IncI1 plasmids carrying *bla*$_{CMY-2}$ originating from the broiler production have been identified. Illustration: S. S. Mo.

The pNVI1292/IncK and pNVI2798/IncI1 plasmids were further subjected to conjugation experiments using *E. coli* (indicator *E. coli* from broiler intestine) and *Serratia* spp. (originating from retail chicken meat and disinfecting footbaths) as recipients in Paper IV. In order to investigate the transferability of these two plasmids in conditions relevant for the broiler production, conjugation experiments were performed at different temperatures and under both planktonic and biofilm modes of growth. Plasmid transfer was observed using colony mating on agar, broth mating and in biofilm at 25°C, 30°C (matings including *Serratia* spp.) and 37°C (matings including *E. coli* only). The pNVI2798/IncI1 plasmid was transferred to the *E. coli* recipient, but no transfer was observed when *S. marcescens* was used as recipient. The pNVI1292/IncK plasmid on the other hand was transferred to both *E.*

coli and *S. marcescens* recipients. Furthermore, the *S. marcescens* transconjugant could transfer the pNVI1292/IncK plasmid on to both *E. coli* and *S. proteamaculans* recipients. Transfer of pNVI1292/IncK, and pNVI2798/IncI1 was observed at suboptimal growth conditions, and also in biofilms. The plasmids could be transferred into bacterial hosts with good biofilm forming abilities, and to *Serratia* spp. (only pNVI1292/IncK).

Overall, the molecular characterization of pAmpC-producing *E. coli* pointed towards a scenario where both horizontal spread of plasmids harbouring $bla_{\text{CMY-2}}$ and clonal expansion of successful *E. coli* clones has occurred in the Norwegian broiler production. Also, the pNVI1292/IncK and pNVI2798/IncI1 plasmids appear to have a large potential for horizontal spread to different hosts and under different conditions. Furthermore, successful IncK and IncI1 plasmids seem to be pandemic in the European broiler production.

10.2.4. Persistence of cephalosporin-resistant *E. coli* in the Norwegian broiler production

Initially, imported breeding animals were suspected as the source of introduction of cephalosporin-resistant *E. coli* into the Norwegian broiler production (NORM/NORM-VET, 2007; Sunde et al., 2009). Identification of broiler flocks with pAmpC-producing *E. coli* scattered throughout Norway supported this theory of a common source (Paper I), as there is little or no contact between farms. The identification of pAmpC-producing *E. coli* ST38 (Paper III), previously detected in the broiler production in several European countries (Börjesson et al., 2013b; Dierikx et al., 2013a; Voets et al., 2013; Agersø et al., 2014; Vogt et al., 2014; Hansen et al., 2016) in addition to the occurrence of $bla_{\text{CMY-2}}$ harbouring plasmids with high homology in the Netherlands (Voets et al., 2013; de Been et al., 2014; Smith et al., 2015), Denmark (Hansen et al., 2016) and Norway (Paper III and unpublished data), also support this assumption. The detection of similar STs and highly similar resistance plasmids may indicate a common source of cephalosporin-resistant *E. coli*, and it is tempting to assume that the sources are the breeding companies supplying the vast majority of the European broiler production. Also, pAmpC-producing *E. coli* have been detected in day-old grandparent animals imported to Sweden (SVARM, 2011), Norway (unpublished data) and the Netherlands (Dierikx et al., 2013b), which further supports this theory. Evidence substantiating vertical transmission of pAmpC-producing *E. coli* throughout the broiler production pyramid has been reported from Sweden (Nilsson et al., 2014). Therefore it seems legitimate to assume that cephalosporin-resistant *E. coli* have emerged in the top of the breeding pyramid as a consequence of cephalosporin use (Agersø

et al., 2014), and subsequently been vertically transferred downwards in the broiler production pyramid.

From January 1st 2012, the BPC voluntarily abandoned the use of cephalosporins in the broiler production (WorldPoultry, 2011). A rapid decrease in the occurrence of cephalosporin-resistant bacteria after discontinuing the use of cephalosporins in day-old chickens has been reported previously (Dutil et al., 2010; MARAN, 2015). Thus, it is likely that this measure implemented by the BPC has contributed to a decrease in the occurrence of cephalosporin-resistant *E. coli* at higher levels of the production pyramid where the ancestors of most Norwegian broilers have their origin (section 6.1. and Figure 1). Furthermore, an action plan against cephalosporin-resistant *E. coli* was initiated by the Norwegian poultry industry in 2014 (Animalia, 2013). The action plan included, amongst other things, sampling of all batches of imported parent animals after hatching, sampling of parent flocks in production, and increasing knowledge and awareness of the importance of biosecurity, including thorough on-farm cleaning and disinfection routines. Also, the Norwegian poultry industry required that antimicrobials were not routinely used at the higher levels of the breeding pyramid, and that the therapeutic use of antimicrobials (if any) had to be documented by the breeding companies. Furthermore, surveillance of the occurrence of cephalosporin-resistant *E. coli* in grandparent flocks supplying the Norwegian market was required. In both 2014 and 2015, a low occurrence of cephalosporin-resistant *E. coli* (5.7 % and 2.4 %, respectively) were detected in samples collected from imported batches of breeding animals. This low occurrence probably reflected the ban of cephalosporin use at higher levels of the broiler production pyramid. In parent flocks in production, the occurrences were 27.1 % in 2014 and 21.7 % in 2015 when the detection method with double selection was used (Animalia, 2015, 2016). These findings indicate that the primary source of cephalosporin-resistant *E. coli*, namely imported breeding animals, currently plays a minor role. It is possible that local recirculation takes place between subsequent parent flocks as suggested for broiler flocks (Paper II), as the occurrence at this level is higher than expected with the low occurrence seen in newly hatched parent animals. The increased focus on a high level of biosecurity including proper cleaning and disinfection routines in the action plan against cephalosporin-resistant *E. coli* address two of the risk factors identified in Paper II. Furthermore, it is assumed that the actions made will also minimize the possibility of carry-over of cephalosporin-resistant *E. coli* from one flock to the next. Therefore, it is likely that both the discontinuation of cephalosporin use

implemented by the BPC and the action plan initiated by the Norwegian poultry industry has played an important role in the significant decrease of cephalosporin-resistant *E. coli* observed during the course of this study (Papers I and II).

Findings in Paper IV emphasised the ability of *bla*$_{CMY-2}$ encoding plasmids to self-transfer to and persist in *Serratia* spp. and *E. coli* hosts with good biofilm forming abilities. Both *Serratia* spp. and bacteria in biofilms have an increased ability to withstand disinfection (Willinghan et al., 1996; Kumar and Anand, 1998; Langsrud et al., 2003; Shi and Zhu, 2009; Srey et al., 2013). Furthermore, biofilms are considered hot-spots for horizontal transfer of genes (Jefferson, 2004; Sørensen et al., 2005), which was also demonstrated in Paper IV. In addition, transconjugants were able to act as secondary plasmid donors. This underlines the possible contribution of environmental bacteria and biofilms in the maintenance and dissemination of plasmids encoding cephalosporin resistance. It is plausible that the existence of *bla*$_{CMY-2}$ carrying plasmids in such hosts might constitute a reservoir of cephalosporin-resistance determinants that can persist between production cycles, as suggested in Paper II. Possibly, intestinal colonisation of pAmpC-producing bacteria and/or horizontal dissemination of *bla*$_{CMY-2}$ carrying plasmids can take place when a new flock is introduced, causing this flock in turn to become positive.

Plasmid stability systems have been reported to play an essential role in the maintenance of IncF plasmids encoding *bla*$_{CTX-M-15}$ in the globally dispersed pathogenic clone *E. coli* ST131, even when antimicrobial selection pressure is absent (Mathers et al., 2015b). Therefore, it is reasonable to assume that the presence of plasmid stability systems on the pNVI1292/IncK (Paper III) and pNVI2798/IncI1 (unpublished data) plasmids are likely to play an important role in the maintenance of these plasmids in *E. coli* and possibly other *Enterobacteriaceae* in the Norwegian broiler production, as the use of antimicrobials is negligible in both parent- and broiler flocks (Animalia, 2015; Refsum, 2015; Animalia, 2016).

Overall, it is tempting to suggest that the observed interrelationship between successful *E. coli* clones (ST38 and ST1158) and IncK plasmids carrying *bla*$_{CMY-2}$ and plasmid stability systems, ensuring their stable segregation within these successful *E. coli* clones, represent an important part of the explanation of the ability of these bacteria to persist. Furthermore, the ability of environmental bacteria and biofilms to act as hosts and reservoirs for plasmids

encoding cephalosporin-resistance is also likely to play a significant role in the maintenance of these plasmids and their hosts in the Norwegian broiler production.

10.2.5. Preventive measures

As mentioned in section 10.2.4, the preventive measures already initiated by the BPC and the Norwegian poultry industry in the action plan against cephalosporin-resistant *E. coli* seem to have had a significant impact on the occurrence at different levels of the production. In order to minimize the occurrence of cephalosporin-resistant *E. coli* in the Norwegian broiler production, continuous awareness of the importance of strict biosecurity, including rigorous cleaning and disinfection routines, is important (Paper II). This will contribute to limit the probability of cross-contamination between contaminated and clean environments, and to prevent recirculation of resistant strains and/or plasmids on farms. Also, it is important to continue the surveillance of imported breeding material to ensure that the contribution of this important source of cephalosporin-resistant *E. coli* is minimized. Furthermore, a continued prudent use of antimicrobials is required in order to keep the situation in Norway as favourable as it is today.

11. Main conclusions

- Cephalosporin-resistant *E. coli* were detected at all levels of the Norwegian broiler production pyramid. Since 2011, a decrease in the occurrence of cephalosporin-resistant *E. coli* in parent- and broiler flocks was observed.

- The risk of occurrence of cephalosporin-resistant *E. coli* can be minimized by implementation of a high level of biosecurity with a minimal number of people entering the broiler house during each production cycle, and intensive washing and disinfection routines.

- All cephalosporin-resistant *E. coli* included in the study displayed an AmpC-phenotype, and the vast majority carried the $bla_{\text{CMY-2}}$ gene.

- pAmpC-producing *E. coli* isolated from broilers displayed a higher genetic diversity than isolates from retail chicken meat. Two *E. coli* sequence types, namely ST38 and ST1158, dominated among isolates from retail chicken meat.

- The plasmids associated with $bla_{\text{CMY-2}}$ were in general self-transmissible and belonged to the incompatibility groups IncK and IncI1.

- Highly similar IncK plasmids were present in *E. coli* of different sequence types. Furthermore, highly similar IncK and IncI1 plasmids were present in *E. coli* originating from the broiler production in Norway and in other European countries.

- Both horizontal transfer of $bla_{\text{CMY-2}}$ harboring plasmids and clonal dissemination of *E. coli* have facilitated the spread of pAmpC-producing *E. coli* in the Norwegian broiler production.

- Plasmids harboring $bla_{\text{CMY-2}}$ (pNVI1292/IncK and pNVI2798/IncI) could be transferred to other hosts in biofilm, and pNVI1292/IncK could be transferred to *Serratia* spp. Transconjugants were able to act as secondary plasmid donors. Transfer occurred at different temperatures, including temperatures sub-optimal for bacterial growth.

- The plasmids pNVI1292/IncK and pNVI2798/IncI1 both carried genes encoding plasmid stability systems, possibly explaining the ability of pAmpC-producing strains to survive within a broiler production without antimicrobial selection pressure.

- *Serratia* spp. and bacteria in biofilms have an increased ability to survive cleaning and disinfection. Therefore, the ability of pNVI1292/IncK and to some extent pNVI2798/IncI1 to transfer into such hosts might indicate that *Serratia* spp. and bacteria in biofilms can constitute a reservoir for resistance plasmids in the broiler production.

12. Future perspectives

- Continuation of in-depth characterization of cephalosporin-resistant *E. coli* in the Norwegian broiler production to keep knowledge of the molecular epidemiology up-to-date.

- Utilization of WGS to facilitate assessment of the genetic similarity between cephalosporin-resistant isolates and their plasmids originating from the broiler production in Norway and other European countries. This would allow identification of clones and/or resistance plasmids that are successful and possibly endemic in the European broiler production, and simplify detection of emerging clones and/or resistance plasmids. Also, comparison with isolates and plasmids originating from other sources, such as human infections, would be easier by the use of WGS. Online tools available for analysis of WGS data will also facilitate efficient typing and surveillance of resistant bacteria.

- Investigation of whether plasmids harboring bla_{CMY-2} are naturally occurring in other *Enterobacteriaceae* in the broiler production, and determine to what extent these plasmids are able to be stably maintained within these hosts. Furthermore, identifying whether biofilms containing pAmpC-producing bacteria and/or resistance plasmids occurs naturally in different parts of the broiler production might give comprehensive information regarding the ability of such strains and/or plasmids to persist in the production.

- Determination of factors causing some broiler farms to have a higher number of positive flocks (i.e. cephalosporin-resistant *E. coli* present) than others. It would be interesting to perform thorough sampling at these farms, including sampling before and after cleaning and disinfection, sampling of the surrounding environment and manure, and sampling of insects and rodents at the farm to determine the extent of contamination with cephalosporin-resistant bacteria. Furthermore, it would be interesting to investigate whether specific clones were more prone to survive on these farms, whether biofilms were present in the broiler houses, and whether these could be reservoirs for pAmpC-producing *E. coli* or plasmids with bla_{CMY-2}. This

could provide increased knowledge on factors affecting the high occurrence of cephalosporin-resistant *E. coli* on certain farms.

- Determination of the efficacy of different cleaning and disinfection strategies on elimination of cephalosporin-resistant bacteria and their plasmids under conditions relevant for the broiler production to identify factors essential for improved control of cephalosporin-resistant *E. coli*.

13. Errata

- Page 15: **Submitted to Preventive Veterinary Medicine after revision** corrected to **Preventive Veterinary Medicine 130, 112-118**
- Page 37: **conjugational** corrected to **conjugal**
- Page 43: **general discussion** corrected to **discussion**
- Page 47: (R Foundation for Statistical Computing, Vienna, Austria) font size corrected from 11 to 12
- Page 51: **selective step** corrected to **selection step**
- Page 55: **hotspots** corrected to **hot-spots**
- Page 63: **some of the parent flocks might be positive** corrected to **some of the parent flocks might have been**
- Page 69: **hotspot** corrected to **hot-spot**

14. References

Aarestrup, F., McDermott, P.F., Kahlmeter, G., 2007. Antimicrobial susceptibility testing. http://www.crl-ar.eu/data/images/pdfer/11%2007%20newsletter%20no%20%202.pdf (last accessed 13.06.2016).

Agersø, Y., Aarestrup, F.M., Pedersen, K., Seyfarth, A.M., Struve, T., Hasman, H., 2012. Prevalence of extended-spectrum cephalosporinase (ESC)-producing *Escherichia coli* in Danish slaughter pigs and retail meat identified by selective enrichment and association with cephalosporin usage. J. Antimicrob. Chemother. 67, 582-588.

Agersø, Y., Jensen, J.D., Hasman, H., Pedersen, K., 2014. Spread of extended spectrum cephalosporinase-producing *Escherichia coli* clones and plasmids from parent animals to broilers and to broiler meat in a production without use of cephalosporins. Foodborne Pathog. Dis. 11, 740-746.

Alonso, G., Baptista, K., Ngo, T., Taylor, D.E., 2005. Transcriptional organization of the temperature-sensitive transfer system from the IncHI1 plasmid R27. Microbiology 151, 3563-3573.

Ambler, R.P., 1980. The structure of beta-lactamases. Philos. Trans. R. Soc. Lond. B Biol. Sci. 289, 321-331.

Animalia, 2013. The poultry industry's action plan against ESBL-producing *E. coli* (Fjørfenæringas handlingsplan mot ESBL-bærende *E. coli*; in Norwegian). http://animalia.no/upload/FIler%20til%20nedlasting/Animalia/Handlingsplan_ESBL_06_desember2013.pdf (last accessed 05.05.2016).

Animalia, 2015. Actions to combat antimicrobial resistance works (Tiltak mot antibiotikaresistens virker; in Norwegian). http://animalia.no/Listesider/Aktuelt-og-fagstoff/Tiltak-mot-antibiotikaresistens-virker-/ (last accessed 31.05.2016).

Animalia, 2016. Continued decrease in the occurrence of resistant bacteria in poultry (Fortsatt nedgang i forekomst av resistente bakterier hos fjørfe; in Norwegian). http://animalia.no/Listesider/Aktuelt-og-fagstoff/Fortsatt-nedgang-i-forekomst-av-resistente-bakterier-hos-fjorfe/ (last accessed 31.05.2016).

Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., McNeil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., Zagnitko, O.,

2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics 9, 75.

Belmar Campos, C., Fenner, I., Wiese, N., Lensing, C., Christner, M., Rohde, H., Aepfelbacher, M., Fenner, T., Hentschke, M., 2014. Prevalence and genotypes of extended spectrum beta-lactamases in *Enterobacteriaceae* isolated from human stool and chicken meat in Hamburg, Germany. Int. J. Med. Microbiol. 304, 678-684.

Bennett, P.M., 1995. The spread of drug resistance. In: Baumberg, S., Young, J.P.W., Wellington, E.M.H., Saunders, J.R. (Eds.), Population genetics of bacteria. Cambridge University Press, Cambridge, 317-344.

Berg, E.S., Wester, A.L., Mo, S.S., Slettemeås, J.S., Steinbakk, M., Dahle, U.R., Samuelsen, O., Simonsen, G.S., Löhr, I.H., Jørgensen, S.B., Sunde, M., 2015. Highly similar cephalosporin resistant *Escherichia coli* and AmpC resistance plasmids found in both patients and poultry meat in Norway. ECCMID conference. Copenhagen, Denmark. http://www.unn.no/getfile.php/UNN%20INTER/Fagfolk/www.antibiotikaresistens.no/K-Res/Poster/2015%20ECCMID%20Sunde%20M%20et%20al.pdf (last accessed 01.06.2016).

Bortolaia, V., Guardabassi, L., Trevisani, M., Bisgaard, M., Venturi, L., Bojesen, A.M., 2010. High diversity of extended-spectrum beta-lactamases in *Escherichia coli* isolates from Italian broiler flocks. Antimicrob. Agents Chemother. 54, 1623-1626.

Bradford, P.A., 2001. Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. Clin. Microbiol. Rev. 14, 933-951.

Bradley, D.E., Taylor, D.E., Cohen, D.R., 1980. Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K-12. J. Bacteriol. 143, 1466-1470.

Briñas, L., Moreno, M.A., Zarazaga, M., Porrero, C., Sáenz, Y., García, M., Dominguez, L., Torres, C., 2003. Detection of CMY-2, CTX-M-14, and SHV-12 beta-lactamases in *Escherichia coli* fecal-sample isolates from healthy chickens. Antimicrob. Agents Chemother. 47, 2056-2058.

Brolund, A., 2014. Overview of ESBL-producing *Enterobacteriaceae* from a Nordic perspective. Infect. Ecol. Epidemiol. 4.

Burmølle, M., Bahl, M.I., Jensen, L.B., Sørensen, S.J., Hansen, L.H., 2008. Type 3 fimbriae, encoded by the conjugative plasmid pOLA52, enhance biofilm formation and transfer frequencies in *Enterobacteriaceae* strains. Microbiology 154, 187-195.

Bush, K., Jacoby, G.A., Medeiros, A.A., 1995. A functional classification scheme for beta-lactamases and its correlation with molecular structure. Antimicrob. Agents Chemother. 39, 1211-1233.

Börjesson, S., Bengtsson, B., Jernberg, C., Englund, S., 2013a. Spread of extended-spectrum beta-lactamase producing *Escherichia coli* isolates in Swedish broilers mediated by an incl plasmid carrying bla(CTX-M-1). Acta Vet. Scand. 55, 3.

Börjesson, S., Egervärn, M., Lindblad, M., Englund, S., 2013b. Frequent occurrence of extended-spectrum beta-lactamase- and transferable AmpC beta-lactamase-producing *Escherichia coli* on domestic chicken meat in Sweden. Appl. Environ. Microbiol. 79, 2463-2466.

Börjesson, S., Jernberg, C., Brolund, A., Edquist, P., Finn, M., Landén, A., Olsson-Liljequist, B., Tegmark Wisell, K., Bengtsson, B., Englund, S., 2013c. Characterization of plasmid-mediated AmpC-producing *E. coli* from Swedish broilers and association with human clinical isolates. Clin. Microbiol. Infect. 19, E309-311.

Börjesson, S., Ny, S., Egervärn, M., Bergström, J., Rosengren, A., Englund, S., Löfmark, S., Byfors, S., 2016. Limited Dissemination of Extended-Spectrum beta-Lactamase- and Plasmid-Encoded AmpC-Producing *Escherichia coli* from Food and Farm Animals, Sweden. Emerg. Infect. Dis. 22, 634-640.

Cantón, R., Novais, A., Valverde, A., Machado, E., Peixe, L., Baquero, F., Coque, T.M., 2008. Prevalence and spread of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in Europe. Clin. Microbiol. Infect. 14 Suppl 1, 144-153.

Carattoli, A., 2008. Animal reservoirs for extended spectrum beta-lactamase producers. Clin. Microbiol. Infect. 14 Suppl 1, 117-123.

Carattoli, A., 2009. Resistance plasmid families in *Enterobacteriaceae*. Antimicrob. Agents Chemother. 53, 2227-2238.

Carattoli, A., 2013. Plasmids and the spread of resistance. Int. J. Med. Microbiol. 303, 298-304.

Carattoli, A., Bertini, A., Villa, L., Falbo, V., Hopkins, K.L., Threlfall, E.J., 2005. Identification of plasmids by PCR-based replicon typing. J. Microbiol. Methods 63, 219-228.

Caroff, N., Espaze, E., Gautreau, D., Richet, H., Reynaud, A., 2000. Analysis of the effects of -42 and -32 *ampC* promoter mutations in clinical isolates of *Escherichia coli* hyperproducing AmpC. J. Antimicrob. Chemother. 45, 783-788.

CDC, 2016. Pulsed-field gel electrophoresis (PFGE). http://www.cdc.gov/pulsenet/pathogens/pfge.html (last accessed 21.05.2016).

Chantziaras, I., Boyen, F., Callens, B., Dewulf, J., 2014. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. J. Antimicrob. Chemother. 69, 827-834.

Christiansson, M., Melin, S., Matussek, A., Löfgren, S., Söderman, J., 2011. MLVA is a valuable tool in epidemiological investigations of *Escherichia coli* and for disclosing multiple carriage. Scand. J. Infect. Dis. 43, 579-586.

Clermont, O., Christenson, J.K., Denamur, E., Gordon, D.M., 2013. The Clermont *Escherichia coli* phylo-typing method revisited: improvement of specificity and detection of new phylo-groups. Environ. Microbiol. Rep. 5, 58-65.

Cohen Stuart, J., van den Munckhof, T., Voets, G., Scharringa, J., Fluit, A., Leverstein-van Hall, M.A., 2012. Comparison of ESBL contamination in organic and conventional retail chicken meat. Int. J. Food Microbiol. 154, 212-214.

Cooper, T.F., Heinemann, J.A., 2000. Postsegregational killing does not increase plasmid stability but acts to mediate the exclusion of competing plasmids. PNAS 97, 12643-12648.

Coque, T.M., Baquero, F., Canton, R., 2008a. Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. Euro Surveill. 13, 1-11.

Coque, T.M., Novais, A., Carattoli, A., Poirel, L., Pitout, J., Peixe, L., Baquero, F., Cantón, R., Nordmann, P., 2008b. Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. Emerg. Infect. Dis. 14, 195-200.

Cortés, P., Blanc, V., Mora, A., Dahbi, G., Blanco, J.E., Blanco, M., López, C., Andreu, A., Navarro, F., Alonso, M.P., Bou, G., Blanco, J., Llagostera, M., 2010. Isolation and characterization of potentially pathogenic antimicrobial-resistant *Escherichia coli* strains from chicken and pig farms in Spain. Appl. Environ. Microbiol. 76, 2799-2805.

Cox, G., Wright, G.D., 2013. Intrinsic antibiotic resistance: mechanisms, origins, challenges and solutions. Int. J. Med. Microbiol. 303, 287-292.

DANMAP, 2011. DANMAP 2010. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Copenhagen. ISSN 1600-2032.

DANMAP, 2012. DANMAP 2011. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Copenhagen. ISSN 1600-2032.

DANMAP, 2013. DANMAP 2012. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Copenhagen. ISSN 1600-2032.

DANMAP, 2014. DANMAP 2013. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Copenhagen. ISSN 1600-2032.

DANMAP, 2015. DANMAP 2014. Use of antimicrobial agents and occurrence of antimicrobial resistance in bacteria from food animals, food and humans in Denmark. Copenhagen. ISSN 1600-2032.

Davies, J., Davies, D., 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. Rev. 74, 417-433.

Day, M.J., Rodriguez, I., van Essen-Zandbergen, A., Dierikx, C., Kadlec, K., Schink, A.K., Wu, G., Chattaway, M.A., DoNascimento, V., Wain, J., Helmuth, R., Guerra, B., Schwarz, S., Threlfall, J., Woodward, M.J., Coldham, N., Mevius, D., Woodford, N., 2016. Diversity of STs, plasmids and ESBL genes among *Escherichia coli* from humans, animals and food in Germany, the Netherlands and the UK. J. Antimicrob. Chemother. 71, 1178-1182.

de Been, M., Lanza, V.F., de Toro, M., Scharringa, J., Dohmen, W., Du, Y., Hu, J., Lei, Y., Li, N., Tooming-Klunderud, A., Heederik, D.J., Fluit, A.C., Bonten, M.J., Willems, R.J., de la Cruz, F., van Schaik, W., 2014. Dissemination of cephalosporin resistance genes between *Escherichia coli* strains from farm animals and humans by specific plasmid lineages. PLoS Genet. 10, e1004776.

den Bakker, H.C., Allard, M.W., Bopp, D., Brown, E.W., Fontana, J., Iqbal, Z., Kinney, A., Limberger, R., Musser, K.A., Shudt, M., Strain, E., Wiedmann, M., Wolfgang, W.J., 2014. Rapid whole-genome sequencing for surveillance of *Salmonella enterica* serovar enteritidis. Emerg. Infect. Dis. 20, 1306-1314.

Dewulf, J., Hoet, B., Minne, D., 2013. Belgian veterinary surveillance of antimicrobial consumtion national consumption report, BelVet-SAC. Ghent University and Federal Agency for Medicines and Health products, , Ghent/Brussels.

Dierikx, C., van der Goot, J., Fabri, T., van Essen-Zandbergen, A., Smith, H., Mevius, D., 2013a. Extended-spectrum-beta-lactamase- and AmpC-beta-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers. J. Antimicrob. Chemother. 68, 60-67.

Dierikx, C., van Essen-Zandbergen, A., Veldman, K., Smith, H., Mevius, D., 2010. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. Vet. Microbiol. 145, 273-278.

Dierikx, C.M., van der Goot, J.A., Smith, H.E., Kant, A., Mevius, D.J., 2013b. Presence of ESBL/AmpC -Producing *Escherichia coli* in the Broiler Production Pyramid: A Descriptive Study. PLoS One 8, e79005.

Dohoo, I., Martin, W., Stryhn, H., 2010a. Cohort studies. In: McPike, S.M. (Ed.), Veterinary epidemiologic research. VER Inc., Prince Edward Island, Canada, 167-180.

Dohoo, I., Martin, W., Stryhn, H., 2010b. Validity in observational studies. In: McPike, S.M. (Ed.), Veterinary epidemiologic research. VER Inc., Prince Edward Island, Canada, 243-270.

Dohoo, I.R., 2014. Bias-is it a problem, and what should we do? Prev. Vet. Med. 113, 331-337.

Doi, Y., Paterson, D.L., Egea, P., Pascual, A., López-Cerero, L., Navarro, M.D., Adams-Haduch, J.M., Qureshi, Z.A., Sidjabat, H.E., Rodriguez-Baño, J., 2010. Extended-spectrum and CMY-type beta-lactamase-producing *Escherichia coli* in clinical samples and retail meat from Pittsburgh, USA and Seville, Spain. Clin. Microbiol. Infect. 16, 33-38.

Doumith, M., Day, M.J., Hope, R., Wain, J., Woodford, N., 2012. Improved Multiplex PCR Strategy for Rapid Assignment of the Four Major *Escherichia coli* Phylogenetic Groups. J. Clin. Microbiol. 50, 3108-3110.

Dutil, L., Irwin, R., Finley, R., Ng, L.K., Avery, B., Boerlin, P., Bourgault, A.M., Cole, L., Daignault, D., Desruisseau, A., Demczuk, W., Hoang, L., Horsman, G.B., Ismail, J., Jamieson, F., Maki, A., Pacagnella, A., Pillai, D.R., 2010. Ceftiofur resistance in *Salmonella enterica* serovar Heidelberg from chicken meat and humans, Canada. Emerg. Infect. Dis. 16, 48-54.

Dziekan, G., Jauregui, I.L., Mathai, E., 2012. The way forward: political commitment to enable options for action. In: WHO (Ed.), The evolving threat of antimicrobial resistance: Options for action. World Health Organization, Geneva, Switzerland, 91-94.

ECDC, 2010. Antimicrobial resistance surveillance in Europe 2009. Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). European Centre for Disease Prevention and Control, Stockholm. ISBN 978-92-9193-227-6.

ECDC, 2015. Antimicrobial resistance surveillance in Europe 2014. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). European Centre for Disease Prevention and Control, Stockholm. ISSN: 2363-2666.

ECDC, EMEA, 2009. The bacterial challenge: Time to react. European Centre for Disease Prevention and Control and European Medicines Agency, Stockholm. ISBN 978-92-9193-193-4

EFSA, 2008. Report from the task force on zoonoses data collection including guidance for harmonized monitoring and reporting of antimicrobial resistance in commensal *Escherichia coli* and *Enterococcus* spp. from food animals. EFSA journal 141, 1-44.

EFSA, 2011. Scientific opinion on the public health risks of bacterial strains producing extended-spectrum beta-lactamases and/or AmpC beta-lactamases in food and food-producing animals. EFSA journal 9, 2322.

EFSA, ECDC, 2016. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food, in 2014. EFSA journal 14, 4380.

EFTA, 2014. Agreement on the European economic area. The European Free Trade Association, http://www.efta.int/media/documents/legal-texts/eea/the-eea-agreement/Main%20Text%20of%20the%20Agreement/EEAagreement.pdf (last accessed 02.05.2016).

Egea, P., López-Cerero, L., Torres, E., Gómez-Sánchez Mdel, C., Serrano, L., Navarro Sánchez-Ortiz, M.D., Rodriguez-Baño, J., Pascual, A., 2012. Increased raw poultry meat colonization by extended spectrum beta-lactamase-producing *Escherichia coli* in the south of Spain. Int. J. Food Microbiol. 159, 69-73.

Egervärn, M., Börjesson, S., Byfors, S., Finn, M., Kaipe, C., Englund, S., Lindblad, M., 2014. *Escherichia coli* with extended-spectrum beta-lactamases or transferable AmpC beta-lactamases and *Salmonella* on meat imported into Sweden. Int. J. Food Microbiol. 171, 8-14.

EMA, 2013. European Surveillance of Veterinary Antimicrobial Consumption, Sales of veterinary antimicrobial agents in 25 EU/EEA countries in 2011. Third ESVAC Report. EMA/236501/2013.

Ewers, C., Bethe, A., Semmler, T., Guenther, T., Wieler, L.H., 2012. Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin. Microbiol. Infect. 18, 646-655.

Ewers, C., Bethe, A., Stamm, I., Grobbel, M., Kopp, P.A., Guerra, B., Stubbe, M., Doi, Y., Zong, Z., Kola, A., Schaufler, K., Semmler, T., Fruth, A., Wieler, L.H., Guenther, S., 2014. CTX-M-15-D-ST648 *Escherichia coli* from companion animals and horses: another pandemic clone combining multiresistance and extraintestinal virulence? J. Antimicrob. Chemother. 69, 1224-1230.

Ewers, C., Grobbel, M., Stamm, I., Kopp, P.A., Diehl, I., Semmler, T., Fruth, A., Beutlich, J., Guerra, B., Wieler, L.H., Guenther, S., 2010. Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum-beta-lactamase-producing *Escherichia coli* among companion animals. J. Antimicrob. Chemother. 65, 651-660.

Fernandez-Astorga, A., Muela, A., Cisterna, R., Iriberri, J., Barcina, I., 1992. Biotic and abiotic factors affecting plasmid transfer in *Escherichia coli* strains. Appl. Environ. Microbiol. 58, 392-398.

Furuya, E.Y., Lowy, F.D., 2006. Antimicrobial-resistant bacteria in the community setting. Nat. Rev. Microbiol. 4, 36-45.

Ghigo, J.M., 2001. Natural conjugative plasmids induce bacterial biofilm development. Nature 412, 442-445.

Giske, C.G., Sundsfjord, A.S., Kahlmeter, G., Woodford, N., Nordmann, P., Paterson, D.L., Cantón, R., Walsh, T.R., 2009. Redefining extended-spectrum beta-lactamases: balancing science and clinical need. J. Antimicrob. Chemother. 63, 1-4.

Grave, K., Kaldhusdal, M.C., Kruse, H., Harr, L.M., Flatlandsmo, K., 2004. What has happened in Norway after the ban of avoparcin? Consumption of antimicrobials by poultry. Prev. Vet. Med. 62, 59-72.

Grave, K., Torren-Edo, J., Muller, A., Greko, C., Moulin, G., Mackay, D., Group, E., 2014. Variations in the sales and sales patterns of veterinary antimicrobial agents in 25 European countries. J. Antimicrob. Chemother. 69, 2284-2291.

Guardabassi, L., Courvalin, P., 2006. Modes of antimicrobial action and mechanisms of bacterial resistance. In: Aarestrup, F.M. (Ed.), Antimicrobial resistance in bacteria of animal origin. ASM Press, Washington DC, 1-18.

Guardabassi, L., Kruse, H., 2008. Principles of prudent and rational use of antimicrobials in animals. In: Guardabassi, L., Jensen, L.B., Kruse, H. (Eds.), Guide to antimicrobial use in animals. Blackwell Publishing Ltd., Oxford, 1-12.

Gupta, S.K., Padmanabhan, B.R., Diene, S.M., Lopez-Rojas, R., Kempf, M., Landraud, L., Rolain, J.M., 2014. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrob. Agents Chemother. 58, 212-220.

Hansen, K.H., Bortolaia, V., Nielsen, C.A., Nielsen, J.B., Schønning, K., Agersø, Y., Guardabassi, L., 2016. Host-specific patterns of genetic diversity among IncI1-Igamma and IncK plasmids encoding CMY-2 beta-lactamase in *Escherichia coli* isolates from humans, poultry meat, poultry and dogs in Denmark. Appl. Environ. Microbiol. Epub ahead of print, doi:10.1128/AEM.00495-16.

Hawkey, P.M., 2015. Multidrug-resistant Gram-negative bacteria: a product of globalization. J. Hosp. Infect. 89, 241-247.

Hawkey, P.M., Jones, A.M., 2009. The changing epidemiology of resistance. J. Antimicrob. Chemother. 64 Suppl 1, i3-10.

Hayes, F., 2003. Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. Science 301, 1496-1499.

Heffron, F., Sublett, R., Hedges, R.W., Jacob, A., Falkow, S., 1975. Origin of the TEM-beta-lactamase gene found on plasmids. J. Bacteriol. 122, 250-256.

Heininger, A., Binder, M., Schmidt, S., Unertl, K., Botzenhart, K., Döring, G., 1999. PCR and blood culture for detection of *Escherichia coli* bacteremia in rats. J. Clin. Microbiol. 37, 2479-2482.

Hiroi, M., Matsui, S., Kubo, R., Iida, N., Noda, Y., Kanda, T., Sugiyama, K., Hara-Kudo, Y., Ohashi, N., 2012a. Factors for occurrence of extended-spectrum beta-lactamase-producing *Escherichia coli* in broilers. J. Vet. Med. Sci. 74, 1635-1637.

Hiroi, M., Yamazaki, F., Harada, T., Takahashi, N., Iida, N., Noda, Y., Yagi, M., Nishio, T., Kanda, T., Kawamori, F., Sugiyama, K., Masuda, T., Hara-Kudo, Y., Ohashi, N., 2012b. Prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in food-producing animals. J. Vet. Med. Sci. 74, 189-195.

Hommelsgård, M., 2016. Victory for the consumers: chicken fed with antibiotics phased out during this year (Seier for forbrukerne: Antibiotikafôret kylling fases ut i løpet av året; in Norwegian). Dagbladet. http://www.dagbladet.no/2016/01/13/tema/mat/narasin/kylling/fjorkre/42735751/ (last accessed 31.05.2016).

Huber, H., Zweifel, C., Wittenbrink, M.M., Stephan, R., 2013. ESBL-producing uropathogenic *Escherichia coli* isolated from dogs and cats in Switzerland. Vet. Microbiol. 162, 992-996.

Huijbers, P.M., de Kraker, M., Graat, E.A., van Hoek, A.H., van Santen, M.G., de Jong, M.C., van Duijkeren, E., de Greeff, S.C., 2013. Prevalence of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in humans living in municipalities with high and low broiler density. Clin. Microbiol. Infect. 19, E256-259.

Huijbers, P.M., Graat, E.A., Haenen, A.P., van Santen, M.G., van Essen-Zandbergen, A., Mevius, D.J., van Duijkeren, E., van Hoek, A.H., 2014. Extended-spectrum and AmpC beta-lactamase-producing *Escherichia coli* in broilers and people living and/or working on broiler farms: prevalence, risk factors and molecular characteristics. J. Antimicrob. Chemother. 69, 2669-2675.

Humeniuk, C., Arlet, G., Gautier, V., Grimont, P., Labia, R., Philippon, A., 2002. Beta-lactamases of *Kluyvera ascorbata*, probable progenitors of some plasmid-encoded CTX-M types. Antimicrob. Agents Chemother. 46, 3045-3049.

Hæggman, S., Löfdahl, S., Burman, L.G., 1997. An allelic variant of the chromosomal gene for class A beta-lactamase K2, specific for *Klebsiella pneumoniae*, is the ancestor of SHV-1. Antimicrob. Agents Chemother. 41, 2705-2709.

Jacoby, G.A., 2009. AmpC beta-lactamases. Clin. Microbiol. Rev. 22, 161-182.

Jakobsen, L., Kurbasic, A., Skjøt-Rasmussen, L., Ejrnaes, K., Porsbo, L.J., Pedersen, K., Jensen, L.B., Emborg, H.D., Agersø, Y., Olsen, K.E., Aarestrup, F.M., Frimodt-Møller, N., Hammerum, A.M., 2010. *Escherichia coli* isolates from broiler chicken meat, broiler chickens, pork, and pigs share phylogroups and antimicrobial resistance with community-dwelling humans and patients with urinary tract infection. Foodborne Pathog. Dis. 7, 537-547.

Jefferson, K.K., 2004. What drives bacteria to produce a biofilm? FEMS Microbiol. Lett. 236, 163-173.

Joensen, K.G., Scheutz, F., Lund, O., Hasman, H., Kaas, R.S., Nielsen, E.M., Aarestrup, F.M., 2014. Real-time whole-genome sequencing for routine typing, surveillance,

and outbreak detection of verotoxigenic *Escherichia coli*. J. Clin. Microbiol. 52, 1501-1510.

Johnson, J.R., Sannes, M.R., Croy, C., Johnston, B., Clabots, C., Kuskowski, M.A., Bender, J., Smith, K.E., Winokur, P.L., Belongia, E.A., 2007. Antimicrobial drug-resistant *Escherichia coli* from humans and poultry products, Minnesota and Wisconsin, 2002-2004. Emerg. Infect. Dis. 13, 838-846.

Kaas, R.S., Leekitcharoenphon, P., Aarestrup, F.M., Lund, O., 2014. Solving the problem of comparing whole bacterial genomes across different sequencing platforms. PLoS One 9, e104984.

Kluytmans, J.A., Overdevest, I.T., Willemsen, I., Kluytmans-van den Bergh, M.F., van der Zwaluw, K., Heck, M., Rijnsburger, M., Vandenbroucke-Grauls, C.M., Savelkoul, P.H., Johnston, B.D., Gordon, D., Johnson, J.R., 2013. Extended-spectrum beta-lactamase-producing *Escherichia coli* from retail chicken meat and humans: comparison of strains, plasmids, resistance genes, and virulence factors. Clin. Infect. Dis. 56, 478-487.

KOORIMP, 2012. Annual Report KOORIMP and KIF (Årsmelding 2011 KOORIMP og KIF; in Norwegian). Animalia, http://www.animalia.no/upload/FIler%20til%20nedlasting/KOORIMP/KOORIMP%20%C3%A5rsmelding%202011%20v6%20%20web.pdf (last accessed 23.05.2016).

Król, J.E., Nguyen, H.D., Rogers, L.M., Beyenal, H., Krone, S.M., Top, E.M., 2011. Increased transfer of a multidrug resistance plasmid in *Escherichia coli* biofilms at the air-liquid interface. Appl. Environ. Microbiol. 77, 5079-5088.

Król, J.E., Wojtowicz, A.J., Rogers, L.M., Heuer, H., Smalla, K., Krone, S.M., Top, E.M., 2013. Invasion of *E. coli* biofilms by antibiotic resistance plasmids. Plasmid 70, 110-119.

Kumar, C.G., Anand, S.K., 1998. Significance of microbial biofilms in food industry: a review. Int. J. Food Microbiol. 42, 9-27.

Langsrud, S., Møretrø, T., Sundheim, G., 2003. Characterization of *Serratia marcescens* surviving in disinfecting footbaths. J. Appl. Microbiol. 95, 186-195.

Laube, H., Friese, A., von Salviati, C., Guerra, B., Käsbohrer, A., Kreienbrock, L., Roesler, U., 2013. Longitudinal Monitoring of extended-spectrum-beta-lactamase/AmpC-Producing *Escherichia coli* in German Broiler Chicken Fattening Farms. Appl. Environ. Microbiol. 79, 4815-4820.

Lazarus, B., Paterson, D.L., Mollinger, J.L., Rogers, B.A., 2015. Do human extraintestinal *Escherichia coli* infections resistant to expanded-spectrum cephalosporins originate from food-producing animals? A systematic review. Clin. Infect. Dis. 60, 439-452.

Leverstein-van Hall, M.A., Dierikx, C.M., Cohen Stuart, J., Voets, G.M., van den Munckhof, M.P., van Essen-Zandbergen, A., Platteel, T., Fluit, A.C., van de Sande-Bruinsma, N., Scharinga, J., Bonten, M.J., Mevius, D.J., group, o.b.o.t.N.E.s., 2011. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin. Microbiol. Infect. 17, 873-880.

Levy, S.B., Marshall, B., 2004. Antibacterial resistance worldwide: causes, challenges and responses. Nat. Med. 10, S122-129.

Liebana, E., Carattoli, A., Coque, T.M., Hasman, H., Magiorakos, A.P., Mevius, D., Peixe, L., Poirel, L., Schuepbach-Regula, G., Torneke, K., Torren-Edo, J., Torres, C., Threlfall, J., 2013. Public health risks of enterobacterial isolates producing extended-spectrum beta-lactamases or AmpC beta-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. Clin. Infect. Dis. 56, 1030-1037.

López-Cerero, L., Egea, P., Serrano, L., Navarro, D., Mora, A., Blanco, J., Doi, Y., Paterson, D.L., Rodríguez-Baño, J., Pascual, A., 2011. Characterisation of clinical and food animal *Escherichia coli* isolates producing CTX-M-15 extended-spectrum beta-lactamase belonging to ST410 phylogroup A. Int. J. Antimicrob. Agents 37, 365-367.

Lovdata, 2001. Regulations regarding keeping of chickens and turkey (Forskrift om hold av høns og kalkun; in Norwegian). FOR-2001-12-12-1494.

Lovdata, 2004. Regulations regarding adjustment of pig- and poultry production (Forskrift om regulering av svine- og fjørfeproduksjon; in Norwegian). FOR-2004-04-01-611.

Lovdata, 2014. Regulation amending regulations regarding adjustment of pig- and poultry production (Forskrift om endring i forskrift om regulering av svine- og fjørfeproduksjonen; in Norwegian). FOR-2014-12-15-1907.

Lyngstad, T.M., Jonsson, M.E., Hofshagen, M., Heier, B.T., 2008. Risk factors associated with the presence of *Campylobacter* species in Norwegian broiler flocks. Poult. Sci. 87, 1987-1994.

Løbersli, I., Haugum, K., Lindstedt, B.A., 2012. Rapid and high resolution genotyping of all *Escherichia coli* serotypes using 10 genomic repeat-containing loci. J. Microbiol. Methods 88, 134-139.

Manges, A.R., 2016. *Escherichia coli* and urinary tract infections: the role of poultry-meat. Clin. Microbiol. Infect. 22, 122-129.

MARAN, 2015. MARAN 2015. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2014., Lelystadt.

Marquer, P., Rabade, T., Forti, R., 2015. Meat production statistics. Eurostat, http://ec.europa.eu/eurostat/statistics-explained/index.php/Meat_production_statistics (last accessed 23.05.2016).

MAST, 2015. ESBL/AmpC-producing *E. coli* in poultry in 2014 (ESBL/AmpC myndandi *E. coli* í alifuglum 2014; in Icelandic). Icelandic food and veterinary authority, http://mast.is/library/Eftirlitsni%C3%B0urst%C3%B6%C3%B0ur/Lyfjathol/LyfjatholESBLskimun2014SHS.pdf (last accessed 23.05.2016).

Mathers, A.J., Peirano, G., Pitout, J.D., 2015a. *Escherichia coli* ST131: The quintessential example of an international multiresistant high-risk clone. Adv. Appl. Microbiol. 90, 109-154.

Mathers, A.J., Peirano, G., Pitout, J.D., 2015b. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant *Enterobacteriaceae*. Clin. Microbiol. Rev. 28, 565-591.

McArthur, A.G., Waglechner, N., Nizam, F., Yan, A., Azad, M.A., Baylay, A.J., Bhullar, K., Canova, M.J., De Pascale, G., Ejim, L., Kalan, L., King, A.M., Koteva, K., Morar, M., Mulvey, M.R., O'Brien, J.S., Pawlowski, A.C., Piddock, L.J., Spanogiannopoulos, P., Sutherland, A.D., Tang, I., Taylor, P.L., Thaker, M., Wang, W., Yan, M., Yu, T., Wright, G.D., 2013. The comprehensive antibiotic resistance database. Antimicrob. Agents Chemother. 57, 3348-3357.

NAA, 2016. Market report 2015. Price- and market assessment of central Norwegian agricultural commodities and processed agricultural products (Markedsrapport 2015. Pris- og markedsvurderinger av sentrale norske landbruksråvarer og RÅK varer; in Norwegian). In: Himle, R. (Ed.) Norwegian Agriculture Agency, https://www.slf.dep.no/no/produksjon-og-marked/frukt-og-gront/marked-og-pris/god-tilgang-av-norske-landbruksvarer-i-2015 (last accessed 29.02.2016).

Naseer, U., Haldorsen, B., Simonsen, G.S., Sundsfjord, A., 2010. Sporadic occurrence of CMY-2-producing multidrug-resistant *Escherichia coli* of ST-complexes 38 and 448, and ST131 in Norway. Clin. Microbiol. Infect. 16, 171-178.

Naseer, U., Haldorsen, B., Tofteland, S., Hegstad, K., Scheutz, F., Simonsen, G.S., Sundsfjord, A., Norwegian, E.S.G., 2009. Molecular characterization of CTX-M-15-

producing clinical isolates of *Escherichia coli* reveals the spread of multidrug-resistant ST131 (O25:H4) and ST964 (O102:H6) strains in Norway. APMIS 117, 526-536.

Naseer, U., Natås, O.B., Haldorsen, B.C., Bue, B., Grundt, H., Walsh, T.R., Sundsfjord, A., 2007. Nosocomial outbreak of CTX-M-15-producing *E. coli* in Norway. APMIS 115, 120-126.

Naseer, U., Sundsfjord, A., 2011. The CTX-M conundrum: dissemination of plasmids and *Escherichia coli* clones. Microb Drug Resist 17, 83-97.

Nesse, L.L., Sekse, C., Berg, K., Johannesen, K.C., Solheim, H., Vestby, L.K., Urdahl, A.M., 2014. Potentially pathogenic *Escherichia coli* can form a biofilm under conditions relevant to the food production chain. Appl. Environ. Microbiol. 80, 2042-2049.

Nicolas-Chanoine, M.H., Blanco, J., Leflon-Guibout, V., Demarty, R., Alonso, M.P., Canica, M.M., Park, Y.J., Lavigne, J.P., Pitout, J., Johnson, J.R., 2008. Intercontinental emergence of *Escherichia coli* clone O25:H4-ST131 producing CTX-M-15. J. Antimicrob. Chemother. 61, 273-281.

Nilsson, O., Börjesson, S., Landén, A., Bengtsson, B., 2014. Vertical transmission of *Escherichia coli* carrying plasmid-mediated AmpC (pAmpC) through the broiler production pyramid. J. Antimicrob. Chemother. 69, 1497-1500.

NL, 2014. The sad story of the Norwegian poulty breedings demise (Den sørgelige historien om den norske fjørfeavlens endelikt; in Norwegian). The federation of Norwegian agricultural cooperatives, http://www.landbruk.no/Nyheter/Nyhetsartikler/Forskning/Den-soergelige-historien-om-den-norske-fjoerfeavlens-endelikt (last accessed 09.05.2016).

NORM/NORM-VET, 2007. NORM/NORM-VET 2006. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo. ISSN:1502-2307.

NORM/NORM-VET, 2012. NORM/NORM-VET 2011. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo. ISSN:1502-2307 (print)/1890-9965 (electronic).

NORM/NORM-VET, 2013. NORM/NORM-VET 2012. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo. ISSN:1502-2307 (print)/1890-9965 (electronic).

NORM/NORM-VET, 2015. NORM/NORM-VET 2014. Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway. Tromsø/Oslo. ISSN:1502-2307 (print)/1890-9965 (electronic).

Norman, A., Hansen, L.H., Sørensen, S.J., 2009. Conjugative plasmids: vessels of the communal gene pool. Phil. Trans. R. Soc. B 364, 2275-2289.

Nortura, 2014. Nortura with initiative to abandon use of narasin in commercial broiler production (Nortura tar inititativ for å kutte narasin i ordinær kyllingproduksjon; in Norwegian). http://www.nortura.no/pressesenter/2014/nortura-tar-intiativ-for-a-kutte-narasin-i-ordinar-kyllingproduksjon/ (last accessed 25.05.2016).

Nortura, 2015. Poultry production in Norway (Fjørfehold i Norge; in Norwegian). http://www.nortura.no/naturlig-kvalitet-fra-norske-bonder/kyllinghold/ (last accessed 09.05.2016).

Nortura, 2016. All Nortura broilers are now reared without narasin- six months before schedule (Nå er alle Nortyra-kyllinger narasinfrie- et halvt år før skjema; in Norwegian). http://www.nortura.no/pressesenter/na-er-alle-nortura-kyllinger-narasinfrie--et-halvt-ar-for-skjema/ (last accessed 16.06.2016).

Novick, R.P., 1987. Plasmid incompatibility. Microbiol. Rev. 51, 381-395.

O'Neill, J., 2016. Tackling drug-resistant infections globally: final report and recommendations. http://amr-review.org/sites/default/files/160518_Final%20paper_with%20cover.pdf (last accessed 23.05.2016).

Olive, D.M., Bean, P., 1999. Principles and applications of methods for DNA-based typing of microbial organisms. J. Clin. Microbiol. 37, 1661-1669.

Overdevest, I., Willemsen, I., Rijnsburger, M., Eustace, A., Xu, L., Hawkey, P., Heck, M., Savelkoul, P., Vandenbroucke-Grauls, C., van der Zwaluw, K., Huijsdens, X., Kluytmans, J., 2011. Extended-spectrum beta-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. Emerg. Infect. Dis. 17, 1216-1222.

Papich, M.G., Riviere, J.E., 2009. Betalactam antibiotics: penicillins, cephalosporins, and related drugs. In: Riviere, J.E., Papich, M.G., Adams, H.R. (Eds.), Veterinary pharmacology and therapeutics. Wiley-Blackwell, Ames, Iowa, 865-893.

Peirano, G., Pitout, J.D., 2010. Molecular epidemiology of *Escherichia coli* producing CTX-M beta-lactamases: the worldwide emergence of clone ST131 O25:H4. Int. J. Antimicrob. Agents 35, 316-321.

Pérez-Pérez, F.J., Hanson, N.D., 2002. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbiol. 40, 2153-2162.

Persoons, D., Dewulf, J., Smet, A., Herman, L., Heyndrickx, M., Martel, A., Catry, B., Butaye, P., Haesebrouck, F., 2012. Antimicrobial use in Belgian broiler production. Prev. Vet. Med. 105, 320-325.

Persoons, D., Haesebrouck, F., Smet, A., Herman, L., Heyndrickx, M., Martel, A., Catry, B., Berge, A.C., Butaye, P., Dewulf, J., 2011. Risk factors for ceftiofur resistance in *Escherichia coli* from Belgian broilers. Epidemiol. Infect. 139, 765-771.

Pfeifer, Y., Cullik, A., Witte, W., 2010. Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. Int. J. Med. Microbiol. 300, 371-379.

Philippon, A., Arlet, G., Jacoby, G.A., 2002. Plasmid-determined AmpC-type beta-lactamases. Antimicrob. Agents Chemother. 46, 1-11.

Pitout, J.D., Laupland, K.B., 2008. Extended-spectrum beta-lactamase-producing *Enterobacteriaceae*: an emerging public-health concern. Lancet Infect. Dis. 8, 159-166.

Poirel, L., Kämpfer, P., Nordmann, P., 2002. Chromosome-encoded Ambler class A beta-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases. Antimicrob. Agents Chemother. 46, 4038-4040.

Pomba, C., da Fonseca, J.D., Baptista, B.C., Correia, J.D., Martínez-Martínez, L., 2009. Detection of the pandemic O25-ST131 human virulent *Escherichia coli* CTX-M-15-producing clone harboring the *qnrB2* and *aac(6')-Ib-cr* genes in a dog. Antimicrob. Agents Chemother. 53, 327-328.

Prescott, J.F., 2014. The resistance tsunami, antimicrobial stewardship, and the golden age of microbiology. Vet. Microbiol. 171, 273-278.

Refsum, T., 2015. Antimicrobial use in the Norwegian poultry production (Antibiotikabehandling i norsk fjørfeproduksjon; in Norwegian). Go' mørning Animalia, http://www.animalia.no/upload/FIler%20til%20nedlasting/Go%C2%B4m%C3%B8rning/2015/GM%204-15%20web.pdf (last accessed 23.05.2016).

Reich, F., Atanassova, V., Klein, G., 2013. Extended-spectrum beta-lactamase- and AmpC-producing enterobacteria in healthy broiler chickens, Germany. Emerg. Infect. Dis. 19, 1253-1259.

Reisner, A., Höller, B.M., Molin, S., Zechner, E.L., 2006. Synergistic Effects in Mixed *Escherichia coli* Biofilms: Conjugative Plasmid Transfer Drives Biofilm Expansion. J. Bacteriol. 188, 3582-3588.

Ribot, E.M., Fair, M.A., Gautom, R., Cameron, D.N., Hunter, S.B., Swaminathan, B., Barrett, T.J., 2006. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog. Dis. 3, 59-67.

Rogers, B.A., Sidjabat, H.E., Paterson, D.L., 2011. *Escherichia coli* O25b-ST131: a pandemic, multiresistant, community-associated strain. J. Antimicrob. Chemother. 66, 1-14.

Sales, J., Vali, L., Hoyle, D.V., Yates, C.M., Amyes, S.G., McKendrick, I.J., 2007. The interaction between dam methylation sites and *Xba*1 restriction digest sites in *Escherichia coli* O157:H7 EDL933. J. Appl. Microbiol. 102, 820-825.

Schaufler, K., Semmler, T., Wieler, L.H., Wöhrmann, M., Baddam, R., Ahmed, N., Müller, K., Kola, A., Fruth, A., Ewers, C., Guenther, S., 2016. Clonal spread and interspecies transmission of clinically relevant ESBL-producing *Escherichia coli* of ST410-another successful pandemic clone? FEMS Microbiol. Ecol. 92.

Schmidt, G.V., Mellerup, A., Christiansen, L.E., Ståhl, M., Olsen, J.E., Angen, O., 2015. Sampling and Pooling Methods for Capturing Herd Level Antibiotic Resistance in Swine Feces using qPCR and CFU Approaches. PLoS One 10, e0131672.

Schwarz, S., Cloeckaert, A., Roberts, M.C., 2006. Mechanisms and spread of bacterial resistance to antimicrobial agents. In: Aarestrup, F.M. (Ed.), Antimicrobial resistance in bacteria of animal origin. ASM Press, Washington DC, 73-98.

Schwarz, S., Kehrenberg, C., Walsh, T.R., 2001. Use of antimicrobial agents in veterinary medicine and food animal production. Int. J. Antimicrob. Agents 17, 431-437.

Shi, X.M., Zhu, X.N., 2009. Biofilm formation and food safety in food industries. Trends Food Sci. Tech. 20, 407-413.

Silbert, S., Boyken, L., Hollis, R.J., Pfaller, M.A., 2003. Improving typeability of multiple bacterial species using pulsed-field gel electrophoresis and thiourea. Diagn. Microbiol. Infect. Dis. 47, 619-621.

Siu, L.K., Lu, P.L., Chen, J.Y., Lin, F.M., Chang, S.C., 2003. High-level expression of ampC beta-lactamase due to insertion of nucleotides between -10 and -35 promoter sequences in *Escherichia coli* clinical isolates: cases not responsive to extended-spectrum-cephalosporin treatment. Antimicrob. Agents Chemother. 47, 2138-2144.

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Catry, B., Herman, L., Haesebrouck, F., Butaye, P., 2008. Diversity of extended-spectrum beta-lactamases and class C beta-lactamases among cloacal *Escherichia coli* Isolates in Belgian broiler farms. Antimicrob. Agents Chemother. 52, 1238-1243.

Smet, A., Rasschaert, G., Martel, A., Persoons, D., Dewulf, J., Butaye, P., Catry, B., Haesebrouck, F., Herman, L., Heyndrickx, M., 2011. *In situ* ESBL conjugation from avian to human *Escherichia coli* during cefotaxime administration. J. Appl. Microbiol. 110, 541-549.

Smith, H., Bossers, A., Harders, F., Wu, G., Woodford, N., Schwarz, S., Guerra, B., Rodríguez, I., van Essen-Zandbergen, A., Brouwer, M., Mevius, D., 2015. Characterization of epidemic IncI1-Igamma plasmids harboring ambler class A and C genes in *Escherichia coli* and *Salmonella enterica* from animals and humans. Antimicrob. Agents Chemother. 59, 5357-5365.

Smith, R., Coast, J., 2013. The true cost of antimicrobial resistance. BMJ 346, f1493.

SN, 2016. Animal husbandry, January 1st, 2016, preliminary figures (Husdyrhald, 1.januar 2016, førebelse tall; in Norwegian). Statistics Norway, http://ssb.no/jord-skog-jakt-og-fiskeri/statistikker/jordhus (last accessed 09.05.2016).

Solheim, H.T., Sekse, C., Urdahl, A.M., Wasteson, Y., Nesse, L.L., 2013. Biofilm as an environment for dissemination of *stx* genes by transduction. Appl. Environ. Microbiol. 79, 896-900.

Srey, S., Jahid, I.K., Ha, S.D., 2013. Biofilm formation in food industries: A food safety concern. Food Control 31, 572-585.

Su, L.H., Chu, C., Cloeckaert, A., Chiu, C.H., 2008. An epidemic of plasmids? Dissemination of extended-spectrum cephalosporinases among *Salmonella* and other *Enterobacteriaceae*. FEMS Immunol. Med. Microbiol. 52, 155-168.

Sunde, M., Simonsen, G.S., Slettemeås, J.S., Böckerman, I., Norström, M., 2015. Integron, plasmid and host strain characteristics of *Escherichia coli* from humans and food included in the Norwegian antimicrobial resistance monitoring programs. PLoS One 10, e0128797.

Sunde, M., Sørum, H., 2001. Self-transmissible multidrug resistance plasmids in *Escherichia coli* of the normal intestinal flora of healthy swine. Microb. Drug Resist. 7, 191-196.

Sunde, M., Tharaldsen, H., Slettemeås, J.S., Norström, M., Carattoli, A., Bjorland, J., 2009. *Escherichia coli* of animal origin in Norway contains a blaTEM-20-carrying plasmid

closely related to blaTEM-20 and blaTEM-52 plasmids from other European countries. J. Antimicrob. Chemother. 63, 215-216.

SVARM, 2011. SVARM 2010 Swedish veterinary antimicrobial resistance monitoring. Uppsala. ISSN 1650-6332.

SVARM, 2012. SVARM 2011 Swedish veterinary antimicrobial resistance monitoring. Uppsala. ISSN 1650-6332.

SWEDRES/SVARM, 2013. SWEDRES/SVARM 2012 Swedish antibiotic utilisation and resistance in human medicine/Swedish veterinary antimicrobial resistance monitoring. Solna/Uppsala. ISSN 1650-6332.

SWEDRES/SVARM, 2014. SWEDRES/SVARM 2013. Use of antimicrobials and occurrence of antimicrobial resistance in Sweden. Solna/Uppsala. ISSN 1650-6332.

SWEDRES/SVARM, 2015. SWEDRES/SVARM 2014. Consumption of antibiotics and occurrence of antibiotic resistance in Sweden. Solna/Uppsala. ISSN 1650-6332.

Sykes, R., 2010. The 2009 Garrod lecture: the evolution of antimicrobial resistance: a Darwinian perspective. J. Antimicrob. Chemother. 65, 1842-1852.

Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., 2005. Studying plasmid horizontal transfer in situ: A critical review. Nat. Rev. Microbiol. 3, 700-710.

Tenover, F.C., 2006. Mechanisms of antimicrobial resistance in bacteria. Am. J. Med. 119, S3-10.

Tenover, F.C., Arbeit, R.D., Goering, R.V., 1997. How to select and interpret molecular strain typing methods for epidemiological studies of bacterial infections: a review for healthcare epidemiologists Infect. Control Hosp. Epidemiol. 18, 426-439.

Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H., Swaminathan, B., 1995. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol. 33, 2233-2239.

Tortora, G.J., Funke, B.R., Case, C.L., 2010. Microbial genetics. In: L., B. (Ed.), Microbiology an introduction. Benjamin Cummings, San Fransisco, CA, USA, 210-245.

Tschudin-Sutter, S., Frei, R., Stephan, R., Hächler, H., Nogarth, D., Widmer, A.F., 2014. Extended-spectrum beta-lactamase (ESBL)-producing *Enterobacteriaceae*: a threat from the kitchen. Infect. Control Hosp. Epidemiol. 35, 581-584.

Unterholzner, S.J., Poppenberger, B., Rozhon, W., 2013. Toxin-antitoxin systems: Biology, identification, and application. Mob. Genet. Elements 3, e26219.

van der Bij, A.K., Peirano, G., Goessens, W.H., van der Vorm, E.R., van Westreenen, M., Pitout, J.D., 2011. Clinical and molecular characteristics of extended-spectrum-beta-lactamase-producing *Escherichia coli* causing bacteremia in the Rotterdam Area, Netherlands. Antimicrob. Agents Chemother. 55, 3576-3578.

Van Melderen, L., Saavedra De Bast, M., 2009. Bacterial toxin-antitoxin systems: more than selfish entities? PLoS Genet. 5, e1000437.

Vincent, C., Boerlin, P., Daignault, D., Dozois, C.M., Dutil, L., Galanakis, C., Reid-Smith, R.J., Tellier, P.P., Tellis, P.A., Ziebell, K., Manges, A.R., 2010. Food reservoir for *Escherichia coli* causing urinary tract infections. Emerg. Infect. Dis. 16, 88-95.

Vittinghoff, E., Glidden, D.V., Shiboski, S.C., McCulloch, C.E., 2012a. Linear regression. In: Gail, M., Krickeberg, K., Samet, J.M., Tsiatis, A., Wong, W. (Eds.), Regression methods in biostatistics. Springer, New York, USA, 69-138.

Vittinghoff, E., Glidden, D.V., Shiboski, S.C., McCulloch, C.E., 2012b. Logistic regression. In: Gail, M., Krickeberg, K., Samet, J.M., Tsiatis, A., Wong, W. (Eds.), Regression Methods in Biostatistics. Springer, New York, USA, 139-202.

VKM, 2015. Assesment of antimicrobial resistance in the food chains in Norway. Scientific Opinion of the Panel on microbiological hazards for the Norwegian Scientific Committee for Food Safety. Oslo. ISBN: 978-82-8259-184-3

Voets, G.M., Fluit, A.C., Scharringa, J., Schapendonk, C., van den Munckhof, T., Leverstein-van Hall, M.A., Stuart, J.C., 2013. Identical plasmid AmpC beta-lactamase genes and plasmid types in *E. coli* isolates from patients and poultry meat in the Netherlands. Int. J. Food Microbiol. 167, 359-362.

Vogt, D., Overesch, G., Endimiani, A., Collaud, A., Thomann, A., Perreten, V., 2014. Occurrence and genetic characteristics of third-generation cephalosporin-resistant *Escherichia coli* in Swiss retail meat. Microb. Drug Resist. 20, 485-494.

Walther-Rasmussen, J., Høiby, N., 2002. Plasmid-borne AmpC beta-lactamases. Can. J. Microbiol. 48, 479-493.

Wasyl, D., Hasman, H., Cavaco, L.M., Aarestrup, F.M., 2012. Prevalence and characterization of cephalosporin resistance in nonpathogenic *Escherichia coli* from food-producing animals slaughtered in Poland. Microb. Drug Resist. 18, 79-82.

Wasyl, D., Hoszowski, A., Zajac, M., Skarzynska, M., 2010. Simple and efficient screening method for the detection of cephalosporin resistant *Escherichia coli*. Bull. Vet. Inst. Pulawy. 54, 147-151.

WHO, 2014. Antimicrobial resistance. Fact sheet No 194. WHO, http://www.who.int/mediacentre/factsheets/fs194/en/ (last accessed 18.03.2016).

Wieler, L.H., Ewers, C., Guenther, S., Walther, B., Lübke-Becker, A., 2011. Methicillin-resistant staphylococci (MRS) and extended-spectrum beta-lactamases (ESBL)-producing *Enterobacteriaceae* in companion animals: nosocomial infections as one reason for the rising prevalence of these potential zoonotic pathogens in clinical samples. Int. J. Med. Microbiol. 301, 635-641.

Wilkins, B.M., 1995. Gene transfer by bacterial conjugation: diversity of systems and functional specializations. In: Baumberg, S., Young, J.P.W., Wellington, E.M.H., Saunders, J.R. (Eds.), Population genetics of bacteria. Cambridge University Press, Cambridge, 59-88.

Willinghan, E.M., Sander, J.E., Thayer, S.G., Wilson, J.L., 1996. Investigation of bacterial resistance to hatchery disinfectants. Avian Dis. 40, 510-515.

Winokur, P.L., Vonstein, D.L., Hoffman, L.J., Uhlenhopp, E.K., Doern, G.V., 2001. Evidence for transfer of CMY-2 AmpC beta-lactamase plasmids between *Escherichia coli* and *Salmonella* isolates from food animals and humans. Antimicrob. Agents Chemother. 45, 2716-2722.

Woerther, P.L., Burdet, C., Chachaty, E., Andremont, A., 2013. Trends in human fecal carriage of extended-spectrum beta-lactamases in the community: toward the globalization of CTX-M. Clin. Microbiol. Rev. 26, 744-758.

Woodford, N., Turton, J.F., Livermore, D.M., 2011. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol. Rev. 35, 736-755.

WorldPoultry, 2011. British Poultry Council to cut antibiotic usage. http://www.worldpoultry.net/Broilers/Health/2011/12/British-Poultry-Council-to-cut-antibiotic-usage-WP009751W/ (last accessed 11.05.2016).

Wu, G., Day, M.J., Mafura, M.T., Nunez-Garcia, J., Fenner, J.J., Sharma, M., van Essen-Zandbergen, A., Rodriguez, I., Dierikx, C., Kadlec, K., Schink, A.K., Chattaway, M., Wain, J., Helmuth, R., Guerra, B., Schwarz, S., Threlfall, J., Woodward, M.J., Woodford, N., Coldham, N., Mevius, D., 2013. Comparative analysis of ESBL-positive *Escherichia coli* isolates from animals and humans from the UK, The Netherlands and Germany. PLoS One 8, e75392.

Wu, S.W., Dornbusch, K., Kronvall, G., Norgren, M., 1999. Characterization and nucleotide sequence of a *Klebsiella oxytoca* cryptic plasmid encoding a CMY-type beta-

lactamase: confirmation that the plasmid-mediated cephamycinase originated from the *Citrobacter freundii* AmpC beta-lactamase. Antimicrob. Agents Chemother. 43, 1350-1357.

Zankari, E., Hasman, H., Cosentino, S., Vestergaard, M., Rasmussen, S., Lund, O., Aarestrup, F.M., Larsen, M.V., 2012. Identification of acquired antimicrobial resistance genes. J. Antimicrob. Chemother. 67, 2640-2644.

Zankari, E., Hasman, H., Kaas, R.S., Seyfarth, A.M., Agersø, Y., Lund, O., Larsen, M.V., Aarestrup, F.M., 2013. Genotyping using whole-genome sequencing is a realistic alternative to surveillance based on phenotypic antimicrobial susceptibility testing. J. Antimicrob. Chemother. 68, 771-777.

Zinsstag, J., Schelling, E., Waltner-Toews, D., Tanner, M., 2011. From "one medicine" to "one health" and systemic approaches to health and well-being. Prev. Vet. Med. 101, 148-156.

14. Enclosed papers I-IV

Paper I

Veterinary Microbiology 171 (2014) 315–320

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Emergence of AmpC-producing *Escherichia coli* in the broiler production chain in a country with a low antimicrobial usage profile

Solveig Sølverød Mo [a,*], Madelaine Norström [a], Jannice Schau Slettemeås [a], Atle Løvland [b], Anne Margrete Urdahl [a], Marianne Sunde [a]

[a] *Norwegian Veterinary Institute, Pb 750 Sentrum, 0106 Oslo, Norway*
[b] *Nortura SA, Pb 33, 1891 Rakkestad, Norway*

ARTICLE INFO

Keywords:
ESBL
AmpC
Broiler
E. coli

ABSTRACT

The aim of this study was to estimate the prevalence of cephalosporin-resistant *Escherichia coli* at the different levels of the Norwegian broiler production pyramid and identify the mechanisms responsible for the resistance phenotype. Samples from all levels of the broiler production pyramid and retail chicken meat (fillets) were included ($n = 649$). The occurrence of cephalosporin-resistant *E. coli* at the different production levels ranged from 8 to 43%. All these isolates had an AmpC-phenotype, and the majority carried the bla_{CMY-2} gene. In addition, a few isolates with up-regulated chromosomal *ampC* were identified. The results show that Norway has a relatively high prevalence of cephalosporin-resistant *E. coli* in the broiler production chain in spite of a very low consumption of antimicrobial agents. Cephalosporins have not been used in the Norwegian broiler production, and it has been hypothesised that import of breeding animals and hatching eggs may be the source of these resistant bacteria. We demonstrate that these bacteria are disseminated in the production pyramid despite the lack of selection pressure from antimicrobial agents.

© 2014 Elsevier B.V. All rights reserved.

1. Introduction

Antimicrobial resistance is recognised as one of the most important global health challenges (Dziekan et al., 2012). In recent years, the prevalence of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* (*E. coli*) has been increasing in both human and veterinary medicine (Carattoli, 2008; Coque et al., 2008; Ewers et al., 2012; Overdevest et al., 2011). In veterinary medicine, poultry are associated with especially high prevalence of ESBL-producing *E. coli* (Ewers et al., 2012), and in some countries the majority of tested broiler flocks are positive, i.e. ESBL producing *E. coli* are present (Dierikx et al., 2013; Reich et al., 2013; Smet et al., 2008).

Since the start of the Norwegian monitoring programme on antimicrobial resistance in bacteria from food, feed and animals (NORM-VET) in year 2000, the occurrence of resistance has been low to moderate (NORM/NORM-VET reports, www.vetinst.no). Until 2006, ESBL-producing *E. coli* had not been detected in any food producing animals in Norway (Sunde et al., 2009). However, in 2006 the first ESBL-producing *E. coli* was isolated from the faeces of a healthy broiler (NORM/NORM-VET, 2007). This could be interpreted as an "early warning" that the broiler production could be a reservoir for ESBL-producing *E. coli* in Norway. The isolate carried an incI1 plasmid with a bla_{TEM-20} gene, and the plasmid

* Corresponding author at: Department of Diagnostic Services, Norwegian Veterinary Institute, Pb 750 Sentrum, 0106 Oslo, Norway. Tel.: +47 23216437; fax: +47 23216485.
E-mail address: solveig.mo@vetinst.no (S.S. Mo).

http://dx.doi.org/10.1016/j.vetmic.2014.02.002
0378-1135/© 2014 Elsevier B.V. All rights reserved.

showed close relation to a plasmid present in *Salmonella* Parathypi B dT+ isolated from poultry in the Netherlands (Sunde et al., 2009). This indicated possible spread of a common resistance plasmid in the broiler production between European countries.

The aim of this study was to estimate the prevalence of cephalosporin-resistant *E. coli* at all levels in the Norwegian broiler production chain using a selective method. Isolates with reduced cephalosporin susceptibility were further investigated for the mechanisms responsible for the resistance phenotype.

2. Materials and methods

2.1. Sampling and bacterial isolates

Samples were collected and analysed from January 2011 to December 2012. The samples were collected under the auspices of a survey conducted on the only grandparent flock imported from Scotland during the study period (in 2011), and a survey including parent and grandparent flocks ($n = 64$ out of 66) associated with the largest poultry cooperation in Norway (Nortura SA) in November 2011. In addition, samples collected in the NORM-VET programme were included in the present study. These comprised of samples from broiler flocks ($n = 252$) in 2011, and Norwegian retail chicken meat ($n = 205$) and all parent flocks ($n = 120$) in 2012 (NORM/NORM-VET, 2012, 2013). In general, one flock was sampled at one time point to represent the status of the flock or meat product. However, the grandparent flock was sampled several times ($n = 8$) at different ages.

Samples were collected from paper linings (day old chickens), meconium, boot swabs, and retail chicken meat. Boot swabs were dissolved in sterile water and plated directly onto MacConkey agar (Beckton, Dickinson and Company, Le Pont de Claire, France) containing 1 mg/L cefotaxime (Duchefa, Haarlem, The Netherlands) and MacConkey agar containing 2 mg/L ceftazidime (Sigma–Aldrich, St. Louis, MO, USA). Overnight pre-enrichment of samples from retail meat was done in MacConkey broth (BD), while meconium and paper linings were enriched in peptone water (Oxoid, Basingstoke, UK). After overnight pre-enrichment, the samples were plated out as described above. Agar plates were incubated at 37 °C for 24–48 h. Colonies with typical morphology were selected and confirmed as *E. coli* using API 20 E (bioMérieux, Marcy L'Etoile, Rhone, France). Isolates from meat samples and parent stock in 2012 were confirmed as *E. coli* by PCR (Heininger et al., 1999).

2.2. Susceptibility testing

E. coli isolates with suspected ESBL/AmpC-production, originating from the MacConkey screening plates, were subjected to further testing. The isolates were tested for resistance to cefotaxime, ceftazidime, cefepime, cefoxitin and synergy to clavulanic acid and cloxacillin using the disc diffusion methodology recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org). *E. coli* ATCC 25922, *E. coli* K5-20 (bla_{CMY-2}) and *E. coli* K8-1 ($bla_{CTX-M-15}$) were included as quality controls. Minimum inhibitory concentrations (MICs) were determined by the use of a broth microdilution method (VetMIC™ GN-mo, National Veterinary Institute, Uppsala, Sweden). Epidemiological cut-off values recommended by EUCAST were applied for categorising the isolates as susceptible or resistant. *E. coli* ATCC 25922 was included as quality control.

2.3. Characterisation of resistance genes

All *E. coli* isolates displaying cephalosporin resistance (with cloxacillin synergy, i.e. augmented zone around cephalosporin discs towards the cloxacillin disc) were subjected to a multiplex PCR for detection of plasmid mediated AmpC encoding genes as previously described (Perez-Perez and Hanson, 2002). Further, the PCR amplicons were sequenced to determine the gene responsible for resistance. Investigation of up-regulated chromosomal *ampC*-production was performed on cephalosporin-resistant isolates with a negative PCR result. Briefly, the promoter region of the chromosomal *ampC* gene was amplified and sequenced in order to detect mutations (Agersø et al., 2012). The sequences were analysed in CLC Main Workbench 6 (CLC bio A/S, Aarhus, Denmark), and interpreted as previously described (Peter-Getzlaff et al., 2011).

2.4. Data management and statistical analysis

Data management and statistical analyses were carried out using SAS Enterprise Guide version 4.3 for Windows (SAS Institute Inc., Cary, NC, USA). Handling of geographical data and generation of the map was performed using ArcGIS 9.3 Software (ESRI, Redlands, CA, USA).

3. Results

All *E. coli* isolates resistant to third generation cephalosporins had a beta-lactam resistance profile with an AmpC-phenotype, displaying resistance to cefoxitin and synergy to cloxacillin.

The bla_{CMY-2} gene was found in all cephalosporin-resistant *E. coli*, except for 15 isolates (originating from parent flocks; $n = 9$, broilers; $n = 4$, and retail meat; $n = 2$). These isolates were investigated for mutations in the *ampC* promoter and attenuator regions. In all these isolates the same sequence variant was found with nucleotide substitutions at position-42 and -18.

The occurrence of bla_{CMY-2} carrying *E. coli* at the different production levels ranged from 8 to 43% (Fig. 1). The highest occurrence was found in broilers (43%), while the occurrence in chicken meat was lower (32%, $p < 0.05$). The occurrence in parent flocks decreased significantly ($p < 0.05$) from 28% in 2011 to 8% in 2012 (Fig. 1). Furthermore, the occurrence of bla_{CMY-2} carrying *E. coli* in parent flocks decreased during 2012, and was detected only in two flocks after March 2012.

Broiler flocks positive for *E. coli* with plasmid-mediated AmpC-production were geographically scattered throughout Norway as illustrated in Fig. 2.

Grandparent stock
Year 2011: 25 % (n=8)

Parent stock
Year 2011: 28 % (n=64)
Year 2012: 8 % (n=120)

Broilers
Year 2011: 43 % (n=252)

Retail meat
Year 2012: 32 % (n=205)

Fig. 1. Prevalence of plasmid mediated AmpC-producing *Escherichia coli* at the different levels of the broiler production pyramid in Norway during the study period (2011–2012).

Resistance to non-beta lactams was observed in some bla_{CMY-2} carrying isolates (Tables 1 and 2). None of the isolates displayed multidrug resistance (resistance to three or more antimicrobial classes), and the resistance patterns were similar in isolates from the different production levels (Table 1).

4. Discussion

This study shows that cephalosporin-resistant *E. coli* is present at all levels of the Norwegian broiler production chain with higher occurrence in broilers than in parent animals and meat. These observed differences should be further investigated in new prevalence studies. A similar study from the Netherlands also revealed higher prevalence of ESBL-producing *E. coli* in broilers than in parent animals. Retail meat was not included. However, the broiler production in the Netherlands differs from that in Norway with larger flocks, different management practices, and higher consumption of antimicrobial agents (Dierikx et al., 2013).

Table 1
Antimicrobial resistance in *Escherichia coli* with plasmid mediated AmpC-production ($n = 203$) isolated from the different levels of the Norwegian broiler production pyramid during 2011–2012.

Isolate origin	Number of isolates (%) carrying *bla*CMY-2	Total number of isolates	Coresistance (%)										
			TET	CHL	FF	SMX	TMP	GEN	STR	KAN	CIP	NAL	COL
Grandparents	2 (25)	8									2 (100)	2 (100)	
Parents (2011)	18 (28)	64				2 (7)			1 (4)				
Parents (2012)	9 (8)	120	1 (11)			3 (33)	2 (22)	1 (11)	1 (11)				
Broilers	108 (43)	252	3 (3)			9 (8)	1 (1)		3 (3)	1 (1)	4 (4)	2 (2)	
Meat	66 (32)	205				4 (6)	2 (3)		2 (3)		1 (2)	1 (2)	

TET, tetracycline; CHL, chloramphenicol; FF, florfenicol; SMX, sulfametoxazole; TMP, trimethoprim; GEN, gentamicin; STR, streptomycin; KAN, kanamycin; CIP, ciprofloxacin; NAL, nalidixic acid; COL, colistin.

Table 2
Minimum inhibitory concentrations (MICs) and antimicrobial resistance in *Escherichia coli* with plasmid mediated AmpC-production ($n = 203$) isolated from all levels of the Norwegian broiler production pyramid during 2011–2012.

Substance	Resistance (%)	[95% CI*]	Distribution (%) of MIC values (mg/L)*														
			0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	≥ 512
Tetracycline	2.0	[0.5-5.0]						42.4	55.7				0.5	1.5			
Chloramphenicol	0	[0.0-1.8]							10.3	83.3	6.4						
Florfenicol	0	[0.0-1.8]								51.2	48.3	0.5					
Ampicillin	100	[98.2-100]											3.4	19.7	44.3	32.5	
Cefotaxime	100	[98.2-100]						0.5		99.5							
Ceftazidime	100	[98.2-100]							0.5	12.8	59.6	25.1	2.0				
Sulfamethoxazole	9.4	[5.7-14.3]									28.6	54.2	6.4	1.5	0.5		8.9
Trimethoprim	2.5	[0.8-5.7]			9.4	36.9	47.3	3.9					2.5				
Gentamicin	0.5	[0.0-2.7]					40.4	54.7	4.4	0.5							
Streptomycin	3.5	[1.4-7.0]								34.5	60.1	2.0	2.5		1.0		
Kanamycin	0.5	[0.0-2.7]									99.5	0.5					
Ciprofloxacin	3.5	[1.4-7.0]	18.7	77.8	1.0		2.0	0.5									
Nalidixic acid	2.5	[0.8-5.7]						2.5	79.3	14.8	1.0				1.5	1.0	
Colistin	0	[0.0-1.8]					97.0	3.0									

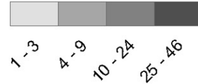


Fig. 2. Distribution of the positive and negative commercial broiler farms tested for plasmid mediated AmpC-producing *Escherichia coli* and the number of commercial broiler farms per municipality in Norway in 2011.

Of special concern is the occurrence of cephalosporin-resistant *E. coli* in retail meat. A close relationship between ESBL-producing *E. coli* isolated from poultry and humans has been identified, suggesting a link between the occurrence of ESBL-producing *E. coli* in retail meat and humans (Overdevest et al., 2011). Also, resistant *E. coli* from human faeces showed a higher degree of similarity to *E. coli* from poultry than to susceptible *E. coli* from humans (Johnson et al., 2007). On the other hand, a limited number of bla_{CMY-2} carrying *E. coli* isolates from broilers and

humans (clinical isolates) in Sweden showed high diversity, indicating distinct populations. However, an IncK plasmid carrying bla_{CMY-2} was identified in both human and broiler isolates, thus transmission of this plasmid cannot be excluded (Börjesson et al., 2013b). It should therefore be an overall goal to keep the prevalence of cephalosporin-resistant *E. coli* in retail chicken meat low in order to limit the probability of zoonotic transmission.

The cephalosporin-resistant *E. coli* isolated from the Norwegian broiler production pyramid in this study have been found to carry the bla_{CMY-2} gene, except for a small number of isolates with up-regulated chromosomal *ampC.* The genetic background for cephalosporin resistance in *E. coli* isolated from broilers and retail chicken meat in other European countries is more heterogeneous, also including genes of the CTX-M, TEM and SHV groups (Ewers et al., 2012). The bla_{CMY-2} gene is also the most frequently reported gene responsible for cephalosporin resistance in *E. coli* from broilers and meat in Sweden and Denmark. However, both $bla_{CTX-M-1}$ (Sweden and Denmark) and bla_{SHV-2} (Denmark) have been reported in recent years (www.danmap.org, www.sva.se). The results indicate that bla_{CMY-2}-carrying *E. coli* is capable of broad dissemination within the Norwegian broiler production.

Previous studies have shown that treatment with third-generation cephalosporins result in selection of bla_{CMY-2}-carrying *E. coli* in cattle (Tragesser et al., 2006). It has also been hypothesised that treatment with other antimicrobial agents, such as amoxicillin, may select ESBL/AmpC-producing isolates (EFSA, 2011). Norway has a low level of antimicrobial usage to food producing animals compared to other European countries (Chantziaras et al., 2013; ESVAC, 2013; Grave et al., 2012). In 2012, roughly 600 broiler farms produced 62.7 million broilers for human consumption (Statistics Norway, www.ssb.no). The total sale of antimicrobial agents to the poultry production was 147.5 kg active substance in 2012, consisting of amoxicillin (131 kg), phenoxymethylpenicillin (16 kg) and enrofloxacin (0.5 kg) (Kari Grave, European Medicines Agency, personal communication). It is estimated that less than 50% of these antimicrobials are prescribed to the broiler production, hence the use of antimicrobial agents, except coccidiostatics (11.890 kg in 2012 (NORM/NORM-VET, 2013)), in the Norwegian broiler production is nearly negligible. Furthermore, there are no commercial preparations containing cephalosporins available for treatment of food producing animals in Norway (www.felleskatalogen.no). Thus, there is no selection pressure from cephalosporin usage, making the high occurrence of resistance surprising. Other factors must therefore play an important role in the emergence and persistence of cephalosporin-resistant *E. coli* in the Norwegian broiler production.

Cephalosporin-resistant *E. coli* have emerged in the broiler production in Sweden and Denmark in a similar manner as in Norway (www.danmap.org, www.sva.se). It has been hypothesised that the introduction of these bacteria may have been through imported breeding animals (Börjesson et al., 2013a; NORM/NORM-VET, 2007; Sunde et al., 2009). This could also explain the emergence of cephalosporin-resistant *E. coli* in the Norwegian broiler industry, as it is dependent on import of parent animals (hatching eggs) and, in some cases, grandparent animals. The fact that positive broiler flocks are geographically scattered throughout Norway might also support this theory. The mechanism behind dissemination of cephalosporin resistant *E. coli* in the Norwegian broiler production should be further investigated. Preliminary typing results and conjugation experiments indicate that the resistant isolates from broilers are genetically diverse, and that the bla_{CMY-2} gene is located on a self-transmissible plasmid in a large proportion of the isolates (data not published).

Interestingly, the occurrence of bla_{CMY-2} carrying *E. coli* in the parent stock was significantly lower in 2012 than in 2011. In addition, only two parent flocks sampled after March 2012 were positive even if the sampling was distributed throughout the year.

When using a non-selective method, which was done in parallel for the samples from broilers and meat (NORM/NORM-VET, 2012, 2013), the occurrence of cephalosporin-resistant *E. coli* was less than 1%. This indicates that even if cephalosporin resistance is present in the Norwegian broiler production chain, it is still at a low level. Methods of quantification has so far not been applied but is expected to be implemented in future surveillance as recommended by the European Commission (SANCO/11591/2012r14).

In conclusion, AmpC-producing *E. coli* is disseminated throughout the Norwegian broiler production pyramid without the presence of selection pressure from antimicrobial use. Other factors must therefore play an important role. A single gene, namely bla_{CMY-2}, has so far been responsible for the plasmid-mediated cephalosporin resistance.

Conflict of interest

None to declare.

Acknowledgements

This work was funded by the Norwegian Food Safety Authority, the Norwegian Veterinary Institute and Nortura SA. Arnfinn Sundsfjord (University Hospital of North Norway) is acknowledged for donation of the quality control strains *E. coli* K8-1 and K5-20. Hanne Fanuelsen and Aina Steihaug Barstad are acknowledged for technical assistance. Karl Eckner is acknowledged for language editing.

References

Agersø, Y., Aarestrup, F.M., Pedersen, K., Seyfarth, A.M., Struve, T., Hasman, H., 2012. Prevalence of extended-spectrum cephalosporinase (ESC)-producing *Escherichia coli* in Danish slaughter pigs and retail meat identified by selective enrichment and association with cephalosporin usage. J. Antimicrob. Chemother. 67, 582–588.

Börjesson, S., Bengtsson, B., Jernberg, C., Englund, S., 2013a. Spread of extended-spectrum beta-lactamase producing *Escherichia coli* isolates in Swedish broilers mediated by an incl plasmid carrying *bla*CTX-M-1. Acta Vet. Scand. 55.

Börjesson, S., Jernberg, C., Brolund, A., Edquist, P., Finn, M., Landén, A., Olsson-Liljequist, B., Wisell, K.T., Bengtsson, B., Englund, S., 2013b. Characterization of plasmid-mediated AmpC-producing *E. coli* from Swedish broilers and association with human clinical isolates. Clin Microbiol. Infect. 19, E309–E311.

Carattoli, A., 2008. Animal reservoirs for extended spectrum beta-lactamase producers. Clin. Microbiol. Infect. 14 Suppl. (1) 117–123.

Chantziaras, I., Boyen, F., Callens, B., Dewulf, J., 2013. Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. J. Antimicrob. Chemother., http://dx.doi.org/10.1093/jac/dkt443.

Coque, T.M., Baquero, F., Canton, R., 2008. Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. Euro Surveill. 13 .

Dierikx, C., van der Goot, J., Fabri, T., van Essen-Zandbergen, A., Smith, H., Mevius, D., 2013. Extended-spectrum-beta-lactamase- and AmpC-beta-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers. J. Antimicrob. Chemother. 68, 60–67.

Dziekan, G., Jauregui, I.L., Mathai, E., 2012. The way forward: political commitment to enable options for action. In: WHO (Eds.), The Evolving Threat of Antimicrobial Resistance: Options for Action. World Health Organization, Geneva, Switzerland.

EFSA, 2011. Scientific opinion on the public health risks of bacterial strains producing extended-spectrum beta-lactamases and/or AmpC beta-lactamases in food and food-producing animals. EFSA Journal 9, 2322.

ESVAC, 2013. European Medicines Agency, European Surveillance of Veterinary Antimicrobial Consumption. Sales of veterinary antimicrobial agents in 25 EU/EEA countries in 2011 (EMA/236501/2013) .

Ewers, C., Bethe, A., Semmler, T., Guenther, T., Wieler, L.H., 2012. Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin. Microbiol. Infect. 18, 646–655.

Grave, K., Greko, C., Kvaale, M.K., Torren-Edo, J., Mackay, D., Muller, A., Moulin, G., Grp, E., 2012. Sales of veterinary antibacterial agents in nine European countries during 200509: trends and patterns. J. Antimicrob. Chemother. 67, 3001–3008.

Heininger, A., Bider, M., Schmidt, S., Unertl, K., Botzenhart, K., Döring, G., 1999. PCR and blood culture for detection of *Escherichia coli* bacteremia in rats. J. Clin. Microbiol. 37, 2479–2482.

Johnson, J.R., Sannes, M.R., Croy, C., Johnston, B., Clabots, C., Kuskowski, M.A., Bender, J., Smith, K.E., Winokur, P.L., Belongia, E.A., 2007. Antimicrobial drug-resistant *Escherichia coli* from humans and poultry products, Minnesota and Wisconsin, 2002–2004. Emerg. Infect. Dis. 13, 838–846.

NORM/NORM-VET, 2007. NORM/NORM-VET 2006. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway (Tromsø/Oslo) .

NORM/NORM-VET, 2012. NORM/NORM-VET 2011. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway (Tromsø/Oslo) .

NORM/NORM-VET, 2013. NORM/NORM-VET 2012. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway (Tromsø/Oslo) .

Overdevest, I., Willemsen, I., Rijnsburger, M., Eustace, A., Xu, L., Hawkey, P., Heck, M., Savelkoul, P., Vandenbroucke-Grauls, C., van der Zwaluw, K., Huijsdens, X., Kluytmans, J., 2011. Extended-spectrum beta-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. Emerg. Infect. Dis. 17, 1216–1222.

Perez-Perez, F.J., Hanson, N.D., 2002. Detection of plasmid-mediated AmpC beta-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbiol. 40, 2153–2162.

Peter-Getzlaff, S., Polsfuss, S., Poledica, M., Hombach, M., Giger, J., Böttger, E.C., Zbinden, R., Bloemberg, G.V., 2011. Detection of AmpC beta-lactamase in *Escherichia coli*: comparison of three phenotypic confirmation assays and genetic analysis. J. Clin. Microbiol. 49, 2924–2932.

Reich, F., Atanassova, V., Klein, G., 2013. Extended-spectrum beta-lactamase- and AmpC-producing Enterobacteria in healthy broilers chickens, Germany. Emerg. Infect. Dis. 19, 1253–1259.

Smet, A., Martel, A., Persoons, D., Dewulf, J., Heyndrickx, M., Catry, B., Herman, L., Haesebrouck, F., Butaye, P., 2008. Diversity of extended-spectrum beta-lactamases and class C beta-lactamases among cloacal *Escherichia coli* Isolates in Belgian broiler farms. Antimicrob. Agents Chemother. 52, 1238–1243.

Sunde, M., Tharaldsen, H., Slettemeas, J.S., Norstrom, M., Carattoli, A., Bjorland, J., 2009. *Escherichia coli* of animal origin in Norway contains a blaTEM-20-carrying plasmid closely related to blaTEM-20 and blaTEM-52 plasmids from other European countries. J. Antimicrob. Chemother. 63, 215–216.

Tragesser, L.A., Wittum, T.E., Funk, J.A., Winokur, P.L., Rajala-Schultz, P.J., 2006. Association between ceftiofur use and isolation of *Escherichia coli* with reduced susceptibility to ceftriaxone from fecal samples of dairy cows. Am. J. Vet. Res. 67, 1696–1700.

Paper II

Risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in Norwegian broiler flocks

Solveig Sølverød Mo[a], Anja Bråthen Kristoffersen[a], Marianne Sunde[ab], Ane Nødtvedt[c], Madelaine Norström[a]

[a] Norwegian Veterinary Institute, Pb 750 Sentrum, 0106 Oslo, Norway

[b] Norwegian Institute of Public Health, Pb 4404 Nydalen, 0403 Oslo, Norway

[c] Norwegian University of Life Sciences, Pb 8146 Dep, 0033 Oslo, Norway

Corresponding author: Solveig Sølverød Mo, solveig.mo@vetinst.no, Phone: +47 23 21 63 07

Solveig Sølverød Mo, solveig.mo@vetinst.no, SSM

Anja Bråthen Kristoffersen, anja.kristoffersen@vetinst.no, ABK

Marianne Sunde, marianne.sunde@vetinst.no, MS

Ane Nødtvedt, ane.nodtvedt@nmbu.no, AN

Madelaine Norström, madelaine.norstrom@vetinst.no, MN

Abstract

A longitudinal study of 27 broiler farms including 182 broiler flocks was performed to determine risk factors for occurrence of cephalosporin-resistant *E. coli* in Norwegian broiler flocks. Information regarding possible risk factors was collected by an online questionnaire and by samples obtained from broiler and parent flocks during the study period. Additional information was provided by the broiler production company. The prevalence of cephalosporin-resistant *E. coli* in parent flocks and broiler flocks sampled in the study was estimated.

Cephalosporin-resistant *E. coli* was detected in 13.8% of the parent flocks and 22.5% of the broiler flocks included in the study.

A multivariable generalized linear model was used to estimate risk factors. The risk for occurrence of cephalosporin-resistant *E. coli* was associated with the status of the previous flock in the broiler house (odds ratio=12.7), number of parent flocks supplying the broiler flock with day-old chickens (odds ratio=6.3), routines for disinfection of floor between production cycles (odds ratio=0.1), and transport personnel entering the room where the broilers are raised (odds ratio=9.3).

Our findings highlights that implementation of a high level of biosecurity with a minimal number of people entering the broiler house during production cycles, as well as rigorous cleaning and disinfection routines between production cycles will contribute to a decrease in the occurrence of cephalosporin-resistant *E. coli* in broiler flocks provided that there is no selection pressure from antimicrobial use in the broiler production.

Keywords: Antimicrobial resistance, AmpC, Biosecurity, Broiler, *E. coli*, Risk factor

1. Introduction

Escherichia coli (*E. coli)* with acquired resistance towards extended-spectrum cephalosporins is an increasing problem worldwide (Carattoli, 2008; Coque et al., 2008; Overdevest et al., 2011; Ewers, 2012). This is worrying, as cephalosporins are regarded as critically important antimicrobials for treatment of human infections (WHO-AGISAR, 2011). Broilers, including retail chicken meat, are associated with especially high occurrences of cephalosporin-resistant *E. coli* (Ewers, 2012). Presence of resistant bacteria in food is unwanted, as this can constitute a reservoir for human acquisition (Winokur et al., 2001; EFSA, 2011; Overdevest et al., 2011). In order to minimize the occurrence of cephalosporin-resistant *E. coli* in broiler flocks and thereby possibly also in retail chicken meat, it is necessary to identify and quantify risk factors for occurrence of cephalosporin-resistant *E. coli* in broiler flocks.

The usage of antimicrobial agents in the Norwegian broiler production is minimal (Mo et al., 2014), with only a single broiler flock treated in 2013 and 2014 (0.02%) (Animalia, 2015; Refsum, 2015) and

four flocks treated in 2015 (0.08%) (Animalia, 2016). Furthermore, only two parent flocks (1.1%), have been treated yearly in the period 2013-2015 (Personal communication, Thorbjørn Refsum, Animalia, (Refsum, 2015)). Findings indicating vertical transmission of both cephalosporin-resistant *E. coli* and quinolone-resistant *E. coli* throughout the broiler production pyramid has been reported from Sweden (Nilsson et al., 2014; Börjesson et al., 2015), and import of breeding animals have been suggested as the probable source of resistant *E. coli* in both Sweden and Norway (NORM/NORM-VET, 2007; Sunde et al., 2009; Börjesson et al., 2013; Nilsson et al., 2014).

Norway is dependent on import of parent animals (imported as hatching eggs) from Sweden to supply the broiler production. Thus, only parent animals and broilers are produced in Norway (Figure 1). One parent flock can supply several broiler flocks, and one broiler flock can receive chickens from several parent flocks per production cycle. Therefore, the potential for spread of newly introduced resistant bacteria is considerable (Dierikx et al., 2013b). In 2014, the Norwegian poultry industry started an action plan against cephalosporin-resistant *E. coli* in the Norwegian broiler production. As part of this project, samples were collected from imported parent animals after hatching. None of the samples collected at the parent hatchery of the largest chicken breeding company in Norway were positive for cephalosporin-resistant *E. coli* in 2014 (Animalia, 2015). However, cephalosporin-resistant *E. coli* were still detected in samples from retail chicken meat and faecal material from broilers collected in 2015 (NORM/NORM-VET, 2015). Therefore it is hypothesized that other factors than antimicrobial use and import of breeding animals contaminated with cephalosporin-resistant *E. coli* must play a role in the maintenance of cephalosporin-resistant *E. coli* in the Norwegian broiler production.

The aim of this study was to identify possible risk factors for occurrence of cephalosporin-resistant *E. coli* in broiler flocks affiliated to the largest broiler production company in Norway. Furthermore, the occurrence of cephalosporin-resistant *E. coli* in sampled parent- and broiler flocks during the study period was estimated.

2. Materials and methods

2.1. Study design and study population

The study was designed as a longitudinal study with each broiler flock as the study unit. The study population consisted of 62 randomly selected commercial broiler producers located in Hedmark county and affiliated to the largest broiler production company in Norway, Nortura Samvirkekylling. The random selection was generated using the command surveyselect in SAS Enterprise Guide 6.1 for Windows (SAS Institute Inc., Cary, NC, USA) from a dataset provided by Nortura SA. Criteria for inclusion in the study were as follows; active commercial broiler producer, farm located in Hedmark county, affiliation to Nortura Samvirkekylling, respond to questionnaire, and providing samples from a minimum of two broiler flocks housed at the farm during the study period.

To calculate the sample size (n), we used the following formula (Thrusfield, 2005) considering a large population size;

$$n = (Z_\alpha^2 \times p \times q)/L^2 \quad (1)$$

We expected a flock prevalence of 30% (p=0.30, q=1-0.30=0.70) based on previous knowledge on occurrence of cephalosporin-resistant *E. coli* in Norwegian broilers (NORM/NORM-VET, 2012). When applying a 95% confidence level (Z_α=1.96) and an acceptable error of 5% (L=0.05), this resulted in a sample size of 323 flocks. We further assumed that each producer would supply samples from an average of 6.5 flocks, proposing a required inclusion of at least 62 producers, as the expected response rate was 80%.

2.2. Data collection

An online questionnaire was designed in order to collect information about potential risk factors for the presence of cephalosporin-resistant *E. coli* in broiler flocks (Supplementary material). The questionnaire was based on a questionnaire previously used for collection of data regarding risk factors for campylobacteriosis in Norwegian broilers (Lyngstad et al., 2008). Furthermore, it was pilot tested on consultants working with broiler producers affiliated to Nortura SA. Before distribution of the questionnaire, a report was sent to the Norwegian Data Protection Authority.

A link to the online questionnaire and a request for participation in the study were sent per e-mail to the 62 selected broiler producers in November 2013, with three weeks' response time. The day after the deadline, reminders were sent to the producers that did not respond (n=35). The same procedure was repeated one week later (n=22). Three weeks after the first reminder, producers that still had not responded to the questionnaire (n=17) were called and reminded again. Only broiler producers completing the questionnaire (n=45) received equipment for sampling. This included boot swabs, prepaid envelopes for sending samples to the Norwegian Veterinary Institute in Oslo for analysis, and a thorough description of how to sample their broiler flocks. Broiler producers were instructed to sample broiler flocks housed at the farm from February 2014 through January 2015. Sampling was performed once during the production period using boot swabs. A flock was defined as broilers housed in a single house at the same farm during the same time period. All sampled flocks consisted exclusively of the hybrid Ross 308.

To obtain results regarding the occurrence of cephalosporin-resistant *E. coli* in the parent flocks providing hatching eggs for the broiler flocks, sampling was performed from November 2013 through October 2014. Samples were collected by the respective producer using boot swabs every four weeks during the production period. Nine parent flocks were not sampled in this study, but information regarding the presence of cephalosporin-resistant *E. coli* in these flocks was available from the poultry industry's action plan (Animalia, 2015). Sampling of these flocks was done once during the

production period using boot swabs, and the samples were analysed using the same detection method in the laboratory. Furthermore, some grandparent flocks provided hatching eggs for a limited number of broiler flocks. Information regarding the occurrence of cephalosporin-resistant *E. coli* in grandparent flocks was provided by Nortura SA.

Broiler producers were reminded per e-mail if the sample was not received within the week the flock was going to be slaughtered, while producers with parent flocks were reminded if the sample was not received five weeks after the previous sample.

Information about ancestry, flock size, slaughter date and slaughter age for all sampled broiler flocks was provided by Nortura SA.

2.3. Detection of cephalosporin-resistant E. coli

Boot swabs were dissolved in MacConkey broth (Beckton, Dickinson and Company, Le Pont de Claire, France) with 1mg/L cefotaxime (Duchefa, Haarlem, The Netherlands) and incubated at 37°C overnight. Subsequently, 10 µL of the broth was plated out on MacConkey agar (BD) supplemented with 1 mg/L cefotaxime, and MacConkey agar supplemented with 2 mg/L ceftazidime (Sigma-Aldrich, St. Louis, MO, USA). Agar plates were incubated at 37°C for 24-48 hours. Colonies with typical morphology were plated on blood agar, incubated at 37°C overnight, and confirmed as *E. coli* using Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF, Bruker Daltonics). The cephalosporin resistance phenotype was investigated using the disk-diffusion method as recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, www.eucast.org). Real-time PCR with previously published primers and probe (Schmidt et al., 2015) was applied to determine the genetic background of acquired cephalosporin resistance. The specificity of the detection method was assumed to be 100%, while a conservative estimate for the sensitivity was assumed to be 95% (Wasyl et al., 2010). The method corresponds to the method recommended by the European Food Safety Authority (EFSA, 2011).

2.4. Case definition and dependent variable

The occurrence of cephalosporin-resistant *E. coli* in a broiler flock was defined as the dependent variable. The status of a flock was defined as positive if *E. coli* from the sample were able to grow on MacConkey agar supplemented with cephalosporins, and an acquired cephalosporin resistance gene was detected by PCR. Otherwise, the flock was defined as negative.

2.5. Description of variables

The questionnaire included 58 multiple choice questions on potential risk factors for occurrence of cephalosporin-resistant *E. coli* in the broiler flock. The main subjects covered were information regarding the size of the production, management factors at the farm and in the broiler house(s), and

areas surrounding the broiler house(s). Information regarding washing and disinfection routines was divided into single binary variables describing whether or not different parts of the house and equipment were washed or disinfected between each production cycle. Also, a binary variable indicating whether or not the disinfection routine was performed between each production cycle was included. For 13 of the variables derived from the questionnaire, two or more categories were merged to accomplish reasonable representation of each category.

The variable “Day sampled” represented the day a certain flock was sampled, and was defined as number of days after 31st January 2014, in accordance with the starting date for sampling of the included broiler flocks. Thus, “Day sampled” represented a variable explaining a possible seasonal variation in occurrence of cephalosporin-resistant *E. coli*.

For ten parent flocks, the status regarding occurrence of cephalosporin-resistant *E. coli* was unknown. To avoid missing data in the analysis, parent status was imputated once based on the distribution of the status of grandparent flocks and the status of the receiving parent flocks. Imputation was performed using the ranbin function in SAS Enterprise Guide 6.1 for Windows. If the status of the grandparent flock was negative, the probability of a receiving parent flock being positive was 0.12. Furthermore, if the status of the grandparent flock was positive, the probability of the receiving parent flock being positive was 0.14. In total, 25 broiler flocks were affected by the imputated parent status. In order to ensure that the imputation did not have a significant effect in the model, sensitivity analysis was performed on three datasets. The status of all parent flocks with an imputated status was changed to negative in the first dataset, positive in the second dataset, while all the 25 broiler flocks affected by the imputation were excluded in the third dataset. Univariable analyses with the status of the broiler flock as outcome and parent status as explanatory variable were performed on each dataset.

A variable describing the “overall status” of the parent flocks supplying hatching eggs for a single broiler flock was determined as follows; if at least one of the parent flocks were positive, the overall status was positive. If all parent flocks were negative, the overall status was negative.

Variables explaining the “total score cleaning” and “total score disinfection” were determined on the basis of how many parts of the broiler house and equipment were washed and disinfected between production cycles. One point was awarded for each part cleaned or disinfected with a maximum score of 11.

In order to distinguish between different houses at the same farm, a variable explaining the House ID was included in the dataset. If the farm only had one house, the House ID was the same as the Farm ID. A variable describing the number of parent flocks (i.e. 1, 2 or ≥3 flocks) supplying hatching eggs to each broiler flock and a variable explaining the size of the broiler flock (>10,000; 10,000-20,000; >20,000 chickens) was derived from the data provided by Nortura SA.

The start of the production period for a broiler flock was calculated as follows;

$$t_{ij} = s_{ij} - a_{ij} \quad (2)$$

where s was the slaughter date of flock i at farm j, and a is the slaughter age of flock i at farm j.

To calculate the number of empty days between two consecutive production cycles, the following formula was used;

$$t_{empty} = t_{ij} - s_{(i-1)j} \quad (3)$$

where t was the start of production of flock i at farm j, and s is the slaughter date of flock i at farm j. The number of empty days between production cycles was divided into three categories; <11 days, 11-20 days and >20 days.

A variable explaining the status of the previous broiler flock in the same house was included on the basis of the samples collected in this study. Consequently, the first flock sampled in each included house (n=37) was excluded from the dataset to avoid missing values for the variable "Status of the previous broiler flock in the same house". In addition, farms or houses where samples from only a single flock were available were excluded in order to enable inclusion of a random nested effect of House ID within Farm ID in the model. The final dataset included 182 observations from 34 different broiler houses.

2.6. Data management and statistical analyses

Data management was performed in SAS Enterprise Guide 6.1 for Windows (SAS Institute Inc.), and statistical analyses were performed in R version 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria).

2.7. Univariable and multivariable analysis

All explanatory variables were fit into separate univariable logistic regression models using the function glm in R with status as the binary outcome variable. In parallel, the variables were also fit into separate univariable logistic regression models using the function glmer in the lme4 library in order to include a nested random effect of House ID within Farm ID. A p-value ≤ 0.20 was set as criterion for considering inclusion of the variable in the multivariable analysis.

"Day sampled" was modelled as a b-spline using the function bs in the R library splines.

The multivariable analysis was divided into two steps. First, variables were sorted into sub-groups, and a multivariable model was built for each sub-group. For each sub-group, associations between pairs of included variables were tested using Pearson Chi-squared tests (two categorical variables),

ANOVA (one categorical and one numerical variable) or Spearman correlation (two numerical variables). If two variables were significantly associated, the variable with the highest biological plausibility or with the strongest association to the outcome was withheld for inclusion in the multivariable analysis. Each sub-model was built by backward selection. Only variables with a p-value ≤ 0.05 were retained in the sub-models. Subsequently, the significant variables from each of the sub-models were included in building of the final predictive model. This model was built as described for the sub-models. Due to the hierarchical structure of the data (i.e. multiple flocks and houses within farms), we performed the modelling with and without a nested random effect of House ID within Farm ID. Finally, all remaining variables, regardless of exclusion in univariable- and sub-models, were tested against the model one by one to investigate if any confounding effects were present. Furthermore, biologically plausible interactions between variables included in the final model were investigated. The models were compared using ANOVA and Akaike information criterion (AIC).

A receiver-operating characteristics (ROC)-curve was used to assess the overall predictive ability of the model, and the area under the curve was calculated. Residuals were plotted against predicted values and variables included in the final model.

Odds ratios and 95% confidence intervals were calculated from the estimated coefficients in the final model, and used as a prediction of the strength of the association between the variable and the outcome.

3. Results

3.1. Response rate

Two broiler producers randomly selected for participation in the project were excluded due to discontinuation of broiler production. The response-rate for the questionnaire was 72.6 % (45/62). However, only 31 of the 45 responders contributed with samples from their broiler flocks, and two of these were excluded due to affiliation to another hatchery. Thus, the final response rate was 46.8 % (29/62). An overview of included and excluded broiler producers is illustrated in Figure 2.

3.2. Descriptive results

Cephalosporin-resistant *E. coli* were detected in samples from eight (13.8 %) parent flocks (n=58). Furthermore, 50 (22.5 %) of all sampled broiler flocks (n=222) and 41 (22.5 %) of the broiler flocks included in the final dataset (n=182) were positive. All isolates displayed an AmpC-phenotype and were found to carry the $bla_{\text{CMY-2}}$ gene encoding cephalosporin resistance.

Five of the included broiler producers (n=27) had more than one house at the farm, of which four producers had two houses and one producer had four houses, leading to a total of 34 houses included in the study. Overall, 28 (82.4 %) of the houses housed only or mainly negative flocks (60-100 % of

flocks negative), while three houses (8.8 %) housed only positive flocks. In three houses (8.8 %), an equal distribution between positive and negative flocks was seen. The number of flocks sampled per house varied from two-eight, and the time span between sampling of consecutive flocks varied from 27-151 days.

The average flock size was approximately 14,000 broilers (range 3,600-30,450), and the mean age of broilers at sampling was 17 days (range 6-32). The mean number of empty days between two production cycles was 22 (range 5-118 days).

The majority of the broiler flocks (n=85, 46.7 %) received day-old chickens from two parent flocks, while 58 (31.9 %) broiler flocks received chickens from a single parent flock, and 39 (21.4 %) received chickens from three or more parent flocks. None of the sampled broiler flocks were subjected to treatment with antimicrobial agents.

3.3. Multivariable analysis

Results from the univariable analyses are presented in supplementary material (Tables S1a-b).

The variance for the nested random effect of House ID within Farm ID did not change when the variable describing floor disinfection was excluded, while the random effect of Farm ID increased from <0.001 to 0.4 (Tables S3a and S3b, supplementary material). For the nested random effect of House ID within Farm ID, the variance increased from <0.001 to 0.12 when excluding the variable describing transport personnel entering the house. The variance for the random effect of Farm ID did not change (Tables S3a and S3c, supplementary material). When comparing the different models including the nested random effect of House ID within Farm ID, the full model provided the best fit for the data (p=0.02 and p=0.01, respectively) (Table S3a, supplementary material). The estimates for the included variables did not change significantly using the different models, but the AIC values were higher for the models including the nested random effect compared to the model excluding the nested random effect (Table 1 and Tables S3a-c, supplementary material). Also, when the final multivariable models including and excluding the nested random effect of House ID within Farm ID were compared by ANOVA, the random effect was not significant (p=1.0). Therefore, the simplest model possible with a high degree of explanation of the data without the nested random effect was chosen. The final multivariable model included the four variables “Status of previous broiler flock in the same house”, “Transport personnel enter the room where the broilers are raised”, “Always disinfect floor between production cycles” and “Number of parent flocks supplying the broiler flock” (Table 1). Disinfection of floor between production cycles reduced the odds of a positive status (OR=0.1, p=0.01, 95 % confidence interval [CI] 0.03-0.6). On the other hand, a positive status of the previous flock (OR=12.7, p<0.001, 95 % CI 4.8-33.5), having transport personnel entering the room where the broilers are raised (OR=9.3, p=0.01, 95 % CI 1.6-55.1) and having three or more parent flocks supplying the broiler flock

with day old chickens (OR=6.3, p=0.01, 95 % CI 1.6-25.0) all increased the odds of a positive status (Table 1).

The area under the ROC curve was 0.86, indicating a good overall fit of the model to the observed data. The plot of residuals against the predicted values revealed no major shortcomings of the model.

4. Discussion

Our study indicated that the variables "status of previous broiler flock in the same house", "transport personnel entering the room where the broilers are raised", "always disinfect floor between production cycles" and number of parent flocks supplying the broiler flock" were associated with a broiler flock being positive for cephalosporin-resistant *E. coli*.

The models including a nested random effect of House ID within Farm ID did not improve the models, as random effect provided the same explanation as the variables describing floor disinfection and transport personnel entering the house. These results led us to the conclusion that the multivariable model excluding the nested random effect provided the simplest and best fit for the data.

We identified a positive status of the previous broiler flock in the same broiler house as the most significant risk factor for occurrence of cephalosporin-resistant *E. coli* in a broiler flock. If the previous flock was positive, the odds of the next flock being positive was approximately thirteen times higher compared to if the previous flock was negative. Also, not always disinfecting the floor between production cycles was identified as a significant risk factor. This suggests that good cleaning and disinfection routines are essential in lowering the risk of transmission of cephalosporin-resistant *E. coli* from one flock to the next. Improvement of hygiene has also previously been suggested as a control measure to prevent spread of cephalosporin-resistant *E. coli*, and to prevent local recirculation of resistant strains in broiler houses (Liebana et al., 2013). Furthermore, we found that some broiler houses included in the study housed mainly positive flocks, while others housed none or only a few positive flocks. These findings enhance the theory that recirculation of cephalosporin-resistant *E. coli* in broiler houses may occur. A previous study from the Netherlands identified cephalosporin-resistant *E. coli* in empty broiler houses even after intensive cleaning and disinfection routines had been performed (Dierikx et al., 2013b). Recirculation of cephalosporin-resistant *E. coli* to consecutive flocks has also been suggested by others (Hiroi et al., 2012; Dierikx et al., 2013a; Laube et al., 2013; Agerso et al., 2014; Nilsson et al., 2014). These findings also underline the importance of sufficient cleaning and disinfection routines to minimize the risk of cephalosporin-resistant *E. coli* surviving in the broiler house and infecting the subsequent flock. However, our findings are in contrast to previous findings in a Belgian study, where it was suggested that a dirty environment may lead to a decrease in occurrence of resistant bacteria due to a more diverse microbiota and a dilution effect by susceptible bacteria (Persoons et al., 2011). Contamination of the environment surrounding the broiler farms with

cephalosporin-resistant *E. coli* has been reported (Laube et al., 2014; Blaak et al., 2015). Thus, there is a possibility of cross-contamination from surrounding areas into the broiler house if hygienic measures are insufficient, or possibly via vectors such as flies (Blaak et al., 2014). The contamination of the surroundings, and thus the risk of cross-contamination will probably be higher on farms with many positive flocks, and might offer an explanation for why we observed some farms where the majority of flocks were positive, while other farms had no or only a few positive flocks.

The variable "Transport personnel enter the room where the broilers are raised" was identified as having a strong positive association with broiler flock status. Furthermore, this variable was not surprisingly, strongly associated with the variable "Average frequency of visitors in the broiler house per production cycle", and in the final model only one could be included. However, both variables represent an increased number of people entering the broiler house, which again might result in an increased risk of cross-contamination of resistant strains between hatchery and broiler house, between broiler houses at different farms, or between surrounding environment and the broiler house.

The odds of a positive status increased when three or more parent flocks supplied day-old chickens for a specific broiler flock. This may be explained by an increased probability of at least one of the supplying parent flocks being positive when the number of parent flocks increase, as only a limited occurrence of cephalosporin-resistant *E. coli* in parent flocks was found in this study. However, as no apparent association was found between status of supplying parent flocks and status of broiler flocks this theory does not represent a full explanation. Another possibility may be that several parent flocks can have a low level of cephalosporin-resistant *E. coli* circulating, and that this low level cannot be detected by the laboratory tests used in our study. However, when several parent flocks with low levels of cephalosporin-resistant *E. coli* supply one broiler flock with day old chickens, the "burden" of cephalosporin-resistant *E. coli* might accumulate, and the broiler flock could be positive. The lack of an association between the status of parent flocks and the status of broiler flocks receiving day-old chickens from these parent flocks was somewhat surprising. Vertical transmission of clonally related cephalosporin-resistant *E. coli* through the broiler production has been substantiated by findings in a study from Sweden (Nilsson et al., 2014), which has a broiler production highly similar to that in Norway. However, a decrease in the occurrence of cephalosporin-resistant *E. coli* in parent flocks has been reported in the Norwegian broiler production (Mo et al., 2014). Also, cephalosporin-resistant *E. coli* was not detected in any of the samples collected in conjunction with hatching of imported parent animals by Nortura Samvirkekylling in 2014 (Animalia, 2015). Thus, it is possible that the initial introduction of cephalosporin-resistant *E. coli* to the Norwegian broiler production was by vertical transmission from breeding animals as previously hypothesized (NORM/NORM-VET, 2007; Sunde et al., 2009), but that recirculation on farms already contaminated with resistant bacteria is more important when the burden of resistant bacteria from supplying parent flocks is low.

Previous studies have pointed at treatment with antimicrobials as an important risk factor for occurrence of cephalosporin-resistant *E. coli* in broiler flocks (Persoons et al., 2011; Agerso et al., 2014). In this study, none of the sampled broiler flocks were treated with antimicrobial agents. Furthermore, the consumption of antimicrobial agents in the Norwegian broiler production is minimal (Mo et al., 2014; Animalia, 2015; Refsum, 2015; Animalia, 2016), and therefore this important risk factor cannot be identified in Norway.

The occurrence of cephalosporin-resistant *E. coli* in broiler flocks and parent flocks in this study was low to moderate compared to other countries (Persoons et al., 2011; Dierikx et al., 2013a; Reich et al., 2013; Agerso et al., 2014; MARAN, 2015). The plasmid-mediated AmpC-gene $bla_{\mathrm{CMY-2}}$, was identified in all cephalosporin-resistant isolates, which is in accordance with previous findings (Mo et al., 2014; NORM/NORM-VET, 2015).

A strength of the study was that the broiler flocks of included producers were sampled throughout a year. Thus, we would be able to identify possible seasonal variations in the occurrence of cephalosporin-resistant *E. coli*. However, no such variation was present in our data. Furthermore, we were able to get an overview of several flocks at each farm, meaning that we could identify whether included producers had houses where the majority of flocks were positive or negative. Also, parent flocks supplying the broiler flocks included in the study were sampled prior to the broiler flocks, allowing us to identify a possible association between status of the parent flocks and status of the broiler flocks. Data on the ancestry of all broiler flocks sampled in the study and the status of grandparent flocks supplying the included flocks was available from Nortura SA. However, this study also had some limitations. Only farms affiliated to a single hatchery were included, and the final response rate was low. In addition, samples were not provided for all flocks on all included farms, as we only received samples from 222 of 275 flocks (80.7%). This led to a smaller sample size than expected. Participation in the study was voluntary, possibly resulting in an over-representation of farmers with interest in issues regarding antimicrobial resistance and bacterial contamination. It is possible that these farmers also were extra careful with their cleaning and disinfection routines, and had better biosecurity routines than an average farmer. However, we still found that infrequent disinfection of floors, and transport personnel entering the broiler house increased the odds of occurrence of cephalosporin-resistant *E. coli* in broiler flocks. Thus, it is possible that the effect of these two variables have been underestimated in the current study. The online questionnaire was answered before the initiation of sampling. Therefore, it cannot be excluded that some of the answers provided might not be applicable for all flocks from the same farm, as routines could have changed during the study period. The direction of any possible bias resulting from this is difficult to estimate. The farms were located in a restricted geographical area, and only one hybrid, namely Ross 308, was represented. Thus, the results might not be directly extrapolated to the entire Norwegian broiler production. However, Nortura SA has the main share in the retail chicken meat market, and Ross 308

is by far the most common hybrid used in the Norwegian broiler production. Although the Norwegian broiler production is quite uniform, both hybrid and hatchery of origin has previously been identified as risk factors for cephalosporin-resistant *E. coli* (Persoons et al., 2011), and a possible effect of these two factors cannot be excluded on the basis of this study. Only one broiler producer reported that floors were not always disinfected between production cycles, and two broiler producers reported that transport personnel occasionally entered the room where the broilers are raised when delivering day-old chickens. Thus, it cannot be excluded that other factors than disinfection of floor and transport personnel entering the broiler house, not identified by the questionnaire, can be the reason that these producers differ from the others. However, it is highly biologically plausible that cephalosporin-resistant *E. coli* can survive in the broiler house if disinfection is not done, and that cross-contamination from hatcheries, other broiler farms or surrounding areas can occur if transport personnel enter the room where the broilers are raised.

5. Conclusion

This is the first study identifying risk factors for occurrence of cephalosporin-resistant *E. coli* in Norwegian broilers. Our results indicate that implementation of a high level of biosecurity with a minimal number of persons entering the broiler house during each production cycle, and intensive washing and disinfection routines will contribute to a decrease in the odds of cephalosporin-resistant *E. coli* being detected in a broiler flock.

Acknowledgements

This work was funded by the Foundation for Research Levy on Agricultural Agreement Research Fund (Matfondavtalen), grant no. 225165. All parent and broiler producers contributing with samples from their flocks are greatly acknowledged. Atle Løvland and Anne-Mette Dagrød (Nortura SA) is acknowledged for providing data on ancestry, flock size, slaughter age and slaughter date for the sampled broiler flocks, and information regarding the Norwegian broiler production pyramid.

References

Agerso, Y., Jensen, J.D., Hasman, H., Pedersen, K., 2014. Spread of extended spectrum cephalosporinase-producing Escherichia coli clones and plasmids from parent animals to broilers and to broiler meat in a production without use of cephalosporins. Foodborne Pathog. Dis. 11, 740-746.

Animalia, 2015. Tiltak mot antibiotikaresistens virker. http://animalia.no/Listesider/Aktuelt-og-fagstoff/Tiltak-mot-antibiotikaresistens-virker-/

Animalia, 2016. Fortsatt nedgang i forekomst av resistente bakterier hos fjørfe. http://animalia.no/Listesider/Aktuelt-og-fagstoff/Fortsatt-nedgang-i-forekomst-av-resistente-bakterier-hos-fjorfe/

Blaak, H., Hamidjaja, R.A., van Hoek, A.H., de Heer, L., de Roda Husman, A.M., Schets, F.M., 2014. Detection of extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli on flies at poultry farms. Appl. Environ. Microbiol. 80, 239-246.

Blaak, H., van Hoek, A.H., Hamidjaja, R.A., van der Plaats, R.Q., Kerkhof-de Heer, L., de Roda Husman, A.M., Schets, F.M., 2015. Distribution, Numbers, and Diversity of ESBL-Producing E. coli in the Poultry Farm Environment. PLoS One 10, e0135402.

Börjesson, S., Bengtsson, B., Jernberg, C., Englund, S., 2013. Spread of extended-spectrum beta-lactamase producing Escherichia coli isolates in Swedish broilers mediated by an incl plasmid carrying bla(CTX-M-1). Acta Vet. Scand. 55, 3.

Börjesson, S., Guillard, T., Landen, A., Bengtsson, B., Nilsson, O., 2015. Introduction of quinolone resistant Escherichia coli to Swedish broiler population by imported breeding animals. Vet. Microbiol.

Carattoli, A., 2008. Animal reservoirs for extended spectrum beta-lactamase producers. Clin. Microbiol. Infect. 14 Suppl 1, 117-123.

Coque, T.M., Baquero, F., Canton, R., 2008. Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. Euro Surveill. 13, 1-11.

Dierikx, C., van der Goot, J., Fabri, T., van Essen-Zandbergen, A., Smith, H., Mevius, D., 2013a. Extended-spectrum-beta-lactamase- and AmpC-beta-lactamase-producing Escherichia coli in Dutch broilers and broiler farmers. J. Antimicrob. Chemother. 68, 60-67.

Dierikx, C.M., van der Goot, J.A., Smith, H.E., Kant, A., Mevius, D.J., 2013b. Presence of ESBL/AmpC -Producing Escherichia coli in the Broiler Production Pyramid: A Descriptive Study. PLoS One 8, e79005.

EFSA, 2011. Scientific opinion on the public health risks of bacterial strains producing extended-spectrum beta-lactamases and/or AmpC beta-lactamases in food and food-producing animals. EFSA journal 9, 2322.

Ewers, C., Bethe, A., Semmler, T., Guenther, T., Wieler, L.H.,, 2012. Extended-spectrum beta-lactamase-producing and AmpC-producing Escherichia coli from livestock and companion animals, and their putative impact on public health: a global perspective. Clin. Microbiol. Infect. 18, 646-655.

Hiroi, M., Matsui, S., Kubo, R., Iida, N., Noda, Y., Kanda, T., Sugiyama, K., Hara-Kudo, Y., Ohashi, N., 2012. Factors for occurrence of extended-spectrum beta-lactamase-producing Escherichia coli in broilers. J. Vet. Med. Sci. 74, 1635-1637.

Laube, H., Friese, A., von Salviati, C., Guerra, B., Kasbohrer, A., Kreienbrock, L., Roesler, U., 2013. Longitudinal Monitoring of Esbl/Ampc-Producing Escherichia Coli in German Broiler Chicken Fattening Farms. Appl. Environ. Microbiol. 79, 4815-4820.

Laube, H., Friese, A., von Salviati, C., Guerra, B., Rosler, U., 2014. Transmission of ESBL/AmpC-producing Escherichia coli from broiler chicken farms to surrounding areas. Vet. Microbiol. 172, 519-527.

Liebana, E., Carattoli, A., Coque, T.M., Hasman, H., Magiorakos, A.P., Mevius, D., Peixe, L., Poirel, L., Schuepbach-Regula, G., Torneke, K., Torren-Edo, J., Torres, C., Threlfall, J., 2013. Public health risks of enterobacterial isolates producing extended-spectrum beta-lactamases or AmpC beta-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. Clin. Infect. Dis. 56, 1030-1037.

Lyngstad, T.M., Jonsson, M.E., Hofshagen, M., Heier, B.T., 2008. Risk factors associated with the presence of Campylobacter species in Norwegian broiler flocks. Poult. Sci. 87, 1987-1994.

MARAN, 2015. MARAN 2015. Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2014. In: Mevius, D.J., Dierikx, C., Veldman, K.T., Wit, B., Pelt, W.v., Heederik, D. (Eds.).

Mo, S.S., Norström, M., Slettemeås, J.S., Løvland, A., Urdahl, A.M., Sunde, M., 2014. Emergence of AmpC-producing Escherichia coli in the broiler production chain in a country with a low antimicrobial usage profile. Vet. Microbiol. 171, 315-320.

Nilsson, O., Börjesson, S., Landen, A., Bengtsson, B., 2014. Vertical transmission of Escherichia coli carrying plasmid-mediated AmpC (pAmpC) through the broiler production pyramid. J. Antimicrob. Chemother. 69, 1497-1500.

NORM/NORM-VET, 2007. NORM/NORM-VET 2006. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo.

NORM/NORM-VET, 2012. NORM/NORM-VET 2011. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo.

NORM/NORM-VET, 2015. NORM/NORM-VET 2014. Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway. Tromsø/Oslo.

Overdevest, I., Willemsen, I., Rijnsburger, M., Eustace, A., Xu, L., Hawkey, P., Heck, M., Savelkoul, P., Vandenbroucke-Grauls, C., van der Zwaluw, K., Huijsdens, X., Kluytmans, J., 2011. Extended-spectrum beta-lactamase genes of Escherichia coli in chicken meat and humans, The Netherlands. Emerg. Infect. Dis. 17, 1216-1222.

Persoons, D., Haesebrouck, F., Smet, A., Herman, L., Heyndrickx, M., Martel, A., Catry, B., Berge, A.C., Butaye, P., Dewulf, J., 2011. Risk factors for ceftiofur resistance in Escherichia coli from Belgian broilers. Epidemiol. Infect. 139, 765-771.

Refsum, T., 2015. Antibiotikabehandling i norsk fjørfeproduksjon. Go' mørning, Animalia, http://www.animalia.no/upload/FIler%20til%20nedlasting/Go%C2%B4m%C3%B8rning/2015/GM%204-15%20web.pdf.

Reich, F., Atanassova, V., Klein, G., 2013. Extended-spectrum beta-lactamase- and AmpC-producing enterobacteria in healthy broiler chickens, Germany. Emerg. Infect. Dis. 19, 1253-1259.

Schmidt, G.V., Mellerup, A., Christiansen, L.E., Stahl, M., Olsen, J.E., Angen, O., 2015. Sampling and Pooling Methods for Capturing Herd Level Antibiotic Resistance in Swine Feces using qPCR and CFU Approaches. PLoS One 10, e0131672.

Sunde, M., Tharaldsen, H., Slettemeås, J.S., Norström, M., Carattoli, A., Bjorland, J., 2009. Escherichia coli of animal origin in Norway contains a blaTEM-20-carrying plasmid closely related to blaTEM-20 and blaTEM-52 plasmids from other European countries. J. Antimicrob. Chemother. 63, 215-216.

Thrusfield, M., 2005. Veterinary Epidemiology. Blackwell Science Ltd. Oxford, UK.

Wasyl, D., Hoszowski, A., Zajac, M., Skarzynska, M., 2010. Simple and efficient screening method for the detection of cephalosporin resistant Escherichia coli. Bull. Vet. Inst. Pulawy. 54, 147-151.

WHO-AGISAR, 2011. Critically Important Antimicrobials for Human Medicine- 3rd rev. World Health Organization, Geneva, Switzerland.

Winokur, P.L., Vonstein, D.L., Hoffman, L.J., Uhlenhopp, E.K., Doern, G.V., 2001. Evidence for transfer of CMY-2 AmpC beta-lactamase plasmids between Escherichia coli and Salmonella isolates from food animals and humans. Antimicrob. Agents Chemother. 45, 2716-2722.

1 **Table 1.** Results from the multivariable generalized model used to identify risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in 182
2 Norwegian broiler flocks originating from 34 houses on 27 farms during February 2014-January 2015. Nested random effect of House ID within Farm ID was
3 not found to be significant (ANOVA, p=1.0), and therefore not included in the final model.

Variable	Estimate	SE	OR (95% CI)	*p*- value
Status of previous broiler flock in the same house				
Negative	0		1	
Positive	2.5	0.5	12.7 (4.8-33.5)	<0.001
Transport personnel enter the room where the broilers are raised				
Never	0		1	
Occasionally	2.2	0.9	9.3 (1.6-55.1)	0.01
Always disinfect floor between production cycles				
No	0		1	
Yes	-2.0	0.8	0.1 (0.03-0.6)	0.01
Number of parent flocks supplying the broiler flock				0.02
1	0		1	
2	1.2	0.6	3.2 (0.9-11.1)	0.06
>2	1.8	0.7	6.3(1.6-25.0)	0.01

4 Null deviance 194 on 181 degrees of freedom, residual deviance 126 on 176degrees of freedom, and AIC=138.0. SE: standard error, OR: odds ratio, CI:
5 confidence interval.

Figure 1. Structure of the broiler production pyramid. Norway is dependent on import of hatching eggs produced by grandparent animals in Sweden. In Norway, only parent animals and broiler are produced.

Figure 2. Flow-chart showing included and excluded broiler producers in a study of risk factors for occurrence of cephalosporin-resistant *E. coli* in Norwegian broiler flocks from February 2014 through January 2015. The inclusion criteria were as follows; 1) Active commercial broiler producer, 2) respond to online questionnaire, 3) provide samples from broiler flocks housed at the farm during the study period, 4) affiliation to the hatchery of Nortura Samvirkekylling

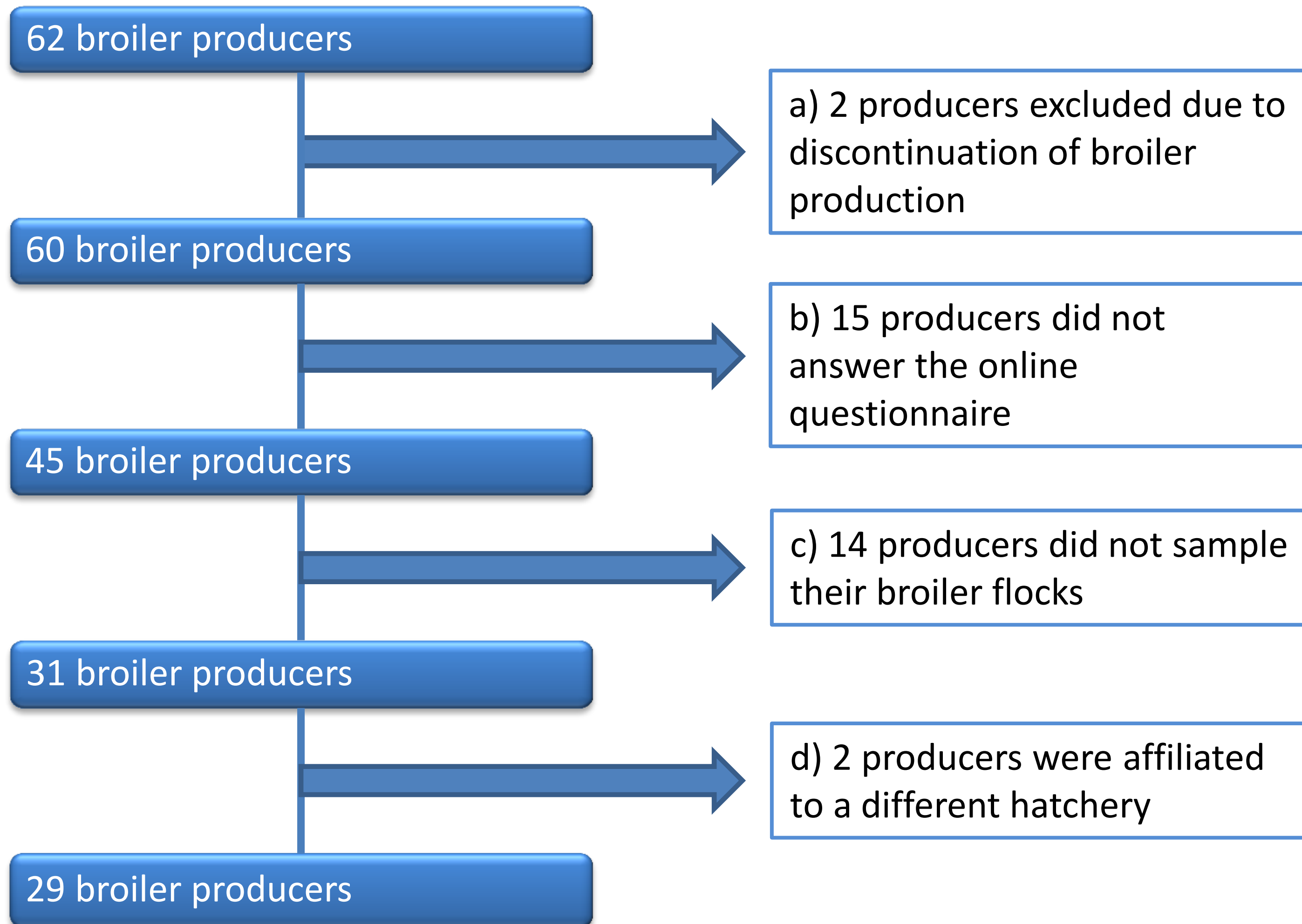
Purebred pedigree and great grandparent animals
(Breeding companies in Scotland or Germany)

Grandparent animals
(Sweden)

Parent animals
Rearing flocks 0-18 weeks
Breeding flocks 18-60 weeks

Broiler chickens

Retail chicken meat


Total number of broiler producers
Number of excluded broiler producers
62 broiler producers
a) 2 producers excluded due to discontinuation of broiler production
60 broiler producers
b) 15 producers did not answer the online questionnaire
45 broiler producers
c) 14 producers did not sample their broiler flocks
31 broiler producers
d) 2 producers were affiliated to a different hatchery
29 broiler producers

Supplementary material

Results from the univariable analyses

A total of 34 variables had a *p*-value ≤ 0.20 in the univariable analysis and were considered for inclusion in the multivariable analysis excluding random effect (Table S1a).When a nested random effect of House ID within Farm ID was included in the univariable analysis, 17 variables had a *p*-value ≤ 0.20 and were considered for inclusion in the multivariable analysis (Table S1b). The variables were divided into sub-groups as shown in Table S2a-b. For sub-models 2-5 at least one pair of variables were significantly associated. All remaining variables had a *p*-value > 0.20, and were not eligible for inclusion in the multivariable analysis.

The sensitivity analysis of the imputation of parent flock status showed that none of the different imputation methods changed the association between parent flock status and broiler flock status.

Data provided in Tables S1a-b represent results from the univariable logistic regression on potential risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in 182 Norwegian broiler flocks during February 2014-January 2015 excluding and including a nested random effect of house (House ID) within farm (Farm ID), respectively. The dataset used for analyses was extracted from the original dataset, as only houses and farms with two or more flocks sampled could be included in analyses with random effect. In order to facilitate comparison of models with and without random effect, the same dataset was used for analysis including and excluding the nested random effect. Table S1a presents results from models run without random effect, while Table S1b present results from models run including a nested random effect of House ID within Farm ID.

Table S1a. Results from the univariable generalized linear model on potential risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in 182 Norwegian broiler flocks originating from 34 houses on 27 farms during February 2014-January 2015. The collection of information regarding possible risk factors were done at both farm level (a) and flock level (b). The overall *p*-values for variables with more than two categories were calculated using ANOVA.

Variable	Negative flocks (No.)	Positive flocks (No.)	OR (95 % CI)	*p*- value	AIC	Overall *p*-value
Number of parent flocks supplying the broiler flock (b)					193.8	0.04
One	50	8				
Two	66	19	1.8 (0.7-4.4)	0.20		
More than two	25	14	3.5 (1.3-9.4)	0.01		
Age of flock (in days) at sampling (b)					190.8	
Range: 6-32			1.1 (1.0-1.2)	0.01		
Mean=17						
Median=19						
Placement of the broiler house (a)					185.5	<0.001
Own separate building	92	36				
Integrated in another building	26	5	0.5 (0.2-1.4)	0.18		
Different for different houses	23	0	<0.001 (0-∞)	0.99		
Floor heating in the broiler house (a)					184.2	<0.001
No	89	16				
Yes	42	25	3.3 (1.6-6.8)	0.001		
Different for different houses	10	0	<0.001 (0-∞)	1.0		

Bucket used to collect dead chickens is in contact with unclean part of the broiler house (a)					195.7	
Never	96	33				
Occasionally	45	8	0.5 (0.2-1.2)	0.13		
Transport personnel enter the room where the broiler are raised (a)					181.9	
Never	138	32				
Occasionally	3	9	12.9 (3.3-50.5)	<0.001		
Storage of the paper used to feed chickens on between production cycles					195.4	0.09
Wrapped in packaging	122	31				
Unpacked	10	8	3.1 (1.1-8.6)	0.03		
Don't use paper	9	2	0.9 (0.2-4.3)	0.87		
Air inlet secured with mesh (a)					193.4	
No	17	1				
Yes	124	40	5.5 (0.7-42.5)	0.10		
Mesh checked between production cycles (a)					191.6	0.01
Don't have mesh	17	1				
Rarely	70	22	5.3 (0.7-42.5)	0.11		
Often	39	7	3.1 (0.3-26.8)	0.31		
Always	15	11	12.5 (1.4-108.3)	0.02		
Water source for the broiler house (a)					187.2	
Public or private waterworks	99	17				
Own private water source	42	24	3.3 (1.6-6.8)	0.001		
Specify type of private water source (a)					193.0	0.03
Drill water	45	23				
Well	5	2	0.8 (0.1-4.3)	0.78		
Lake/pond	19	2	0.2 (0.04-1.0)	0.04		
Not private water source	72	14	0.4 (0.2-0.8)	0.01		
UV equipment in the broiler house (a)					194.6	
No	68	13				
Yes	73	28	2.0 (1.0-4.2)	0.06		
Alarm if UV equipment fails (a)					196.5	0.15

No	36	15				
Yes	37	13	0.8 (0.4-2.0)	0.70		
Don't have UV equipment	68	13	0.5 (0.2-1.1)	0.07		
Bacteriological sampling of UV equipment yearly (a)					197.7	0.29
No	64	23				
Yes	13	5	1.1 (0.3-3.3)	0.91		
Don't have UV equipment	64	13	0.6 (0.3-1.2)	0.14		
Tractor used when litter material is spread in the house (a)					193.5	
No	64	11				
Yes	77	30	2.3 (1.1-4.9)	0.04		
Distance to nearest lake, pond, pool or swamp (a)					190.0	0.01
0-200 meters	25	10				
200m-1 km	69	9	0.3 (0.1-0.9)	0.03		
>1 km	47	22	1.2 (0.5-2.9)	0.73		
Dog present at the farm (a)					182.5	
No	15	16				
Yes	126	25	0.2 (0.1-0.4)	<0.001		
Horse present at the farm (a)					193.3	
No	111	38				
Yes	30	3	0.3 (0.1-1.0)	0.05		
Caretakers live at the farm (a)					192.4	
No	13	10				
Yes	128	31	0.3 (0.1-0.8)	0.01		
Average frequency of visitors in the broiler house per production cycle (a)					173.9	
No visitors	95	10				
One visitor or more	46	31	6.4 (2.9-14.2)	<0.001		
Observed rodents in the room where the broilers are raised (a)					196.6	
No	122	32				
Yes	19	9	1.8 (0.7-4.4)	0.19		
Same tools used for cleaning and disinfectin in more than one house (a)					197.3	0.23

No	24	3			
Yes	45	13	2.3 (0.6-8.9)	0.22	
Only have one house	72	25	2.8 (0.8-10.0)	0.12	
Design of the hygiene barrier (a)					194.5
Own room	80	30			
Limited part of the service room	61	11	0.5 (0.2-1.0)	0.06	
Ground outside doors and entrance to the broiler house (a)					194.0
Hard (asphalt, concrete etc.)	108	37			
Gravel	33	4	0.4 (0.1-1.1)	0.06	
Disinfection always performed between production cycles (a)					196.1
No	29	13			
Yes	112	28	0.6 (0.3-1.2)	0.14	
Always disinfect floor between production cycles (a)					186.6
No	4	8			
Yes	137	33	0.1 (0.03-0.4)	<0.001	
Always disinfect walls between production cycles (a)					189.9
No	12	11			
Yes	129	30	0.3 (0.1-0.6)	0.003	
Always disinfect ceiling between production cycles (a)					195.4
No	86	19			
Yes	55	22	1.8 (0.9-3.6)	0.10	
Always disinfect ventilation system between production cycles (a)					196.5
No					
Yes			1.6 (0.8-3.2)	0.20	
Always disinfect footwear between production cycles (a)					195.7
No	78	13			
Yes	63	28	0.6 (0.3-1.1)	0.11	
Total score cleaning (a)					195.8
Range:9-11			1.7 (0.8-3.3)	0.16	
Mean=11					

Median=11						
Size of broiler flock (b)					198.3	0.38
<10,000	50	10				
10,000-20,000	65	23	1.8 (0.8-4.1)	0.18		
>20,000	26	8	1.5 (0.5-4.4)	0.42		
Status of previous broiler flock in the same house (b)					147.6	
Negative	129	15				
Positive	12	26	18.6 (7.8-44.4)	<0.001		
Empty period between production cycles (b)					195.9	0.11
< 11 days	21	4				
11-20 days	60	25	2.2 (0.7-7.0)	0.19		
>20 days	60	12	1.1 (0.3-3.6)	0.94		

Null-model: AIC=196.2. Null model including nested random effect of broiler house and broiler farm: AIC=168.9. OR: odds ratio, CI: confidence interval.

Table S1b. Results from the univariable generalized linear model including a nested random effect of House ID within Farm ID on potential risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in 182 Norwegian broiler flocks originating from 34 houses on 27 farms during February 2014-January 2015. The collection of information regarding possible risk factors were done at both farm level (a) and flock level (b). The overall *p*-values for variables with more than two categories were calculated using ANOVA.

Variables	Negative flocks (No.)	Positive flocks (No.)	OR (95 % CI)	*p*-value	AIC	Overall *p*-value
Number of parent flocks supplying the broiler flock (b)					168.2	0.09
One	50	8				
Two	66	19	2.2 (0.6-7.6)	0.23		
More than two	25	14	5.0 (1.1-22.8)	0.03		
Floor heating in the broiler house (a)						
No	89	16			167.3	0.06
Yes	42	25	4.3 (0.9-19.6)	0.06		
Different for different houses	10	0	<0.001 (0-∞)	1.0		
Transport personnel enter the room where the broiler are raised (a)					165.5	
Never	138	32				
Rarely	3	9	38.5 (1.6-943.5)	0.02		
Mesh checked between production cycles (a)					170.6	0.23
Don't have mesh	17	1				
Rarely	70	22	6.2 (0.2-211.5)	0.31		
Often	39	7	4.5 (0.12-171.3)	0.42		
Always	15	11	30.3 (0.7-1328.9)	0.08		
Water source for the broiler house (a)					166.1	
Public or private waterworks	99	17				
Own private watersource	42	24	6.6 (1.3-33.9)	0.02		
Specify type of private water source (a)					171.8	
Drillwater	45	23				0.36
Well	5	2	1.0 (0.02-59.3)	1.0		
Lake/pond	19	2	0.2 (0.01-2.9)	0.22		

Not private watersource	72	14	0.2 (0.03-1.2)	0.09		
UV equipment in the broiler house (a)					169.0	
No	68	13				
Yes	73	28	3.8 (0.6-24.4)	0.16		
Alarm if UV equipment fails (a)					170.5	0.29
No	36	15				
Yes	37	13	0.5 (0.04-4.5)	0.50		
Don't have UV equipment	68	13	0-2 (0.02-1.6)	0.13		
Dog present at the farm (a)					164.5	
No	15	16				
Yes	126	25	0.08 (0.01-0.6)	0.01		
Horse present at the farm (a)					169.0	
No	111	38				
Yes	30	3	0.2 (0.02-2.2)	0.18		
Caretakers live at the farm (a)					169.7	
No	13	10				
Yes	128	31	0.2 (0.02-2.2)	0.19		
Average frequency of visitors in the broiler house per production cycle (a)					162.4	
No visitors	95	10				
One visitor or more	46	31	9.6 (2.2-42.2)	0.003		
Ground outside doors and entrance to the broiler house (a)					168.8	
Hard (asphalt, concrete etc)	108	37				
Gravel	33	4	0.2 (0.01-2.1)	0.17		
Always disinfect floor between production cycles (a)					167.8	
No	4	8				
Yes	137	33	0.04 (0.002-1.2)	0.06		
Always disinfect walls between production cycles (a)					168.4	
No	12	11				
Yes	129	30	0.1 (0.01-1.3)	0.08		
Always disinfect ceiling between production cycles (a)					168.5	

No	86	19			
Yes	55	22	4.4 (0.6-31.2)	0.13	
Status of previous broiler flock in the same house (b)					150.7
Negative	129	15			
Positive	12	26	15.4 (5.5-42.8)	<0.001	

OR: odds ratio, CI: confidence interval.

Data provided in Tables S2a-b shows the sub-grouping of variables for building a multivariable generalized logistic regression model. Table S2a presents sub-grouping for a multivariable model without random effect, while Table S2b present sub-grouping for a multivariable model run including a nested random effect of House ID within Farm ID.

Table 2a. Sub-grouping of variables for building a multivariable generalized model in a study on risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in 182 Norwegian broiler flocks originating from 34 houses on 27 farms during February 2014- January 2015.

Sub-model	Description	Variables included
Sub-model 1	Flock specific variables	Status of previous broiler flock in the same house, Number of parent flocks supplying the broiler flock, Size of broiler flock, Age of broiler flock at sampling, Empty period between production cycles
Sub-model 2	House specific variables	Location of broiler house(s), Floor heating in the broiler house, Storage of the paper used to feed chickens on, Air inlet secured with mesh, Mesh checked between production cycles, Water source for the broiler house, Type of private water source, UV equipment in the broiler house, Alarm if UV equipment fails, Bacteriological sampling of UV equipment yearly, Ground outside doors and entrance to the broiler house
Sub-model 3	Biosecurity	Bucket used to collect dead chickens is in contact with the unclean part of the broiler house, Transport personnel enter the room where the broiler are raised, Tractor used when litter material is spread in the house, Average frequency of visitors in the broiler house per production period, Same tools used to clean and disinfect several broiler houses, Empty period between production cycles, Observed rodents in the room where the broilers are raised
Sub-model 4	Surroundings	Distance to nearest lake, pond, pool or swamp, Dog present at the farm, Horse present at the farm, Caretakers live at the farm
Sub-model 5	Cleaning and disinfection	Design of hygiene barrier, Always disinfect between production cycles, Always disinfect floors between production cycles, Always disinfect walls between production cycles, Always disinfect ceiling between production cycles, Always disinfect ventilation system between production cycles, Always disinfect footwear between production cycles, Total score cleaning

Table 2b. Sub-grouping of variables for building a multivariable generalized model including a nested random effect of House ID within Farm ID in a study on risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in 182 Norwegian broiler flocks originating from 34 houses on 27 farms during February 2014- January 2015.

Sub-model	**Description**	**Variables included**
Sub-model 1	Flock specific variables	Status of previous broiler flock in the same house, Number of parent flocks supplying the broiler flock
Sub-model 2	House specific variables	Floor heating in the broiler house, Mesh checked between production cycles, Water source for the broiler house, Type of private water source, UV equipment in the broiler house, Alarm if UV equipment fails, Ground outside doors and entrance to the broiler house
Sub-model 3	Biosecurity	Transport personnel enter the room where the broiler are raised, Average frequency of visitors in the broiler house per production period,
Sub-model 4	Surroundings	Dog present at the farm, Horse present at the farm, Caretakers live at the farm
Sub-model 5	Cleaning and disinfection	Always disinfect floors between production cycles, Always disinfect walls between production cycles, Always disinfect ceiling between production cycles,

Data provided in Tables 3a-c present results from the final multivariable model including a nested random effect of House ID within Farm ID, and the changes in the variation of random effect when the variables "Transport personnel enter the room where the broilers are raised" and "Always disinfect floor between production cycles" are removed from the model, respectively.

Table S3a. Results from the multivariable generalized model including a nested random effect of House ID within Farm ID used to identify risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in 182 Norwegian broiler flocks originating from 34 houses on 27 farms during February 2014-January 2015.

Variable	Estimate	SE	OR (95 % CI)	*p*-value
Status of previous broiler flock in the same house				
Negative	0		1	
Positive	2.5	0.5	12.7 (4.8-33.5)	<0.001
Transport personnel enter the room where the broilers are raised				
Never	0		1	
Occasionally	2.2	0.9	9.4 (1.6-55.1)	0.01
Always disinfect floor between production cycles				
No	0		1	
Yes	-2.0	0.8	0.1 (0.03-0.6)	0.01
Number of parent flocks supplying the broiler flock				0.02
1	0		1	
2	1.2	0.6	3.2 (0.9-11.1)	0.06
>2	1.8	0.7	6.3 (1.6-25.0)	0.01

AIC: 142.0, Variance for random effect of House ID:Farm ID=<0.001, variance for random effect of Farm ID: <0.001

ANOVA comparing this model to the final model excluding the nested random effect of House ID within Farm ID; *p*=1.0.

ANOVA comparing this model to the model excluding the variable describing floor disinfection; *p*=0.01.

ANOVA comparing this model to the model excluding the variable describing transport personnel entering the house; *p*=0.02.

Table S3b. Results from the multivariable generalized model including a nested random effect of House ID within Farm ID used to identify risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in 182 Norwegian broiler flocks originating from 34 houses on 27 farms during February 2014- January 2015. Variable describing disinfection of floors excluded.

Variable	Estimate	SE	OR (95 % CI)	*p*-value
Status of previous broiler flock in the same house				
Negative	0		1	
Positive	2.7	0.5	15.4 (5.5-43.6)	<0.001
Transport personnel enter the room where the broilers are raised				
Never	0		1	
Occasionally	2.2	1.1	9.2 (1..01-80.2)	0.04
Number of parent flocks supplying the broiler flock				0.06
1	0		1	
2	1.0	0.6	2.8 (0.8-9.5)	0.10
>2	1.6	0.7	4.8 (1.2-19.5)	0.03

AIC: 146.0, Variance for random effect of House ID:Farm ID=<0.001, variance random effect of Farm ID: 0.4

Table S3c. Results from the multivariable generalized model including a nested random effect of House ID within Farm ID used to identify risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in 182 Norwegian broiler flocks originating from 34 houses on 27 farms during February 2014- January 2015. Variable describing transport personnel entering the room excluded.

Variable	Estimate	SE	OR (95 % CI)	*p*-value
Status of previous broiler flock in the same house				
Negative	0		1	
Positive	2.7	0.5	15.6 (5.6-43.6)	<0.001
Always disinfect floor between production cycles				
No	0		1	
Yes	-1.8	0.9	0.2 (0.02-1.0)	0.05
Number of parent flocks supplying the broiler flock				0.02
1	0		1	
2	1.1	0.6	3.1 (0.9-10.7)	0.08
>2	1.9	0.7	6.4 (1.6-25.5)	0.01

AIC: 146.8, Variance for random effect of House ID:Farm ID=0.12, variance for random effect of Farm ID: <0.001

Paper III

RESEARCH ARTICLE

Plasmid and Host Strain Characteristics of *Escherichia coli* Resistant to Extended-Spectrum Cephalosporins in the Norwegian Broiler Production

Solveig Sølverød Mo[1]*, Jannice Schau Slettemeås[1], Einar Sverre Berg[2], Madelaine Norström[3], Marianne Sunde[1,4]

1 Department of Diagnostic Services, Norwegian Veterinary Institute, Oslo, Norway, **2** Department of Foodborne Infections, Norwegian Institute of Public Health, Oslo, Norway, **3** Department of Health Surveillance, Norwegian Veterinary Institute, Oslo, Norway, **4** Department of Bacteriology and Immunology, Norwegian Institute of Public Health, Oslo, Norway

* solveig.mo@vetinst.no

OPEN ACCESS

Citation: Mo SS, Slettemeås JS, Berg ES, Norström M, Sunde M (2016) Plasmid and Host Strain Characteristics of *Escherichia coli* Resistant to Extended-Spectrum Cephalosporins in the Norwegian Broiler Production. PLoS ONE 11(4): e0154019. doi:10.1371/journal.pone.0154019

Editor: Jose Luis Balcazar, Catalan Institute for Water Research (ICRA), SPAIN

Received: January 5, 2016

Accepted: April 7, 2016

Published: April 25, 2016

Copyright: © 2016 Mo et al. This is an open access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its supporting files. The plasmid nucleotide sequence is available in NCBI with accession number KU312044, http://www.ncbi.nlm.nih.gov/.

Funding: The study was funded by a grant from the Research Council of Norway, www.forskningsradet.no, grant no. 225165/E40. The funders had no role in study design, data collection and analysis, descision to publish, or preparation of the manuscript.

Abstract

Escherichia coli resistant to extended-spectrum cephalosporins have been detected in the Norwegian broiler production, despite the fact that antimicrobial agents are rarely used. The genetic mechanism responsible for cephalosporin resistance is mainly attributed to the presence of the bla_{CMY-2} gene encoding a plasmid-mediated AmpC-beta-lactamase (pAmpC). The aim of this study was to characterize and compare bla_{CMY-2} containing *Escherichia coli* isolated from the intestinal flora of broilers and retail chicken meat (fillets) to identify possible successful clones and/or resistance plasmids widespread in the Norwegian broiler production. Methods used included PCR based phylotyping, conjugation experiments, plasmid replicon typing, pulsed-field gel electrophoresis, multiple locus variable-number tandem-repeats analysis and whole genome sequencing. The nucleotide sequence of an IncK plasmid carrying bla_{CMY-2} was determined. Intestinal isolates displayed a higher degree of genetic diversity than meat isolates. A cluster of genetically related isolates belonging to ST38, phylogroup D, carrying bla_{CMY-2} containing IncK plasmids was identified. Furthermore, genes encoding plasmid stability systems (*relBE/stbDE* and *pndAC)* were identified on the IncK plasmid. Single nucleotide polymorphism (SNP) analysis of a subset of isolates confirmed a close genetic relationship within the two most prevalent STs. The IncK plasmids within these two STs also shared a high degree of similarity. Cephalosporin-resistant *E*. *coli* with the same genetic characteristics have been identified in the broiler production in other European countries, and the IncK plasmid characterized in this study showed close homology to a plasmid isolated from retail chicken meat in the Netherlands. The results indicate that both clonal expansion and horizontal transfer of bla_{CMY-2} containing plasmids contribute to dissemination of cephalosporin resistant *E*. *coli* in the broiler production. The presence of plasmid stability systems may explain why the IncK plasmid containing bla_{CMY-2} is maintained and disseminated in the Norwegian broiler production in absence of selection pressure from the use of antimicrobial agents.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

In the last decade, *Escherichia coli* resistant to extended-spectrum cephalosporins have emerged globally in both human and veterinary medicine [1–4]. This trend is concerning, as cephalosporins are defined as critically important for treatment of human infections [5]. Cephalosporin resistance in *E. coli* is primarily mediated by the spread and acquisition of genes encoding extended-spectrum beta-lactamases (ESBLs) and/or plasmid-mediated AmpC-beta-lactamases (pAmpC) [6]. These genes are usually located on plasmids [7], and thus have a large potential for dissemination in the bacterial population through horizontal spread. Furthermore, clonal spread also plays a significant role in the dissemination of cephalosporin-resistant *E. coli* [2], exemplified by the global spread of *E. coli* sequence type 131 (ST131) containing the $bla_{CTX\text{-}M\text{-}15}$ gene [8, 9].

In veterinary medicine, poultry have been associated with high rates of cephalosporin-resistant *E. coli* [3]. Recently, cephalosporin-resistant *E. coli* have also been detected in the Norwegian broiler production pyramid [10–13]. However, the situation in Norway is unique, as the use of antimicrobials is minimal, and therefore selection pressure is virtually absent [10–13]. Only a single broiler flock was treated with antimicrobial agents in 2013 and 2014 [14, 15], and four flocks were treated in 2015 [16]. Also, a previous study has shown that the $bla_{CMY\text{-}2}$ gene, encoding a pAmpC-beta-lactamase, is responsible for cephalosporin resistance in the vast majority of the resistant isolates [10]. In other countries, a more heterogeneous genetic background for cephalosporin resistance is present, also including genes of the $bla_{CTX\text{-}M}$, bla_{SHV} and bla_{TEM} groups [3]. Furthermore, fresh conventional chicken meat available at retail is produced in Norway, and the import of chicken meat is very limited [17]. Thus, consumers may be exposed to *E. coli* carrying $bla_{CMY\text{-}2}$ through handling of raw chicken meat, or by consumption of undercooked chicken meat. The occurrence of cephalosporin resistant *E. coli* in other food products seems to be of less importance in Norway [11–13, 18]. Import of breeding animals and hatching eggs has been suggested as the likely source of the pAmpC-producing *E. coli* present in the Norwegian broiler production pyramid [10, 19, 20].

It has been hypothesized that animals and food contaminated with ESBL/pAmpC-producing *E. coli* may constitute a reservoir for human acquisition of such bacteria. An increasing number of studies highlight the similarities between isolates and resistance elements from animals and/or meat and humans [4, 21–28]. In order to minimize a possible zoonotic transfer of resistant bacteria, it should be an overall goal to keep the occurrence of ESBL/AmpC-producing *E. coli* in animals and food at the lowest possible level.

The aim of this study was to characterize and compare pAmpC-producing *E. coli* isolated from the intestinal flora of broilers and retail chicken meat (fillets) in order to identify possible successful clones and/or resistance plasmids widespread in the Norwegian broiler production. Furthermore, the nucleotide sequence of a frequently occurring IncK plasmid carrying $bla_{CMY\text{-}2}$ was determined. This knowledge is important in order to evaluate a possible "spill-over" effect to humans and the environment, and in evaluation of the epidemiology of $bla_{CMY\text{-}2}$ carrying plasmids in the Norwegian and European broiler production.

Materials and Methods

Bacterial isolates

A total of 232 *E. coli* with pAmpC-production previously collected within the frame of the Norwegian monitoring programme for antimicrobial resistance in bacteria from food, feed and animals (NORM/NORMVET) were included in the study. The isolates were collected from the intestinal flora of healthy broiler chickens by the use of boot swabs and from retail

chicken meat (fillets) in 2011 (n = 108, boot swabs), 2012 (n = 66, retail meat) and 2014 (n = 58, retail meat). Methods for isolation and species determination are described previously [11–13]. The included isolates represented all pAmpC-producing *E. coli* isolated from broiler in 2011 and retail chicken meat in 2012 and 2014. All isolates were known to harbour the $bla_{\text{CMY-2}}$ gene. Intestinal isolates originated from pieces of boot swabs collected in the Norwegian *Salmonella* control programme for live animals. Five samples were collected each week during January to November 2011. One piece of boot swab was analysed per flock, and maximum one isolate was obtained per flock. Meat isolates originated from samples collected at retail in counties Oslo, Akershus and Vestfold following a proportionate stratified sampling scheme throughout 2012 and 2014, representing the market share from the three largest chicken meat producers in Norway. Due to the centralised structure of the Norwegian meat production, samples collected in the three counties represent production units in all parts of Norway with broiler production. The chicken meat producers are affiliated to different supermarkets rather than different parts of the country and a representative proportion of samples from each meat producer according to their market shares were obtained by collecting samples from different supermarkets. Each meat isolate represent one unique sample. All isolates had been subjected to susceptibility testing (VetMIC or Sensititre® TREK) and the minimum inhibitory concentrations (MICs) to several antimicrobial agents were known [11–13].

Determination of phylogroups

All isolates were subjected to phylotyping using multiplex PCR as described previously [29]. The isolates were classified into phylogroup A, B1, B2 or D. An isolate belonging to the B2 group, (*E. coli* 2003500827) [30] producing amplicons with all four primer sets, was included as positive control in each PCR run.

Conjugation experiments

Transferability of $bla_{\text{CMY-2}}$ containing resistance plasmids was determined by conjugation experiments. Quinolone susceptible isolates were subjected to conjugation with quinolone-resistant *E. coli* DH5α (CCUG 32825) as the recipient. Quinolone-resistant isolates were subjected to conjugation with *E. coli* OneShot$^{\text{TM}}$ cells with pCR$^{\text{TM}}$ II vector encoding kanamycin resistance (Invitrogen™, LifeTechnologies, Thermo Fisher Scientific Inc., Waltham, MA, USA). Overnight cultures of recipient and donor was mated 500 μL: 10 μL in four mL Luria Bertani (LB) broth (Merck, Damstadt, Germany), and incubated at 37°C. From the respective matings, 100 μL broth was plated out on Mueller Hinton agar (Difco, Becton Dickinson and company, Sparks, MD) supplemented with 0,5 mg/L cefotaxime and 20 mg/L nalidixic acid (*E. coli* DH5α) or 0,5 mg/L cefotaxime and 50 mg/L kanamycin (*E. coli* OneShot$^{\text{TM}}$), and incubated at 37°C for 24–48 hours. Sampling of the matings was done using the following strategy: The first samples were plated out after four hours incubation. If no transconjugants were identified, samples were also plated out after 24 hours incubation. Furthermore, a new mating was prepared and samples plated out after two and six hours if no transconjugants were identified in the first conjugation experiment. Presumptive transconjugants were plated on blood agar and lactose-saccharose-bromthymol blue agar to inspect the colony morphology. All donor strains were lactose fermenters, while the recipient strains did not ferment lactose. In addition, the recipient strain grew with small characteristic colonies on blood-agar. The transconjugants were subjected to Real-Time-PCR with a previously published probe [31] to confirm conjugative transfer of $bla_{\text{CMY-2}}$.

PCR-based replicon typing

PCR-based replicon typing (PBRT) was performed on all transconjugants to determine the incompatibility group of plasmids transferred from the donor isolates. The replicon typing was carried out using the PBRT KIT-PCR- based replicon typing (Diatheva, Fano, Italy), according to the manufacturers' instruction. Positive and negative controls were included in each PCR reaction.

Pulsed-Field gel electrophoresis

All isolates were subjected to Pulsed-Field Gel Electrophoresis (PFGE) after digestion with the *Xba*I restriction enzyme (Sigma, St Louis, MO). A protocol recommended by Pulse Net [32] was used with minor alterations as previously described [30]. Lambda Ladder PFG marker (New England Biolabs, Hitchin, UK) was included in each run. The banding patterns were evaluated using UPGMA cluster analysis in BioNumerics version 6.6 (BioNumerics, Applied Maths, Sint-Marten-Latem, Belgium) with tolerance 1% and optimization 1%, and visual inspection. Banding patterns with ≥97% similarity were defined as identical, while banding patterns with ≥80% similarity were defined as belonging to the same PFGE cluster.

Multiple locus variable-number tandem-repeat analysis

A subset of meat isolates (n = 20) that were non-typeable by PFGE were subjected to multiple locus variable-number tandem-repeat analysis (MLVA) to investigate the genetic relatedness between these isolates. MLVA of 10 loci was conducted as previously described [33]. The PCR products were separated by capillary electrophoresis on a 3130*xl* Genetic Analyzer (Applied Biosystems Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA). Peaks were analysed using Peak Scanner Software (Applied Biosystems) with respect to colour and size. Allele numbers were determined based on the fragment size. If no fragment was detected, the allele number was set to zero. Intestinal isolates that were non-typeable by PFGE were not subjected to further genotyping.

Whole genome sequencing

In order to do further characterization of isolates within the two largest clusters identified in this study, we selected relevant isolates for whole genome sequencing (WGS). These included five isolates representing the largest PFGE cluster, of which three isolates were from faeces and two isolates from meat. Also, five isolates from meat displaying a banding pattern highly similar to the largest PFGE cluster were included. Finally, five meat isolates with highly similar MLVA profiles, representing the second largest cluster, were subjected to WGS. One isolate from 2011 and one isolate from 2012 were sequenced twice to serve as controls of reproducibility. DNA was isolated manually by the use of the Wizard Genomic Purification Kit (Promega Corporation, Madison, WI) (isolates from 2011 and 2012) or automated by the use of MagNA Pure LS Total Nucleic acid Isolation kit in a MagNA Pure LS instrument (Roche Diagnostics, Mannheim, Germany) (isolates from 2014). Library construction and WGS was performed by BGI (BGI Tech Solutions Co. Ltd., Hong Kong) and by the use of a HiSeq 2500 Illumina platform. Furthermore, the sequence servicing centre provided trimmed, cleaned paired- end read sets and assembled contigs by use of SOAPdenovo software version 2.04 (http://sourceforge.net/projects/soapdenovo2/files/SOAPdenovo2/). The processed sequence data were analysed *in silico* with regard to multi-locus sequence type (ST) [34], serotype, acquired resistance genes, plasmid replicon types and virulence genes by the use of tools available online at www.genomicepidemiology.org from Center for Genomic Epidemiology (CGE), DTU, Denmark.

Also, a SNP tree was constructed using CSI Phylogeny 1.1 [35] available from CGE, to investigate the number of SNP differences between the isolates subjected to WGS. An *E. coli* isolated from retail meat in 2012 (2012-01-1292) was used as reference. A selection of *E. coli* reference genomes available from NCBI was also included. The analysis was run using default settings.

Furthermore, the nucleotide sequence of one frequently occurring IncK plasmid was reconstructed. The plasmid was present in one of the isolates within the largest PFGE cluster (isolate no 2012-01-1292). A transconjugant from the conjugation experiment containing the plasmid (1292DH5α) was subjected to WGS, and the draft sequence of the plasmid was identified in an assembled contig. This was done by aligning each contig to an assembled sequence of *E. coli* DH1 (accession no. CP001637.1) in CLC Genomics (CLC Bio, Qiagen, Aarhus, Denmark). One scaffold did not align to the DH1sequence, and was assumed to represent the IncK plasmid sequence. To ensure sufficient quality of the plasmid sequence, Sanger sequencing was performed to cover areas with inadequate quality, regions containing repeat sequences, and each side of the scaffold to determine if it could be closed into a circular sequence. Plasmid DNA was extracted using Qiagen Plasmid Plus Maxi Kit (Qiagen, Venlo, The Netherlands) and used as template directly in the sequencing reactions. Primers for sequencing relevant regions were designed using CLC Genomics. BigDye Terminator v3.1/1.1. cycle sequencing kit (Applied Biosystems) was used to determine the nucleotide sequences. The reactions were subjected to capillary electrophoresis on a 3130*xl* Genetic Analyser (Applied Biosystems), and further analysis was performed in CLC Main Workbench (CLC Bio, Qiagen). Open reading frames (ORFs) on the plasmid sequence were identified using CLC Genomics. Annotations were performed by a combination of automated annotation using RAST v4.0 [36] and manual annotation in CLC Main Workbench. The plasmid was designated pNVI1292.

To investigate if similar IncK plasmids were present in the other isolates subjected to WGS, we used CSI Phylogeny with pNVI1292 as reference [35].

Results

Phylogenetic grouping and genetic relationship

The results from the phylotyping experiments showed that phylogroup D dominated among isolates from both broilers (56%) and meat (84%). Seven isolates from broilers (6%) and six isolate from meat (5%) belonged to phylogroup B2. Furthermore, 31 broiler isolates (29%) and two meat isolates (2%) grouped into phylogroup B1, while nine (8%) broiler isolates and 12 (10%) meat isolates belonged to phylogroup A. The distribution of phylogroups is shown in Table 1.

PFGE and subsequent cluster analysis revealed that the intestinal isolates showed a higher diversity than the meat isolates (S1 and S2 Figs). Furthermore, a main cluster including 69 isolates was identified when all isolates were analysed together. This cluster included 20 of 108 intestinal isolates (2011), and 49 of 124 meat isolates (43 of 66 from 2012 and 6 of 58 from 2014). The main cluster is indicated in the dendrogram based on the PFGE banding pattern of all isolates included in the study (S3 Fig). Within the main cluster, 25 isolates displayed identical banding patterns. Of these, 21 isolates originated from meat and four from the intestinal flora. *E. coli* with this banding pattern was isolated from meat samples originating from two different manufacturers, and thus different abattoirs. A proportion of the isolates were non-typeable (n = 50), including 30 intestinal isolates and 20 meat isolates.

Of the 20 meat isolates subjected to MLVA (all non-typeable by PFGE), 15 had highly similar MLVA profiles indicating close genetic relationship. All these isolates belonged to phylogroup D. Of the remaining five isolates, two belonged to phylogroup D, and both displayed unique MLVA profiles. The remaining three isolates grouped into phylogroup A and displayed

Table 1. Distribution of phylogroups, conjugative bla_{CMY-2} containing plasmids and replicon types of conjugative plasmids among pAmpC-producing *Escherichia coli* isolated from broiler faeces and retail chicken meat in Norway 2011, 2012 and 2014.

Origin	Replicon- type	A	B1	B2	D	Total
Broiler 2011	IncK	3	6	1	32	42
	IncI1	3	20	4	25	52
	IncK and IncI1	1	0	2	3	6
	NTP	2	5	0	1	8
Total broiler 2011		9	31	7	61	108
Retail chicken meat 2012	IncK	4	0	1	56	61
	IncI1	1	0	0	0	1
	NTP	0	0	0	4	4
Total retail chicken meat 2012		5	0	1	60	66
Retail chicken meat 2014	IncK	6	0	5	28	39
	IncK and IncI1	1	0	0	0	1
	NTP	0	2	0	16	18
Total retail chicken meat 2014		7	2	5	44	58
Overall total		21	33	13	165	232

NTP: Non-transferable plasmid

doi:10.1371/journal.pone.0154019.t001

closely related MLVA profiles, but differed substantially from the other isolates subjected to MLVA (S4 Fig).

Transferability and replicons of bla_{CMY-2} containing plasmids

Conjugative plasmids carrying bla_{CMY-2} were identified in 202 of 232 isolates (87%). A similar occurrence of conjugative IncI1 and IncK plasmids was observed among intestinal isolates from broilers. However, in isolates originating from meat, IncK plasmids were identified in all but one isolate (n = 101, 99%) with conjugative plasmids (Table 1). In addition to IncK and IncI1 plasmids, IncFII and IncFIB plasmids were identified in a few transconjugants (S2 Fig).

In silico typing of WGS data

Isolates within the main PFGE cluster were identified as belonging to ST38 and serotype O7:H18. Five isolates with a highly similar PFGE pattern but grouping just outside the main PFGE cluster were also identified as belonging to ST38, serotype O7:H18. Isolates exhibiting nearly identical MLVA patterns (n = 5) belonged to ST1158 and serotype O17/77:H34. Among the ST38 strains, three isolates contained the bla_{TEM-1} gene in addition to $bla_{CMY-2.}$ No additional acquired resistance genes were identified in the remaining isolates subjected to WGS. The WGS data further showed that 14 isolates carried the virulence factors *iss* (increased serum survival) and *iha* (adhesion siderophore). Eleven isolates carried *gad* (glutamate decarboxylase), and nine isolates carried *iroN* (enterobactin siderophore) and *cma* (colicin M). Also, a few isolates carried *celb* (endonuclease colicin) and *astA* (heat-stable enterotoxin) [37, 38]. The SNP analysis (S5 Fig) showed that isolates within the main cluster, including both intestinal and meat isolates had a limited number of SNP differences (2–14 SNPs). Between isolates in the main cluster and isolates with highly similar PFGE banding patterns outside the main cluster (ST38), 138–150 SNP differences were observed. Isolates that were non-typeable by PFGE but displayed highly similar MLVA profiles (ST1158), differed by 8–63 SNPs. More than 10352 SNP differences were observed between isolates in the two main clusters defined by PFGE and MLVA (ST38 and ST1158) (S1 Table).

Plasmid characterization

The nucleotide sequence of an IncK plasmid harboured by an ST38 isolate grouping within the main PFGE cluster (1292DH5α), designated pNVI1292, was determined and characterized. The plasmid was 79.3 kB in size, and the majority of the genes encoded were associated with conjugal transfer or transcription. The $bla_{\text{CMY-2}}$ gene was flanked by IS*Ecp1* downstream and *blc* and *sugE* upstream. This genetic organization has previously been described in other plasmids from *E. coli* and *Salmonella enterica* [39]. The insertion sequence IS*Ecp1* is known to function both as a transposase [40], and as a strong promotor for expression of genes located downstream [41]. The *blc* gene encodes a membrane lipoprotein [42], while *sugE* encodes an efflux pump mediating resistance to quaternary ammonium compounds when overexpressed [43]. In addition, two plasmid stability systems, namely *relBE/stbDE* and *pndAC* were present on the plasmid sequence (Fig 1). The plasmid sequence showed close homology to *Escherichia*

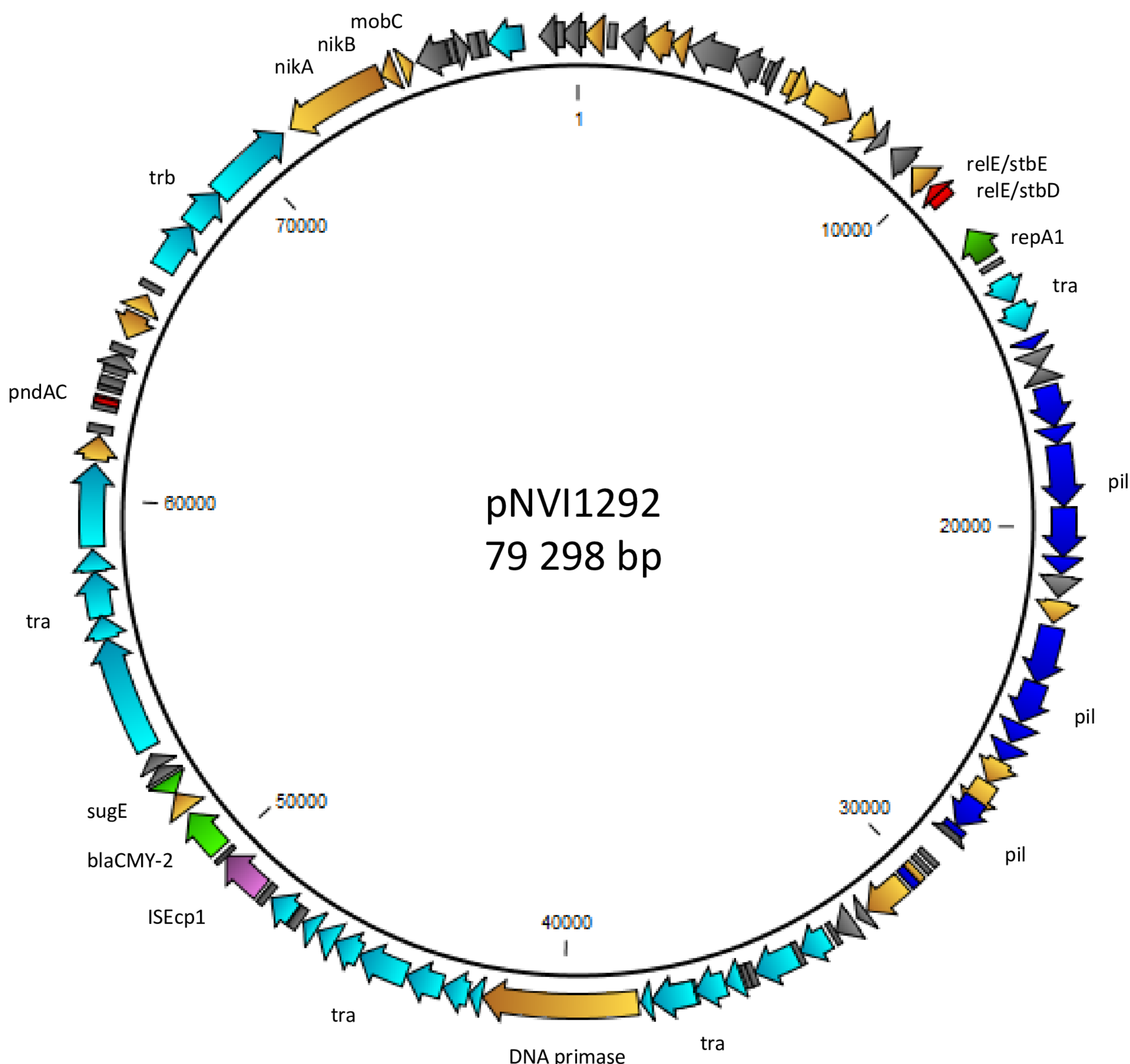


Fig 1. Structure of the *bla*$_{\text{CMY-2}}$ containing plasmid pNVI1292 isolated from 1292DH5α. Light blue colour indicate transfer associated genes, blue colour indicate pilus associated genes, light green colour indicate resistance genes, red colour indicate plasmid stability systems, purple colour indicate insertion sequence, dark green colour indicate replication associated genes, orange colour indicate other proteins, and grey colour indicate hypothetical proteins.

doi:10.1371/journal.pone.0154019.g001

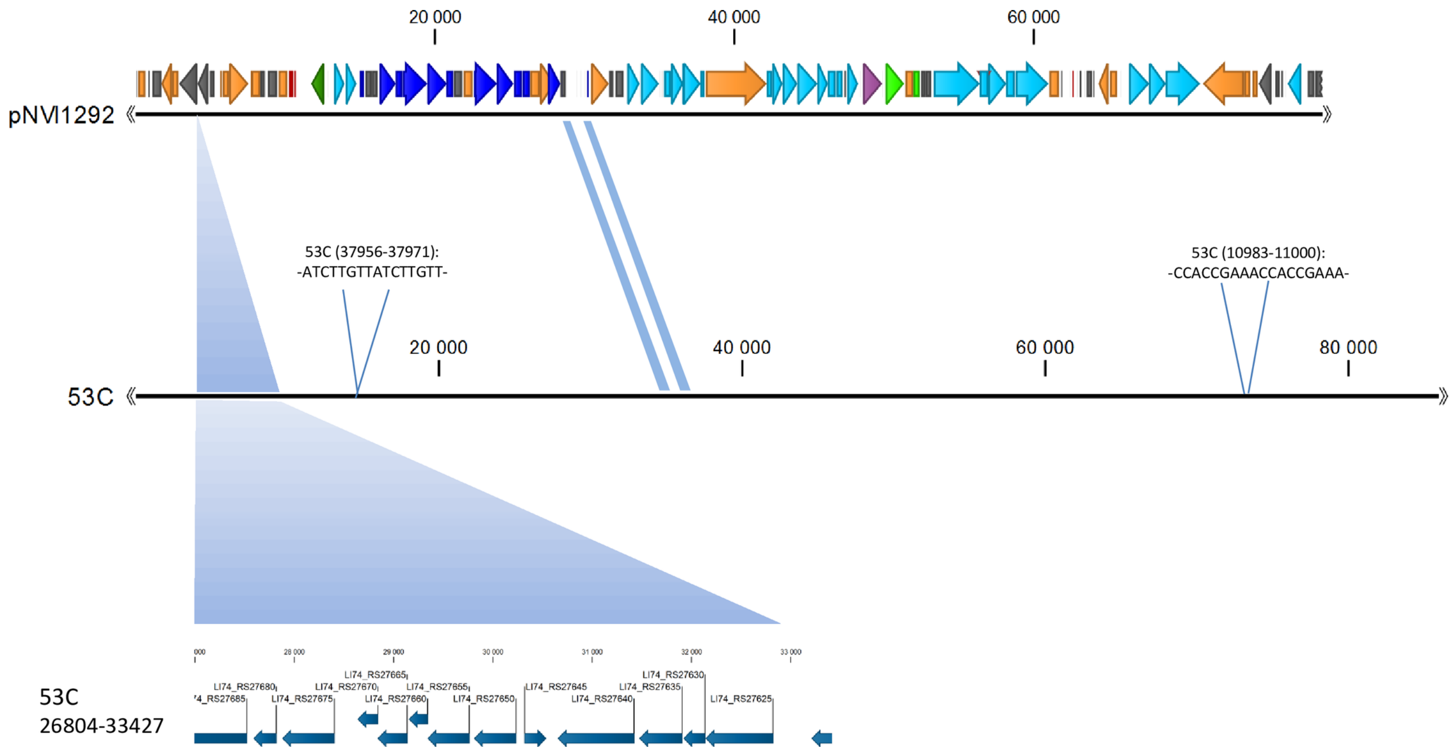


Fig 2. Alignment of plasmid pNVI1292 from 1292DH5α and *Escherichia coli* 53C. A 6.6 kB sequence present in the plasmid from *Escherichia coli* 53C but absent in the pNVI1292 plasmid is shown in the bottom of the figure. Differences in the nucleotide sequence between the two plasmids are marked with blue shadings.

doi:10.1371/journal.pone.0154019.g002

coli 53C plasmid unnamed 3 (Accession number NZ_JXMX01000007.1) isolated from retail chicken meat in the Netherlands in 2010 [22, 25]. However, the pNVI1292 plasmid lacked a 6.6 kb sequence compared to the plasmid from the Netherlands (Fig 2). This 6.6 kb sequence was not found in any of the isolates subjected to WGS in this study.

The nucleotide sequence of the pNVI1292 plasmid from 1292DH5α has been submitted to GenBank (KU312044).

Analysis of the plasmid sequences from all isolates subjected to WGS (ST38 and ST1158) by the use of CSI Phylogeny showed that a 75.6 kb part of the nucleotide sequence from the pNVI1292 plasmid from 1292DH5α was present in all the isolates (S2 Table). This indicates that plasmids within ST38 and ST1158 share a high degree of similarity.

Discussion

Our genotyping studies showed that cephalosporin resistant *E. coli* isolated from the intestinal flora of chickens in Norway is more diverse than those isolated from retail chicken meat. Two large clusters of genetically related isolates from meat were identified, belonging to ST38 and ST1158, respectively. In other studies, a more diverse genetic composition of cephalosporin-resistant *E. coli* has been identified in chicken meat samples [21, 23, 44, 45]. To our best knowledge, there are no other studies demonstrating the dominance of particular cephalosporin resistant clones in chicken meat over a prolonged time period. Only one pAmpC gene, namely *bla*$_{CMY-2}$ was detected in cephalosporin resistant *E. coli* isolated from chicken and chicken meat in Norway [10]. Worldwide, *bla*$_{CMY-2}$ is associated with pAmpC- producing *E. coli* isolated from poultry [3]. In Europe, *bla*$_{CMY-2}$ is frequently linked to IncA/C and IncI1 plasmids in both human and

poultry isolates [7], however an association with IncK plasmids has recently been reported [22, 24, 44, 46–48]. An equal distribution between IncK and IncI1 plasmids carrying bla_{CMY-2} was seen among pAmpC-producing *E. coli* isolated from faecal samples. However, in isolates originating from meat samples, the majority were found to carry IncK plasmids. This may be explained partly by the abundance of *E. coli* ST38 and ST1158 carrying IncK plasmids in Norway. SNP analysis of isolates subjected to WGS showed few SNP differences between ST38 isolates with highly similar PFGE banding patterns. Furthermore, few SNP differences between isolates originating from broilers and retail meat, and isolates collected in different years was seen. It is a perception that *E. coli* isolates from food-borne outbreaks have been shown to differ by 10–20 SNPs [49].Until now, clonal dissemination of cephalosporin-resistant *E. coli* has mostly been described for human adapted *E. coli* variants [8, 9]. However, our results showing few SNP differences highlight that clonal dissemination of pAmpC-producing strains has occurred in the Norwegian broiler production. Most of the bla_{CMY-2} carrying plasmids were conjugative *in vitro*. This indicates a large potential for horizontal transfer of these resistance plasmids in the bacterial population. The fact that the genetic diversity was smaller among meat isolates compared to intestinal isolates, and that IncK plasmids are dominating, indicate that a selection step favouring these variants and plasmids may occur from live animals to retail meat. Possibly, these variants and/or plasmids harbour a selection advantage throughout the production pyramid.

Our genotyping results revealed that a considerable proportion of the meat isolates belonged to ST38. This ST has also been identified in *E. coli* carrying bla_{CMY-2} on an IncK plasmid isolated from broilers and retail chicken meat in the Netherlands [22, 50], broilers and parent animals in Denmark [48], and retail chicken meat originating from Sweden, Finland and Denmark [46]. Thus, *E. coli* ST38 seems to be a variant commonly associated with cephalosporin resistance in the European broiler production pyramid. *E. coli* ST38 is associated with both uropathogenic *E. coli* (UPEC) and entero-aggregative *E. coli* (EAEC) in humans, but these isolates often carry other cephalosporin resistance genes on plasmids different than those found in isolates from broilers and retail chicken meat [51–53]. However, recent findings in Norway revealed a few *E. coli* ST38 carrying bla_{CMY-2} on IncK plasmids in human UPEC isolates. These isolates and plasmids were highly similar to isolates and plasmids from retail chicken meat. The results indicate that chicken meat may be a possible source for human acquisition of resistance plasmids and pAmpC-producing *E. coli* [54]. However, further studies are required to determine the route of transmission and whether other possible sources exist for human acquisition of these bacteria. Among isolates from 2014 we identified ST1158 as an emerging ST. Isolates within this ST were non-typeable by PFGE, but the majority displayed highly similar MLVA profiles. This finding may indicate a shift of the clonality of the pAmpC-producing *E. coli* isolated from chicken meat. This may be due to introduction of new pAmpC-producing *E. coli* strains through import of hatching eggs. ST38 was still prevalent in meat isolates from 2014. *E. coli* ST1158 has also been reported in retail chicken meat from Switzerland, but carrying $bla_{CTX-M-1}$ and not bla_{CMY-2} [45]. To our best knowledge, this is the first report of *E. coli* ST1158 carrying bla_{CMY-2} on an IncK plasmid.

The pNVI1292 plasmid characterized in this study was highly similar to a plasmid isolated from *E. coli* in Dutch chicken meat in 2010. It may therefore be argued that this plasmid is disseminated and successful in the European broiler production. The pNVI1292 plasmid was isolated in 2012, and highly similar plasmids were identified in *E. coli* isolated from chicken in 2014. This suggests that the IncK plasmid has been circulating in the European broiler production for several years. Furthermore, the WGS data supported the hypothesis that related IncK plasmids are widespread and successful in the Norwegian broiler production. Overall, the results from the plasmid characterization and genotyping experiments strengthen the theory that import of hatching eggs is the original source of pAmpC-producing *E. coli* in the Norwegian broiler production.

The presence of the plasmid stability systems *relBE/stbDE* and *pndAC* represents a selection advantage for the plasmids due to post-segregational killing of plasmid-free daughter cells. Also, the addiction modules ensure plasmid stability during replication by elimination of other compatible plasmids from the bacteria [55]. This may represent an explanation of how pAmpC-producing *E. coli* can be maintained in the Norwegian broiler production in spite of the absence of selection pressure from antimicrobial use.

Phylotyping revealed that the majority of the isolates from both broilers and retail chicken meat belonged to phylogroup D. Also, a small number of isolates were identified as belonging to phylogroup B2. A small study from Denmark also showed that phylogroup D dominated among *E. coli* isolated from the broiler production pyramid and retail meat [48]. Also, phylogroup D and bla_{CMY-2} has previously been shown to have a close association with *E. coli* isolated from poultry in Spain [56]. The phylogroups D and B2 are associated with human extraintestinal pathogenic *E. coli* (ExPEC) strains [57]. Also, ExPEC associated virulence factors were detected among isolates subjected to WGS, namely *iss*, *iha*, *iroN* and *astA* [58, 59]. Thus, there are indications that pAmpC-producing *E. coli* from broilers and retail chicken meat in Norway may have pathogenic potential.

In conclusion, both clonal expansion and horizontal transfer of bla_{CMY-2} carrying plasmids are likely to contribute to the dissemination of pAmpC-producing *E. coli* in the Norwegian broiler production. The presence of the plasmid stability systems *relBE/stbDE* and *pndAC* is likely to play a significant role in the broad dissemination and maintenance of IncK plasmids carrying bla_{CMY-2}, as it represents a selection advantage for isolates carrying this plasmid. We also suggest that highly related bla_{CMY-2} carrying IncK plasmids are disseminated and successful in the European broiler production.

Supporting Information

S1 Fig. Pulsed-Field gel electrophoresis (PFGE) banding patterns for intestinal isolates. Dendrogram based on UPGMA cluster analysis of PFGE banding patterns of pAmpC-producing *E. coli* isolated from and faecal samples from healthy broiler chickens, 2011. The red line indicates 80% similarity of banding patterns, and the blue line indicates 97% similarity of banding patterns. Isolates subjected to whole genome sequencing are indicated by *. Isolates that were PFGE non-typeable are shown as a straight line in the bottom of the dendrogram.
(PDF)

S2 Fig. Pulsed-Field gel electrophoresis (PFGE) banding patterns for meat isolates. Dendrogram based on UPGMA cluster analysis of PFGE banding patterns of pAmpC- producing *E. coli* isolated from retail chicken meat, 2012 and 2014. The red line indicates 80% similarity of banding patterns, and the blue line indicates 97% similarity of banding patterns. Isolates subjected to whole genome sequencing are indicated by *. Isolates that were PFGE non-typeable are shown as a straight line in the bottom of the dendrogram.
(PDF)

S3 Fig. Dendrogram including Pulsed-Field gel electrophoresis (PFGE) banding patterns of all isolates included in the study. The red line denotes 80% similarity of banding patterns, while the blue line denotes 97% similarity of banding patterns. The green box highlights the main cluster including 69 isolates. Isolates that were PFGE non-typeable are shown as a straight line in the bottom of the dendrogram. Isolates subjected to whole genome sequencing are indicated by *.
(PDF)

S4 Fig. Minimum-spanning-tree analysis of 20 meat isolates subjected to Multiple locus variable-number tandem-repeats analysis (MLVA). Green colour represents isolates belonging to phylogroup D, while blue colour represents isolates belonging to phylotype B1. The main cluster of highly related MLVA profiles, including 15 isolates, is indicated by the red circle.
(TIF)

S5 Fig. Single nucleotide polymorphism (SNP) tree of isolates subjected to whole genome sequencing and NCBI *E. coli* reference genomes. Retail meat isolates are highlighted with yellow, isolates from broiler faeces are highlighted with green, while NCBI *E. coli* reference genomes are highlighted with blue. Isolate 2012-01-1292 was used as reference. Isolates 2012-01-1292ctrl and 2011-01-2112-2ctrl were included as control of sequence reproducibility.
(PDF)

S1 Table. Number of single nucleotide polymorphisms (SNPs) between pairs of isolates subjected to whole genome sequencing. Isolate 2012-01-1292 was used as reference. Isolates 2012-01-1292ctrl and 2011-01-2112-2ctrl were included as control of sequence reproducibility.
(XLSX)

S2 Table. Percentage similarity between IncK plasmids subjected to whole genome sequencing. The assembled plasmid sequence from pNVI1292 was used as reference (79.3 kb). Percentage of reference genome covered by all isolates was 95.3% (75.6 kb). Number of single nucleotide polymorphisms (SNPs) between pairs of plasmids is shown.
(XLSX)

Acknowledgments

Bjørg Kvitle is acknowledged for technical assistance. Parts of these data were presented at the Society for Applied Microbiology Summer Conference, Brighton, UK, in July 2014, and at the 6th Symposium on Antimicrobial Resistance in Animals and the Environment, Tours, France, in July 2015.

Author Contributions

Conceived and designed the experiments: MN MS. Performed the experiments: SSM JSS ESB MS. Analyzed the data: SSM JSS ESB MN MS. Contributed reagents/materials/analysis tools: SSM JSS ESB MN MS. Wrote the paper: SSM JSS ESB MN MS.

References

1. Carattoli A. Animal reservoirs for extended spectrum beta-lactamase producers. Clin Microbiol Infect. 2008; 14 Suppl 1:117–23. Epub 2007/12/25. doi: 10.1111/j.1469-0691.2007.01851.x PMID: 18154535.
2. Coque TM, Baquero F, Canton R. Increasing prevalence of ESBL-producing Enterobacteriaceae in Europe. Euro Surveill. 2008; 13(47):1–11. Epub 2008/11/22. PMID: 19021958.
3. Ewers C, Bethe A., Semmler T., Guenther T., Wieler L.H.,. Extended-spectrum beta-lactamase-producing and AmpC-producing Escherichia coli from livestock and companion animals, and their putative impact on public health: a global perspective. Clin Microbiol Infect. 2012; 18:646–55. doi: 10.1111/j.1469-0691.2012.03850.x PMID: 22519858
4. Overdevest I, Willemsen I, Rijnsburger M, Eustace A, Xu L, Hawkey P, et al. Extended-spectrum beta-lactamase genes of Escherichia coli in chicken meat and humans, The Netherlands. Emerg Infect Dis. 2011; 17(7):1216–22. Epub 2011/07/19. doi: 10.3201/eid1707.110209 PMID: 21762575; PubMed Central PMCID: PMC3381403.
5. WHO-AGISAR. Critically Important Antimicrobials for Human Medicine- 3rd rev. Geneva, Switzerland: World Health Organization, 2011.

6. Pfeifer Y, Cullik A, Witte W. Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. Int J Med Microbiol. 2010; 300(6):371–9. doi: 10.1016/j.ijmm.2010.04.005 PMID: 20537585.
7. Carattoli A. Resistance plasmid families in Enterobacteriaceae. Antimicrob Agents Chemother. 2009; 53(6):2227–38. doi: 10.1128/AAC.01707-08 PMID: 19307361; PubMed Central PMCID: PMC2687249.
8. Coque TM, Novais A, Carattoli A, Poirel L, Pitout J, Peixe L, et al. Dissemination of clonally related Escherichia coli strains expressing extended-spectrum beta-lactamase CTX-M-15. Emerg Infect Dis. 2008; 14(2):195–200. Epub 2008/02/09. doi: 10.3201/eid1402.070350 PMID: 18258110; PubMed Central PMCID: PMC2600198.
9. Woerther PL, Burdet C, Chachaty E, Andremont A. Trends in human fecal carriage of extended-spectrum beta-lactamases in the community: toward the globalization of CTX-M. Clin Microbiol Rev. 2013; 26(4):744–58. doi: 10.1128/CMR.00023-13 PMID: 24092853; PubMed Central PMCID: PMCPMC3811232.
10. Mo SS, Norström M, Slettemeås JS, Løvland A, Urdahl AM, Sunde M. Emergence of AmpC-producing Escherichia coli in the broiler production chain in a country with a low antimicrobial usage profile. Vet Microbiol. 2014; 171(3–4):315–20. doi: 10.1016/j.vetmic.2014.02.002 PMID: 24629773.
11. NORM/NORM-VET. NORM/NORM-VET 2011. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo: 2012 ISSN: 1890- 9965 (electronic).
12. NORM/NORM-VET. NORM/NORM-VET 2012. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo: 2013 ISSN:1502-2307 (print) / 1890–9965 (electronic).
13. NORM/NORM-VET. NORM/NORM-VET 2014. Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway. Tromsø/Oslo: 2015 ISSN: 1502-2307 (print) / 1890–9965 (electronic)
14. Animalia. Tiltak mot antibiotikaresistens virker http://animalia.no/Listesider/Aktuelt-og-fagstoff/Tiltak-mot-antibiotikaresistens-virker-/ 2015 [12.01.2016]. Available from: http://animalia.no/Listesider/Aktuelt-og-fagstoff/Tiltak-mot-antibiotikaresistens-virker-/.
15. Refsum T. Antibiotikabehandling i norsk fjørfeproducksjon. Go' mørning http://wwwanimaliano/upload/FIler%20til%20nedlasting/Go%C2%B4m%C3%B8rning/2015/GM%204-15%20webpdf. 2015.
16. Animalia. Fortsatt nedgang i forekomst av resistente bakterier hos fjørfe http://animalia.no/Listesider/Aktuelt-og-fagstoff/Fortsatt-nedgang-i-forekomst-av-resistente-bakterier-hos-fjorfe/ 2016 [03.03.2016]. Available from: http://animalia.no/Listesider/Aktuelt-og-fagstoff/Fortsatt-nedgang-i-forekomst-av-resistente-bakterier-hos-fjorfe/.
17. NorwegianAgricultureAgency. Markedsrapport 2015. Pris- og markedsvurderinger av sentrale norske landbruksråvarer og RÅK varer https://www.slf.dep.no/no/produksjon-og-marked/frukt-og-gront/marked-og-pris/god-tilgang-av-norske-landbruksvarer-i-20152016 [29.02.2016]. Available from: https://www.slf.dep.no/no/produksjon-og-marked/frukt-og-gront/marked-og-pris/god-tilgang-av-norske-landbruksvarer-i-2015.
18. NORM/NORM-VET. NORM/NORM-VET 2013. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo: 2014 ISSN: 1502-2307 (print)/ 1890–9965 (electronic).
19. Sunde M, Tharaldsen H, Slettemeås JS, Norström M, Carattoli A, Bjorland J. Escherichia coli of animal origin in Norway contains a blaTEM-20-carrying plasmid closely related to blaTEM-20 and blaTEM-52 plasmids from other European countries. J Antimicrob Chemother. 2009; 63(1):215–6. Epub 2008/10/31. doi: 10.1093/jac/dkn445 PMID: 18971216.
20. NORM/NORM-VET. NORM/NORM-VET 2006. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo: 2007.
21. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Voets GM, van den Munckhof MP, van Essen-Zandbergen A, et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect. 2011; 17(6):873–80. Epub 2011/04/06. doi: 10.1111/j.1469-0691.2011.03497.x PMID: 21463397.
22. Voets GM, Fluit AC, Scharringa J, Schapendonk C, van den Munckhof T, Leverstein-van Hall MA, et al. Identical plasmid AmpC beta-lactamase genes and plasmid types in E. coli isolates from patients and poultry meat in the Netherlands. Int J Food Microbiol. 2013; 167(3):359–62. doi: 10.1016/j.ijfoodmicro.2013.10.001 PMID: 24184615.
23. Doi Y, Paterson DL, Egea P, Pascual A, Lopez-Cerero L, Navarro MD, et al. Extended-spectrum and CMY-type beta-lactamase-producing Escherichia coli in clinical samples and retail meat from Pittsburgh, USA and Seville, Spain. Clin Microbiol Infect. 2010; 16(1):33–8. doi: 10.1111/j.1469-0691.2009.03001.x PMID: 19681957.
24. Börjesson S, Jernberg C, Brolund A, Edquist P, Finn M, Landen A, et al. Characterization of plasmid-mediated AmpC-producing E. coli from Swedish broilers and association with human clinical isolates.

Clinical Microbiology and Infection. 2013; 19(7):E309–11. doi: 10.1111/1469-0691.12192 PMID: 23581796.

25. de Been M, Lanza VF, de Toro M, Scharringa J, Dohmen W, Du Y, et al. Dissemination of cephalosporin resistance genes between Escherichia coli strains from farm animals and humans by specific plasmid lineages. PLoS Genet. 2014; 10(12):e1004776. doi: 10.1371/journal.pgen.1004776 PMID: 25522320; PubMed Central PMCID: PMCPMC4270446.

26. Hammerum AM, Larsen J, Andersen VD, Lester CH, Skovgaard Skytte TS, Hansen F, et al. Characterization of extended-spectrum beta-lactamase (ESBL)-producing Escherichia coli obtained from Danish pigs, pig farmers and their families from farms with high or no consumption of third- or fourth-generation cephalosporins. J Antimicrob Chemother. 2014. doi: 10.1093/jac/dku180 PMID: 24908045.

27. Kluytmans JA, Overdevest IT, Willemsen I, Kluytmans-van den Bergh MF, van der Zwaluw K, Heck M, et al. Extended-spectrum beta-lactamase-producing Escherichia coli from retail chicken meat and humans: comparison of strains, plasmids, resistance genes, and virulence factors. Clin Infect Dis. 2013; 56(4):478–87. doi: 10.1093/cid/cis929 PMID: 23243181.

28. Lazarus B, Paterson DL, Mollinger JL, Rogers BA. Do human extraintestinal Escherichia coli infections resistant to expanded-spectrum cephalosporins originate from food-producing animals? A systematic review. Clin Infect Dis. 2015; 60(3):439–52. doi: 10.1093/cid/ciu785 PMID: 25301206.

29. Doumith M, Day M. J., Hope R., Wain J., Woodford N.,. Improved Multiplex PCR Strategy for Rapid Assignment of the Four Major *Escherichia coli* Phylogenetic Groups. J Clin Microbiol. 2012; 50 (9):3108–10. doi: 10.1128/JCM.01468-12 PMID: 22785193

30. Sunde M, Simonsen GS, Slettemeås JS, Böckerman I, Norström M. Integron, plasmid and host strain characteristics of Escherichia coli from humans and food included in the Norwegian antimicrobial resistance monitoring programs. PLoS One. 2015; 10(6):e0128797. Epub 5 June 2015. doi: 10.1371/journal.pone.0128797 PMID: 26047499

31. Schmidt GV, Mellerup A, Christiansen LE, Stahl M, Olsen JE, Angen O. Sampling and Pooling Methods for Capturing Herd Level Antibiotic Resistance in Swine Feces using qPCR and CFU Approaches. PLoS One. 2015; 10(6):e0131672. doi: 10.1371/journal.pone.0131672 PMID: 26114765; PubMed Central PMCID: PMC4483237.

32. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, et al. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of Escherichia coli O157:H7, Salmonella, and Shigella for PulseNet. Foodborne Pathog Dis. 2006; 3(1):59–67. doi: 10.1089/fpd.2006.3.59 PMID: 16602980.

33. Lobersli I, Haugum K, Lindstedt BA. Rapid and high resolution genotyping of all Escherichia coli serotypes using 10 genomic repeat-containing loci. J Microbiol Methods. 2012; 88(1):134–9. doi: 10.1016/j.mimet.2011.11.003 PMID: 22088357.

34. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in Escherichia coli: an evolutionary perspective. Mol Microbiol. 2006; 60(5):1136–51. doi: 10.1111/j.1365-2958.2006.05172.x PMID: 16689791; PubMed Central PMCID: PMCPMC1557465.

35. Kaas RS, Leekitcharoenphon P, Aarestrup FM, Lund O. Solving the problem of comparing whole bacterial genomes across different sequencing platforms. PLoS One. 2014; 9(8):e104984. doi: 10.1371/journal.pone.0104984 PMID: 25110940; PubMed Central PMCID: PMCPMC4128722.

36. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST Server: rapid annotations using subsystems technology. BMC Genomics. 2008; 9:75. doi: 10.1186/1471-2164-9-75 PMID: 18261238; PubMed Central PMCID: PMCPMC2265698.

37. Anjum MF, Mafura M, Slickers P, Ballmer K, Kuhnert P, Woodward MJ, et al. Pathotyping Escherichia coli by using miniaturized DNA microarrays. Appl Environ Microbiol. 2007; 73(17):5692–7. doi: 10.1128/AEM.00419-07 PMID: 17630299; PubMed Central PMCID: PMCPMC2042074.

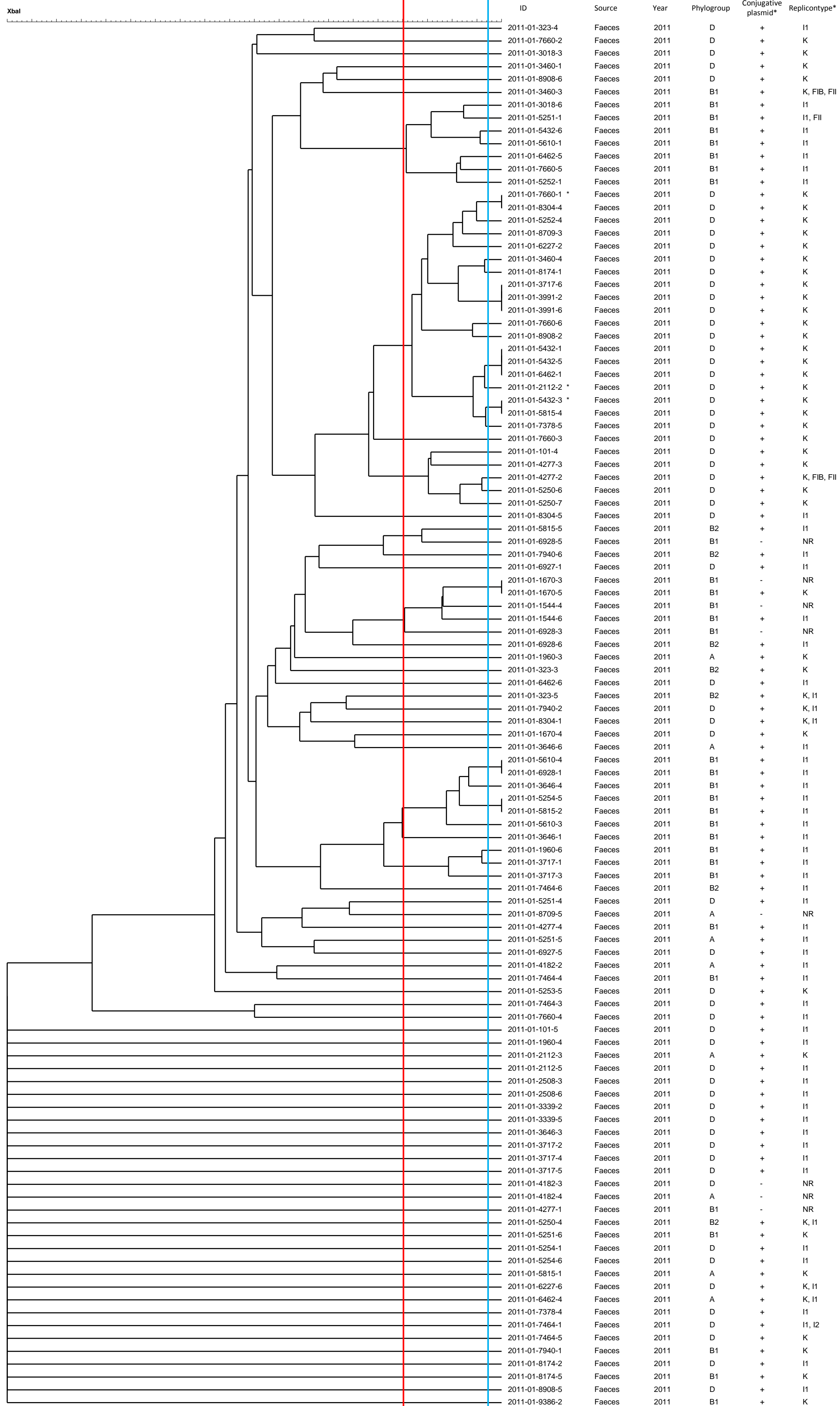
38. Johnson JR, Johnston B, Clabots C, Kuskowski MA, Castanheira M. Escherichia coli sequence type ST131 as the major cause of serious multidrug-resistant E. coli infections in the United States. Clin Infect Dis. 2010; 51(3):286–94. doi: 10.1086/653932 PMID: 20572763.

39. Kang MS, Besser TE, Call DR. Variability in the region downstream of the blaCMY-2 beta-lactamase gene in Escherichia coli and Salmonella enterica plasmids. Antimicrob Agents Chemother. 2006; 50 (4):1590–3. doi: 10.1128/AAC.50.4.1590–1593.2006 PMID: 16569893; PubMed Central PMCID: PMC1426964.

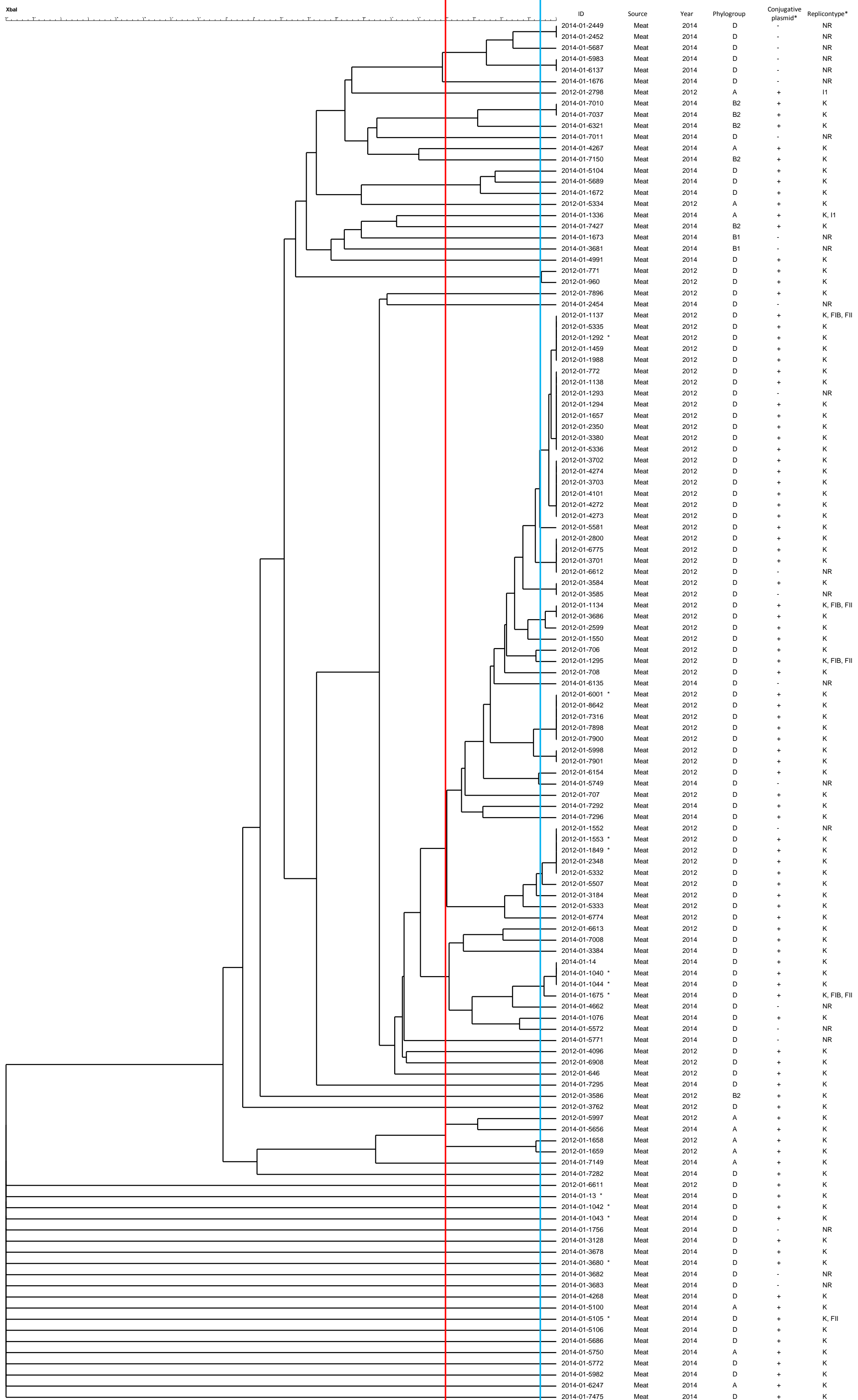
40. Poirel L, Lartigue MF, Decousser JW, Nordmann P. ISEcp1B-mediated transposition of blaCTX-M in Escherichia coli. Antimicrob Agents Chemother. 2005; 49(1):447–50. doi: 10.1128/AAC.49.1.447–450.2005 PMID: 15616333; PubMed Central PMCID: PMCPMC538921.

41. Hossain A, Reisbig MD, Hanson ND. Plasmid-encoded functions compensate for the biological cost of AmpC overexpression in a clinical isolate of Salmonella typhimurium. J Antimicrob Chemother. 2004; 53(6):964–70. doi: 10.1093/jac/dkh240 PMID: 15140855.

42. Bishop RE. The bacterial lipocalins. Biochim Biophys Acta. 2000; 1482(1–2):73–83. PMID: 11058749.
43. Chung YJ, Saier MH, Jr. Overexpression of the Escherichia coli sugE gene confers resistance to a narrow range of quaternary ammonium compounds. J Bacteriol. 2002; 184(9):2543–5. PMID: 11948170; PubMed Central PMCID: PMC135002.
44. Egervarn M, Borjesson S, Byfors S, Finn M, Kaipe C, Englund S, et al. Escherichia coli with extended-spectrum beta-lactamases or transferable AmpC beta-lactamases and Salmonella on meat imported into Sweden. Int J Food Microbiol. 2014; 171:8–14. doi: 10.1016/j.ijfoodmicro.2013.11.005 PMID: 24296257.
45. Vogt D, Overesch G, Endimiani A, Collaud A, Thomann A, Perreten V. Occurrence and genetic characteristics of third-generation cephalosporin-resistant Escherichia coli in Swiss retail meat. Microb Drug Resist. 2014; 20(5):485–94. doi: 10.1089/mdr.2013.0210 PMID: 24773305.
46. Börjesson S, Egervarn M, Lindblad M, Englund S. Frequent occurrence of extended-spectrum beta-lactamase- and transferable ampc beta-lactamase-producing Escherichia coli on domestic chicken meat in Sweden. Appl Environ Microbiol. 2013; 79(7):2463–6. doi: 10.1128/AEM.03893-12 PMID: 23354705; PubMed Central PMCID: PMC3623214.
47. Dierikx C, van Essen-Zandbergen A, Veldman K, Smith H, Mevius D. Increased detection of extended spectrum beta-lactamase producing Salmonella enterica and Escherichia coli isolates from poultry. Vet Microbiol. 2010; 145(3–4):273–8. doi: 10.1016/j.vetmic.2010.03.019 PMID: 20395076.
48. Agerso Y, Jensen JD, Hasman H, Pedersen K. Spread of extended spectrum cephalosporinase-producing Escherichia coli clones and plasmids from parent animals to broilers and to broiler meat in a production without use of cephalosporins. Foodborne Pathog Dis. 2014; 11(9):740–6. doi: 10.1089/fpd.2014.1742 PMID: 24972048.
49. Manges AR. Escherichia coli and urinary tract infections: the role of poultry-meat. Clin Microbiol Infect. 2016; 22(2):122–9. doi: 10.1016/j.cmi.2015.11.010 PMID: 26679924.
50. Dierikx C, van der Goot J, Fabri T, van Essen-Zandbergen A, Smith H, Mevius D. Extended-spectrum-beta-lactamase- and AmpC-beta-lactamase-producing Escherichia coli in Dutch broilers and broiler farmers. J Antimicrob Chemother. 2013; 68(1):60–7. Epub 2012/09/06. doi: 10.1093/jac/dks349 PMID: 22949623.
51. Chattaway MA, Jenkins C, Ciesielczuk H, Day M, DoNascimento V, Day M, et al. Evidence of evolving extraintestinal enteroaggregative Escherichia coli ST38 clone. Emerg Infect Dis. 2014; 20(11):1935–7. doi: 10.3201/eid2011.131845 PMID: 25340736; PubMed Central PMCID: PMC4214294.
52. Naseer U, Haldorsen B, Simonsen GS, Sundsfjord A. Sporadic occurrence of CMY-2-producing multidrug-resistant Escherichia coli of ST-complexes 38 and 448, and ST131 in Norway. Clin Microbiol Infect. 2010; 16(2):171–8. doi: 10.1111/j.1469-0691.2009.02861.x PMID: 19548922.
53. Brolund A, Edquist PJ, Makitalo B, Olsson-Liljequist B, Soderblom T, Wisell KT, et al. Epidemiology of extended-spectrum beta-lactamase-producing Escherichia coli in Sweden 2007–2011. Clin Microbiol Infect. 2014; 20(6):O344–52. doi: 10.1111/1469-0691.12413 PMID: 24118431.
54. Berg ES, Wester AL, Mo SS, Slettemeas JS, Steinbakk M, Dahle UR, et al. Highly similar cephalosporin resistant Escherichia coli and AmpC resistance plasmids found in both patients and poultry meat in Norway. ECCMID; Copenhagen, Denmark: http://www.unn.no/getfile.php/UNN%20INTER/Fagfolk/www.antibiotikaresistens.no/K-Res/Poster/2015%20ECCMID%20Sunde%20M%20et%20al.pdf; 2015.
55. Unterholzner SJ, Poppenberger B, Rozhon W. Toxin-antitoxin systems: Biology, identification, and application. Mob Genet Elements. 2013; 3(5):e26219. doi: 10.4161/mge.26219 PMID: 24251069; PubMed Central PMCID: PMCPMC3827094.
56. Cortes P, Blanc V, Mora A, Dahbi G, Blanco JE, Blanco M, et al. Isolation and characterization of potentially pathogenic antimicrobial-resistant Escherichia coli strains from chicken and pig farms in Spain. Appl Environ Microbiol. 2010; 76(9):2799–805. doi: 10.1128/AEM.02421-09 PMID: 20228098; PubMed Central PMCID: PMC2863447.
57. Johnson JR, Delavari P, Kuskowski M, Stell AL. Phylogenetic distribution of extraintestinal virulence-associated traits in Escherichia coli. J Infect Dis. 2001; 183(1):78–88. doi: 10.1086/317656 PMID: 11106538.
58. Dale AP, Woodford N. Extra-intestinal pathogenic Escherichia coli (ExPEC): Disease, carriage and clones. J Infect. 2015. doi: 10.1016/j.jinf.2015.09.009 PMID: 26409905.
59. Pitout JD. Extraintestinal Pathogenic Escherichia coli: A Combination of Virulence with Antibiotic Resistance. Front Microbiol. 2012; 3:9. doi: 10.3389/fmicb.2012.00009 PMID: 22294983; PubMed Central PMCID: PMCPMC3261549.



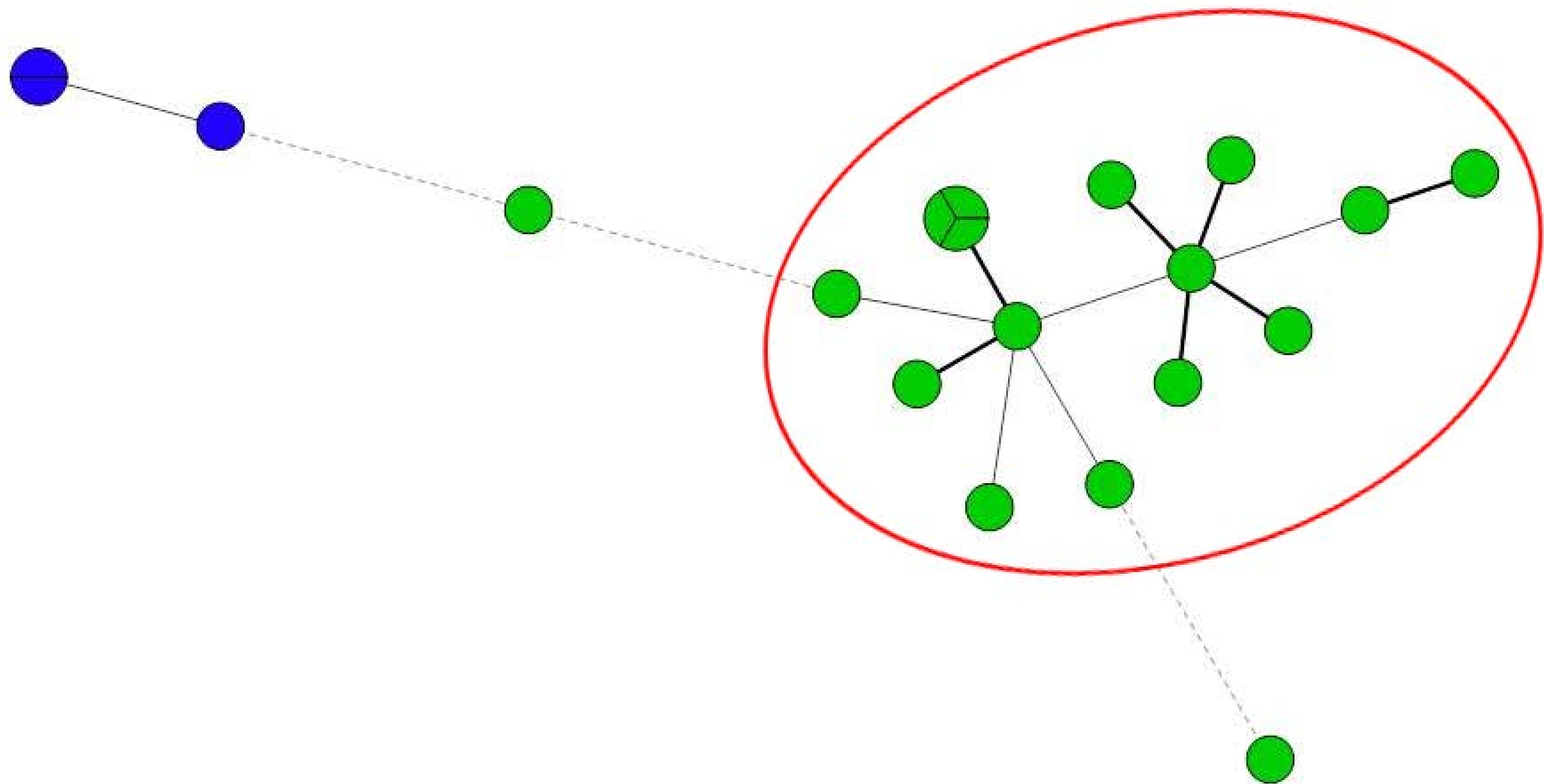
*Transferability of plasmids carrying *bla*$_{CMY-2}$. Replicon type refers to plasmid replicon (s) in transconjugant. NR: not relevant.



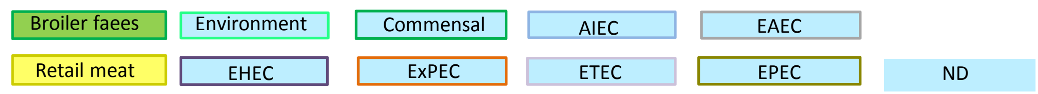
*Transferability of plasmids carrying bla_{CMY-2}. Replicon type refers to plasmid replicon (s) in transconjugant. NR: not relevant.

ID	Source	Year	Phylogroup	Conjugative plasmid*	Replicontype*

*Transferability of plasmids carrying bla_{CMY-2}. Replicon type refers to plasmid replicon (s) in transconjugant. NR: not relevant.



2014-01-1040
2014-01-1675
2014-01-1044
E_coli_O157-H7_str_Sakai_EHEC
E_coli_O55_H7_str_CB9615
E_coli_O55_H7_str_CB9615_EPEC
E_coli_O103-H2_str_12009_EHEC
E_coli_KO11FL
E_coli_SE11_coms
E_coli_55989_EAEC
E_coli_O104-H4_str_2011C-3493
E_coli_O111-H-str_11128_EHEC
E_coli_HS_coms
E_coli_B_str_REL606
E_coli_H10407_ETEC
E_coli_K-12_substr_MG1655_coms
E_coli_O127_H6_E2348-69_EPEC
E_coli_O127-H6_str_E2348-69
E_coli_SE15_coms
E_coli_O83-H1_str_NRG_857C_AIEC
E_coli_ABU-83972_ExPEC
E_coli_CFT073_ExPEC
E_coli_ED1a_coms
E_coli_UTI89_ExPEC
E_coli_SMS-3-5_environ
E_coli_IAI39_ExPEC
E_coli_O7_K1_str_CE10_ExPEC
E_coli_UMN026_ExPEC
2014-01-3680
2014-01-0013
2014-01-1043
2014-01-1042
2014-01-5105
2011-01-7660
2012-01-1553
2012-01-1849
2011-01-2112
2011-01-2112_ctrl
2011-01-5432
2012-01-1292
2012-01-1292_ctrl
2012-01-6001



0.05

S1 Table

	2011-01-2112	2011-01-2112_ctrl	2011-01-5432	2011-01-7660	2012-01-1292	2012-01-1292_ctrl	2012-01-1553	2012-01-1849	2012-01-6001
2011-01-2112	0	0	8	140	6	6	145	143	10
2011-01-2112_ctrl	0	0	8	140	6	6	145	143	10
2011-01-5432	8	8	0	140	4	4	145	143	10
2011-01-7660	140	140	140	0	138	138	85	83	142
2012-01-1292	6	6	4	138	0	0	143	141	8
2012-01-1292_ctrl	6	6	4	138	0	0	143	141	8
2012-01-1553	145	145	145	85	143	143	0	2	147
2012-01-1849	143	143	143	83	141	141	2	0	145
2012-01-6001	10	10	10	142	8	8	147	145	0
2014-01-0013	10364	10364	10364	10412	10362	10362	10411	10409	10366
2014-01-1040	13	13	13	145	11	11	150	148	9
2014-01-1042	10354	10354	10354	10414	10352	10352	10431	10429	10356
2014-01-1043	10355	10355	10355	10415	10353	10353	10432	10430	10357
2014-01-1044	9	9	9	141	7	7	146	144	5
2014-01-1675	14	14	14	146	12	12	151	149	10
2014-01-3680	10364	10364	10364	10412	10362	10362	10411	10409	10366
2014-01-5105	10369	10369	10369	10417	10367	10367	10416	10414	10371
reference/1-10539	12	12	10	144	6	6	149	147	14

min: 0 max: 10432

Percentage of reference genome covered by all isolates: 52.7659708609038

2879076 positions was found in all analyzed genomes.

Size of reference genome: 5456312

2014-01-0013	2014-01-1040	2014-01-1042	2014-01-1043	2014-01-1044	2014-01-1675	2014-01-3680	2014-01-5105	reference/1-10539
10364	13	10354	10355	9	14	10364	10369	12
10364	13	10354	10355	9	14	10364	10369	12
10364	13	10354	10355	9	14	10364	10369	10
10412	145	10414	10415	141	146	10412	10417	144
10362	11	10352	10353	7	12	10362	10367	6
10362	11	10352	10353	7	12	10362	10367	6
10411	150	10431	10432	146	151	10411	10416	149
10409	148	10429	10430	144	149	10409	10414	147
10366	9	10356	10357	5	10	10366	10371	14
0	10369	60	59	10365	10370	8	9	10364
10369	0	10359	10360	6	3	10369	10374	17
60	10359	0	11	10355	10360	62	63	10354
59	10360	11	0	10356	10361	61	62	10355
10365	6	10355	10356	0	7	10365	10370	13
10370	3	10360	10361	7	0	10370	10375	18
8	10369	62	61	10365	10370	0	11	10364
9	10374	63	62	10370	10375	11	0	10369
10364	17	10354	10355	13	18	10364	10369	0

S2 Table

	2011-01-2112-2/1-32	2011-01-5432-3/1-32	2011-01-7660-1/1-32	2012-01-1292/1-32	2012-01-1553/1-32	2012-01-1849/1-32
2011-01-2112-2/1-32	0	1	3	1	0	14
2011-01-5432-3/1-32	1	0	4	0	1	13
2011-01-7660-1/1-32	3	4	0	4	3	17
2012-01-1292/1-32	1	0	4	0	1	13
2012-01-1553/1-32	0	1	3	1	0	14
2012-01-1849/1-32	14	13	17	13	14	0
2012-01-6001/1-32	3	4	6	4	3	17
2014-01-0013/1-32	10	9	11	9	10	22
2014-01-1040/1-32	0	1	3	1	0	14
2014-01-1042/1-32	8	9	9	9	8	22
2014-01-1043/1-32	9	10	10	10	9	23
2014-01-1044/1-32	1	2	4	2	1	15
2014-01-1675/1-32	0	1	3	1	0	14
2014-01-3680/1-32	9	10	10	10	9	23
2014-01-5105/1-32	8	9	9	9	8	22
reference/1-32	1	0	4	0	1	13

min: 0 max: 23

Percentage of reference genome covered by all isolates: 95.3239678175994

75590 positions was found in all analyzed genomes.

Size of reference genome: 79298

2012-01-6001/1-32	2014-01-0013/1-32	2014-01-1040/1-32	2014-01-1042/1-32	2014-01-1043/1-32	2014-01-1044/1-32	2014-01-1675/1-32
3	10	0	8	9	1	0
4	9	1	9	10	2	1
6	11	3	9	10	4	3
4	9	1	9	10	2	1
3	10	0	8	9	1	0
17	22	14	22	23	15	14
0	13	3	11	12	4	3
13	0	10	4	5	11	10
3	10	0	8	9	1	0
11	4	8	0	1	9	8
12	5	9	1	0	10	9
4	11	1	9	10	0	1
3	10	0	8	9	1	0
12	5	9	1	2	10	9
11	4	8	0	1	9	8
4	9	1	9	10	2	1

2014-01-3680/1-32	2014-01-5105/1-32	reference/1-32
9	8	1
10	9	0
10	9	4
10	9	0
9	8	1
23	22	13
12	11	4
5	4	9
9	8	1
1	0	9
2	1	10
10	9	2
9	8	1
0	1	10
1	0	9
10	9	0

Paper IV

Transfer potential of plasmids encoding extended-spectrum cephalosporin resistance in *Escherichia coli* from poultry into different *Enterobacteriaceae* under various conditions

Solveig Sølverød Mo[1], Marianne Sunde[1,2], Hanna Karin Ilag[3], Solveig Langsrud[4], Even Heir[4]

1) Department of Diagnostic Services, Norwegian Veterinary Institute, Oslo, Norway

2) Department of Bacteriology and Immunology, Norwegian Institute of Public Health, Ås, Norway

3) Department of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, ÅS, Norway

4) Nofima, The Norwegian Institute for Food, Fishery and Aquaculture Research, Ås, Norway

Manuscript in preparation

Abstract

Escherichia coli displaying resistance to extended-spectrum cephalosporins (ESC) have been detected in the Norwegian broiler production. Characterization of isolates have identified that the majority of isolates harboured self-transmissible IncK or IncI1 plasmids with *bla*$_{\text{CMY-2}}$. Also, recent investigations have indicated that local recirculation of ESC-resistant strains on broiler farms is more likely than continued re-introduction from parent flocks. Little is known about the ability of these plasmids to transfer to other environmental bacteria or in biofilms. Some environmental bacteria and biofilms have an increased ability to withstand disinfection, and could potentially act as a reservoir for resistance plasmids in the broiler production chain. The aim of this study was to determine the transferability of two well characterized *bla*$_{\text{CMY-2}}$ carrying plasmids belonging to the incompatibility groups IncK and IncI1, respectively, into *E. coli* and *Serratia* spp. recipients. Experiments were performed at different temperatures and under both planktonic and biofilm modes of growth. This was done in order to mimic conditions relevant for the broiler production

chain. Self-transfer of plasmids was observed in colony- and broth matings and in biofilm at different temperatures, including temperatures sub-optimal for bacterial growth. The IncK plasmid was able to self-transfer into *Serratia marcescens*. Furthermore, transconjugant strains were able to act as secondary donors of this plasmid. The findings indicate a large potential for horizontal transfer of *bla*$_{\text{CMY-2}}$ carrying plasmids. Transfer to *Serratia* spp. and hosts with good biofilm forming abilities and with potential to act as secondary plasmid donors to new hosts might contribute to maintenance of these resistance plasmids in the food chain.

Introduction

During the course of the last decade, an increasing occurrence of *Escherichia coli* displaying resistance to extended-spectrum cephalosporins (ESC) has been observed in both human and veterinary medicine (Cantón et al., 2008; Carattoli, 2008; Coque et al., 2008; Pitout and Laupland, 2008; Overdevest et al., 2011; Ewers et al., 2012; ECDC, 2015; EFSA and ECDC, 2016). Broilers and broiler products have been reported to be highly associated with ESC-resistant *E. coli* (Carattoli, 2008; Coque et al., 2008; Overdevest et al., 2011; Ewers et al., 2012). In 2011, a selective method for detection of ESC-resistant *E. coli* was implemented in NORM-VET, the Norwegian monitoring programme for antimicrobial resistance in bacteria from the veterinary and food production sectors (NORM/NORM-VET, 2012). Subsequently, ESC-resistant *E. coli* have been shown to be widely distributed in the Norwegian broiler production and in retail chicken meat. All isolates have displayed an AmpC-phenotype, mainly mediated by plasmids carrying *bla*$_{\text{CMY-2}}$, while a small proportion of isolates were resistant due to up-regulation of chromosomal *ampC* (NORM/NORM-VET, 2012, 2013; Mo et al., 2014; NORM/NORM-VET, 2015). As the use of antimicrobial agents in the Norwegian broiler production is almost absent (Animalia, 2015; Refsum, 2015; Animalia, 2016), this was a surprising finding. Import of breeding animals has been suggested as the probable source of introduction of ESC-resistant *E. coli* into the Norwegian broiler production (NORM/NORM-VET, 2007; Sunde et al., 2009). Therefore, the Norwegian poultry industry initiated an action plan against ESC-resistant *E. coli* in 2014, including sampling of each batch of imported breeding animals after hatching, sampling of parent flocks in production, and requirement of no routine use of antimicrobials in the higher levels of the production pyramid (Animalia, 2013). The number of imported batches positive for ESC-resistant *E. coli* was low in both 2014 and 2015 (6 % and 2 %, respectively), while the occurrence in parent flocks in production decreased from 27 % in

2014 to 22 % in 2015 (Animalia, 2015, 2016). Also, there was no statistical association between the status of parent flocks (i.e. ESC-resistant *E. coli* present or absent) and the status of the broiler flocks receiving day-old chickens from these parent flocks in a study recently performed in Norway (Mo et al., 2016a). Therefore, a continued introduction of new ESC-resistant strains and subsequent vertical spread in the production is considered of less importance. Having good disinfection routines, i.e. always disinfecting the floors in the broiler house between production cycles, lowered the odds of ESC-resistant *E. coli* occurring in broiler flocks (odds ratio [OR]=0.1). However, if the previous flock in the same house was positive, the odds of the subsequent flock also being positive was much higher than if the previous flock was negative (OR=12.7). These results indicate a possible recirculation of ESC-resistant *E. coli* on farms between production cycles (Mo et al., 2016a), facilitating persistence of ESC-resistant *E. coli* and their associated resistance plasmids in the Norwegian broiler production.

We have previously shown that ESC-resistant *E. coli* from the Norwegian broiler production usually carry $bla_{\text{CMY-2}}$ on self-transmissible plasmids belonging to incompatibility (Inc) groups K or I1. Also, highly similar IncK plasmids have been detected in *E. coli* of different multi locus sequence types (STs) in Norway (Mo et al., 2016b). IncK and IncI1 plasmids with $bla_{\text{CMY-2}}$ have also been detected in ESC-resistant *E. coli* isolated from the broiler production chain in several other European countries (Dierikx et al., 2010; Börjesson et al., 2013a; Börjesson et al., 2013b; Dierikx et al., 2013; Voets et al., 2013; Agersø et al., 2014; Egervärn et al., 2014). A high degree of similarity between IncK (Hansen et al., 2016; Mo et al., 2016b) and IncI1 (unpublished data) plasmids with $bla_{\text{CMY-2}}$ isolated from the broiler production in Norway and other European countries have been detected. These findings suggest that these IncK and IncI1 plasmids might be common and successful in the European broiler production. Occurrence of *E. coli* harbouring IncK or IncI1 plasmids with $bla_{\text{CMY-2}}$ at several levels of the broiler production pyramid (Mo et al., 2016b) could facilitate horizontal spread and distribution to other bacterial hosts in the environment. This might also play an important role in the epidemiology and maintenance of ESC resistance in the Norwegian broiler production.

Biofilms are considered as the natural way of living for most bacteria, and provide protection of the bacteria under sub-optimal conditions. The coexistence of bacteria in biofilms enable close interactions between bacteria, often involving different species (Davey and O'Toole G, 2000), and thus biofilms have been suggested as a hot-spot for horizontal

gene transfer (Jefferson, 2004; Sørensen et al., 2005). Both conjugative transfer of plasmids and transduction of bacteriophages between bacteria in biofilm have been reported (Król et al., 2011; Solheim et al., 2013).

The aim of this study was to determine the transferability of two well characterized *bla*$_{CMY-2}$ carrying plasmids, belonging to the incompatibility groups IncK and IncI1, respectively. The plasmids originated from *E. coli* isolated from retail chicken meat. Transfer experiments were performed with other *E. coli* and *Serratia* spp. as recipient strains, at different temperatures and under both planktonic and biofilm modes of growth, reflecting conditions relevant for the broiler production chain.

Materials and Methods

Bacterial isolates

Plasmid donor isolates used included *E. coli* 2012-01-1292 (designated *E. coli* 1292) carrying a recently characterized IncK plasmid (pNVI1292/IncK, accession number KU312044) (Mo et al., 2016b) and *E. coli* 2012-01-2798 (designated *E. coli* 2798) carrying an IncI1 plasmid (pNVI2798/IncI1). Both donor strains were known to harbour *bla*$_{CMY-2}$, and originated from retail chicken meat collected in 2012 as part of the NORM-VET programme (NORM/NORM-VET, 2013). Potential recipient strains included a selection of *E. coli* from broiler faeces and retail chicken meat (n=14) resistant to nalidixic acid (Nal^R), and *Serratia* spp. from food processing units and retail chicken meat (n=18) (Table 1). The *Serratia* spp. isolates were rifampicin resistant (Rif^R) (n=15) or made rifampicin resistant (n=3) as previously described (Heir et al., 2010).

Selection of mating pairs

Initially, the two included donor strains were mated with all the included recipient strains using colony mating on agar to identify mating pairs where conjugal transfer of the plasmids occurred. In addition, *S. marcescens* transconjugants were mated with *E. coli* (n=7) and *S. proteamaculans* (n=1) to determine if they could act as secondary donors of pNVI1292/IncK. Pre-cultures of donor and recipient strains were grown on tryptone soy agar plates (TSA, Oxoid LTD, Basingstoke, England) at 30°C (*Serratia* spp.) or 37°C (*E. coli*) overnight. One colony of the recipient and one colony of the donor were mixed on a TSA plate and incubated at 30°C. Matings were sampled after four, 24 and 48 hours by swiping a loop through the colonies. Samples were plated on Mueller Hinton (MH) agar

(Difco, Sparks, MD, USA) supplemented with two different antimicrobials used to select for transconjugant strains. Mating pairs used in the subsequent conjugation experiments were selected on the basis of the ability of the recipient strains to receive the pNVI1292/IncK and/or the pNVI2798/IncI1 plasmids (Table 2).

Conjugation experiments

Conjugation experiments were performed with selected mating pairs by the use of colony mating, broth mating, and conjugation in biofilm. Conjugation experiments in biofilm were performed in triplicates. All experiments were performed at 12°C, 25°C and 30°C (*Serratia* spp.) or 37°C (*E. coli*).

Colony mating: Pre-cultures of donor and recipient strains were inoculated on TSA and grown at 25°C for three days. Before the colony mating was performed, the agar plates were incubated at the temperature used in the subsequent experiment for one hour. Colony matings were prepared and sampled as described above. Sampling was performed after four (30°C and 37°C), 24 and 48 hours (all matings).

Broth mating: Pre-cultures of donor and recipient strains used in experiments at 25°C, 30°C and 37°C were grown separately in Luria Bertani (LB) broth (Oxoid LTD) at 25°C for three days. Thereafter, they were moved and incubated at the temperature used in the subsequent experiment for one hour before broth mating was performed. Pre-cultures used in experiments at 12°C were incubated at 12°C for three days. Broth mating was conducted as previously described (Sunde and Sørum, 2001). Briefly, 1 mL of the donor and 1 mL of the recipient was mixed in 4 mL fresh LB broth. Sampling was performed after four (30°C and 37°C), 24 and 48 hours (all matings) by plating 100 µL of the broth on MH agar supplemented with two different antimicrobials used to select for transconjugant strains.

Conjugation in biofilm: Pre-cultures of recipient strains were grown in LB without NaCl at 30°C overnight. Subsequently, biofilms of each recipient strain were established on autoclaved coupons of stainless steel (AISI 304) as previously described (Nesse et al., 2014). Pre-cultures of donor strains for conjugation experiments in biofilm were prepared as described for broth mating. However, LB without NaCl was used instead of LB. The steel coupons with established biofilms were rinsed in peptone water to remove loosely attached cells and then transferred to tubes with cultures of the donor strain (Król et al., 2011). Sampling was performed after four (30°C and 37°C), 24 and 48 hours (all matings).

Sampling of biofilms included harvesting the whole biofilm. The steel coupon was rinsed in peptone water and transferred to a glass tube with 15 mL peptone water. The biofilm was detached by scraping with a swab followed by 15 minutes of sonication at 42 kHz in 25°C. One mL of the samples were diluted ten-fold and the dilution series were plated out on three different MH agar plates supplemented with different antimicrobials in order to quantify number of donors, recipients and transconjugants in the sample. The transfer frequencies of plasmids in biofilms were calculated as number of transconjugants/number of recipients (T/R).

All MH agar plates were incubated at 30°C (*Serratia* spp.) or 37°C (*E. coli*) for 24 hours.

Confirmation of transconjugant strains

Donor and recipient strains were differentiated by the use of matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF, Bruker Daltonics) (*E. coli*-*S. marcescens* matings and *S. marcescens*-*S. proteamaculans* matings) or by phylotyping (*E. coli*- *E. coli* matings) (Doumith et al., 2012), as donor and recipient strains belonged to different phylotypes. Transfer of the plasmid to the recipient strains was confirmed by real-time PCR detection of bla_{CMY-2} with previously published primers and probe (Schmidt et al., 2015). Positive and negative controls were included in each run.

Antimicrobial susceptibility testing

Recipient strains included in the conjugation experiments were subjected to antimicrobial susceptibility testing before and after acquisition of the pNVI1292/IncK or pNVI2798/IncI1 plasmids. Minimum inhibitory concentrations (MICs) for relevant beta-lactam antimicrobials were determined by broth microdilution (EUVSEC2, Sensititre TREK, Thermo Scientific).

Results

In the initial colony mating experiments, self-transfer of pNVI1292/IncK from *E. coli* 1292 was observed to 7/14 *E. coli* recipients and 4/18 *Serratia* spp. In addition, the plasmid was self-transferred from *S. marcescens* transconjugants to the same seven *E. coli* recipients, and to the *S. proteamaculans* recipient. The pNVI2798/IncI1 plasmid from *E. coli* 2798 was self-transferred to 12/14 *E. coli* recipients. No self-transfer of pNVI2798/IncI1 to Serratia

spp. recipients was observed. Seven mating pairs were selected and subjected to conjugation experiments using colony- and broth mating, and in biofilm (Table 2).

Plasmid transfer was observed in colony mating, broth mating and in biofilm at different temperatures, including low temperatures sub-optimal for bacterial growth (Table 3). No plasmid transfer was observed at 12°C (data not shown).

When self-transfer of plasmids occurred in biofilm, the transfer frequencies of the the pNVI1292/ IncK plasmid (T/R) ranged from $5x10^{-7}$-$8x10^{-2}$, with the highest transfer frequencies observed between *E. coli*-*E. coli* mating pairs (Table 4). Furthermore, the pNVI1292/IncK plasmid was transferred from *E. coli* to *S. marcescens* and from the *S. marcescens* transconjugant to an *E. coli* recipient and from the *S. marcescens* transkonjugant to an *S. proteamaculans* recipient in biofilm. The transfer frequencies observed for the pNVI2798/IncI1 plasmid ranged from $4x10^{-5}$-$2x10^{-2}$. The pNVI2798/IncI1 plasmid was not subjected to conjugation experiments with *S. marcescens* as recipient in biofilm on steel coupons, as no transfer was observed after the initial colony mating. In general, the transfer frequencies were higher at 30°C/37°C compared to 25°C. Confirmed routes for horizontal transfer of the pNVI1292/IncK plasmid are illustrated in Figure 1.

Counting the number of donors, recipients and transconjugants in the biofilm experiments revealed that the total number of bacteria was in general somewhat lower at 12°C compared to 25°C and 30/37°C (data not shown).

None of the recipient strains were resistant to any beta-lactam antimicrobials prior to the conjugation experiments. All recipient strains displayed a beta-lactam resistance profile corresponding to an AmpC-phenotype after acquisition of either the pNVI1292/IncK or pNVI2798/IncI1 plasmid (Table 5).

Discussion

IncK and IncI1 plasmids harbouring $bla_{\text{CMY-2}}$ are commonly occurring in ESC-resistant *E. coli* in the European broiler production (Dierikx et al., 2010; Börjesson et al., 2013a; Börjesson et al., 2013b; Dierikx et al., 2013; Voets et al., 2013; Agersø et al., 2014; Egervärn et al., 2014). However, little is known about the potential of these plasmids to self-transfer into environmental bacteria, and to what extent the plasmid transfer can occur. *Serratia* spp. are environmental bacteria that are commonly occurring in the broiler production (Turtura et al., 1990; Schwaiger et al., 2012; Säde et al., 2013). Furthermore,

Serratia spp. have been shown to have the ability to survive and multiply in the presence of disinfectants (Willinghan et al., 1996; Langsrud et al., 2003). Therefore, we chose to include *Serratia* spp. in our experiments to investigate if these environmental bacteria could be a potential reservoir for resistance plasmids in the broiler production. Also, we chose to perform conjugation experiments in biofilm, as these are considered hot-spots for horizontal gene transfer (Jefferson, 2004; Sørensen et al., 2005) and are known to exist in the food production chain (Kumar and Anand, 1998).

The pNVI1292/IncK plasmid was highly similar to a plasmid present in *E. coli* from retail chicken meat in the Netherlands (Mo et al., 2016b), plasmids originating from parent- and broiler flocks in Denmark, and retail chicken meat produced in and imported to Denmark (Hansen et al., 2016). Furthermore, the pNVI2798/IncI1 plasmid was similar to IncI1 plasmids isolated from poultry in The Netherlands (Smith et al., 2015), sharing 96.8 kb corresponding to 97 % nucleotide sequence homology (unpublished data). The plasmid stability systems *relBE/stbDE* and *pndAC* were present on both pNVI1292/IncK (Mo et al., 2016b) and on pNVI2798/IncI1 (unpublished data).

Self-transfer of pNVI1292/IncK and pNVI2798/IncI1 was observed in colony matings, broth matings and in biofilm at 25°C and 30/37°C. Self-transfer of plasmids has also been reported to happen in fluids without nutrients (Fernandez-Astorga et al., 1992; Sunde and Sørum, 2001), and it is tempting to speculate that this might be possible for the plasmids investigated in this study as well. Thus, the plasmid transfer observed in broth mating could indicate that conditions present in freshly packed chicken meat (meat juice) and possibly other environments in the production where fluids or humid conditions are present might favour self-transfer of resistance plasmids. Furthermore, the presence of biofilm at several levels of the food production (Kumar and Anand, 1998), and the ability of plasmids to self-transfer in biofilm indicates that horizontal gene transfer in biofilm might take place at many levels of the broiler production.

We have demonstrated the ability of the pNVI1292/IncK plasmid harbouring $bla_{\text{CMY-2}}$ to transfer from *E. coli* to *S. marcescens* under different conditions considered relevant for the broiler production. Also, we demonstrated that the pNVI1292/IncK plasmid can be transferred from the *S. marcescens* transconjugant back to intestinal *E. coli* and from the *S. marcescens* transconjugant to *S. proteamaculans*. The relatively high transfer frequencies observed from *S. marcescens* transconjugants to *E. coli* recipients indicate the ability of

environmental bacteria to be efficient contributors in the dissemination of IncK resistance plasmids. The ability of *Serratia* spp. to acquire and harbour such resistance plasmids support the hypothesis that *Serratia* spp. might act as a reservoir for plasmid-mediated ESC resistance. The potential of these plasmids to be harboured by bacteria with enhanced ability to survive through cleaning and disinfection routines applied in the production chain might also play a part in the maintenance and persistence of ESC-resistant bacteria in the food chain. *Serratia* spp. that survive cleaning and disinfection might transfer the plasmid back to *E. coli* and possibly on to other *Enterobacteriaceae* when such bacteria are introduced to the environment after disinfection. It is assumed that the presence of plasmid stability systems on the pNVI1292/IncK and pNVI2798/IncI1 plasmids will facilitate their stability in new hosts. However, further experiments should be performed to confirm this theory.

Bacteria in biofilm might have an increased ability to survive cleaning and disinfection (Kumar and Anand, 1998; Shi and Zhu, 2009; Srey et al., 2013), meaning that they might serve as a reservoir for contamination of the environment after disinfection. All the isolates included in this study had good biofilm forming abilities (data not shown). Thus, it is tempting to suggest that survival of ESC-resistant bacteria in biofilm, together with conjugative transfer of plasmids, is also a part of the puzzle when it comes to the maintenance and dissemination of ESC-resistant bacteria and plasmids in the broiler production.

Interestingly, the pNVI2798/IncI1 plasmid included in this study was not capable of self-transfer from *E. coli* to *Serratia* spp. in any of our experiments. The reason for this is unknown, but might be associated with the conditions used in the present studies. Only a limited number of strains and mating combinations were investigated, so the possibility of self-transfer of pNVI2798/IncI1 plasmids to *Serratia* spp. can therefore not be excluded. Further experiments should be performed in order to determine whether pNVI1292/IncK has a higher ability of self-transfer to and maintenance within different hosts relevant for the broiler production than pNVI2798/IncI1.

In general, we observed increased transfer of plasmids at 30/37°C compared to 25°C, which is in correspondence with previous findings (Fernandez-Astorga et al., 1992). No plasmid transfer was observed at 12°C for any of the mating pairs. Low temperatures have been reported to decrease conjugal transfer of plasmids in broth matings (Fernandez-Astorga et al., 1992). Possibly, this is due to a lower cell density observed in biofilms at 12°C, or lower

growth rate of the bacteria at this temperature. This has also been suggested previously (Händel et al., 2015). These findings might indicate that conjugal transfer is a limited problem in parts of the food-production where low temperatures are applied, such as meat-processing. The findings suggest that keeping a low temperature in processing units might hamper the growth of certain bacteria, but also reduce or inhibit horizontal transfer of plasmids. However, self-transfer of plasmids have been reported to take place at 8°C (Fernandez-Astorga et al., 1992). Therefore, we cannot exclude the possibility that the plasmids can be transferred to and maintained within hosts that can facilitate further dissemination of the plasmids under conditions that are sub-optimal for *E. coli*. Thus, it is important to investigate to which extent other bacteria than *E. coli* are able to contribute to dissemination of resistance plasmids, and how various environmental conditions affect transfer and maintenance of them. Such knowledge will be crucial for a deeper understanding of the epidemiology of plasmids mediating ESC-resistance in the broiler production. The fact that horizontal transfer of plasmids occurs at 25°C might indicate a possible relevance in parts of the production with higher temperatures.

In the biofilm experiments, the number of transconjugants increased with time, which has also been described previously (Król et al., 2013). However, with the method used here, it is not possible to determine if this was due to an increased number of conjugative transfers from donor to recipient, if transconjugants started to act as recipients as well, if transconjugant strains started to expand in the biofilm, or a combination of the three. For some mating pairs, transconjugants were observed after 24 hours but not after 48 hours. This could be due to a low transfer frequency. Possibly, the number of transconjugants was around the detection limit, resulting in inconsistent results for consecutive samplings.

In this study we have demonstrated the ability of plasmids of poultry origin encoding ESC resistance to self-transfer into different hosts of *E. coli* and *Serratia* spp. Transfer occurred in suboptimal conditions and in biofilm, indicating a large potential for horizontal transfer of these resistance plasmids. Transfer to hosts with good biofilm forming abilities and with potential to act as secondary plasmid donors to new hosts, might contribute to maintenance of the resistance plasmid through the food chain.

Conflict of interest

None to declare

Acknowledgements

This work was funded by the Foundation for Research Levy on Agricultural Agreement Research Fund (Matfondavtalen), grant no. 225165. Anette Wold Åsli is acknowledged for excellent technical assistance.

References

Agersø, Y., Jensen, J.D., Hasman, H., Pedersen, K., 2014. Spread of extended spectrum cephalosporinase-producing *Escherichia coli* clones and plasmids from parent animals to broilers and to broiler meat in a production without use of cephalosporins. Foodborne Pathog. Dis. 11, 740-746.

Animalia, 2013. The poultry industry's action plan against ESBL-producing *E. coli* (Fjørfenæringas handlingsplan mot ESBL-bærende *E. coli*; in Norwegian). http://animalia.no/upload/FIler%20til%20nedlasting/Animalia/Handlingsplan_ESBL_06_desember2013.pdf (last accessed 05.05.2016).

Animalia, 2015. Actions to combat antimicrobial resistance works (Tiltak mot antibiotikaresistens virker; in Norwegian). http://animalia.no/Listesider/Aktuelt-og-fagstoff/Tiltak-mot-antibiotikaresistens-virker-/ (last accessed 31.05.2016).

Animalia, 2016. Continued decrease in the occurrence of resistant bacteria in poultry (Fortsatt nedgang i forekomst av resistente bakterier hos fjørfe; in Norwegian). http://animalia.no/Listesider/Aktuelt-og-fagstoff/Fortsatt-nedgang-i-forekomst-av-resistente-bakterier-hos-fjorfe/ (last accessed 31.05.2016).

Börjesson, S., Egervärn, M., Lindblad, M., Englund, S., 2013a. Frequent occurrence of extended-spectrum beta-lactamase- and transferable ampc beta-lactamase-producing *Escherichia coli* on domestic chicken meat in Sweden. Appl. Environ. Microbiol. 79, 2463-2466.

Börjesson, S., Jernberg, C., Brolund, A., Edquist, P., Finn, M., Landén, A., Olsson-Liljequist, B., Tegmark Wisell, K., Bengtsson, B., Englund, S., 2013b. Characterization of plasmid-mediated AmpC-producing *E. coli* from Swedish broilers and association with human clinical isolates. Clin. Microbiol. Infect. 19, E309-311.

Cantón, R., Novais, A., Valverde, A., Machado, E., Peixe, L., Baquero, F., Coque, T.M., 2008. Prevalence and spread of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in Europe. Clin. Microbiol. Infect. 14 Suppl 1, 144-153.

Carattoli, A., 2008. Animal reservoirs for extended spectrum beta-lactamase producers. Clin. Microbiol. Infect. 14 Suppl 1, 117-123.

Coque, T.M., Baquero, F., Canton, R., 2008. Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe. Euro Surveill. 13, 1-11.

Davey, M.E., O'Toole G, A., 2000. Microbial biofilms: from ecology to molecular genetics. Microbiol. Mol. Biol. Rev. 64, 847-867.

Dierikx, C., van der Goot, J., Fabri, T., van Essen-Zandbergen, A., Smith, H., Mevius, D., 2013. Extended-spectrum-beta-lactamase- and AmpC-beta-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers. J. Antimicrob. Chemother. 68, 60-67.

Dierikx, C., van Essen-Zandbergen, A., Veldman, K., Smith, H., Mevius, D., 2010. Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. Vet. Microbiol. 145, 273-278.

Doumith, M., Day, M.J., Hope, R., Wain, J., Woodford, N., 2012. Improved Multiplex PCR Strategy for Rapid Assignment of the Four Major *Escherichia coli* Phylogenetic Groups. J. Clin. Microbiol. 50, 3108-3110.

ECDC, 2015. Antimicrobial resistance surveillance in Europe 2014. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). European Centre for Disease Prevention and Control, Stockholm. ISSN: 2363-2666.

EFSA, ECDC, 2016. The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food, in 2014. EFSA journal 14, 4380.

Egervärn, M., Börjesson, S., Byfors, S., Finn, M., Kaipe, C., Englund, S., Lindblad, M., 2014. *Escherichia coli* with extended-spectrum beta-lactamases or transferable AmpC beta-lactamases and *Salmonella* on meat imported into Sweden. Int. J. Food Microbiol. 171, 8-14.

Ewers, C., Bethe, A., Semmler, T., Guenther, T., Wieler, L.H., 2012. Extended-spectrum beta-lactamase-producing and AmpC-producing *Escherichia coli* from livestock and companion animals, and their putative impact on public health: a global perspective. Clin. Microbiol. Infect. 18, 646-655.

Fernandez-Astorga, A., Muela, A., Cisterna, R., Iriberri, J., Barcina, I., 1992. Biotic and abiotic factors affecting plasmid transfer in *Escherichia coli* strains. Appl. Environ. Microbiol. 58, 392-398.

Hansen, K.H., Bortolaia, V., Nielsen, C.A., Nielsen, J.B., Schønning, K., Agersø, Y., Guardabassi, L., 2016. Host-specific patterns of genetic diversity among IncI1-Igamma and IncK plasmids encoding CMY-2 beta-lactamase in *Escherichia coli* isolates from humans, poultry meat, poultry and dogs in Denmark. Appl. Environ. Microbiol.

Heir, E., Holck, A.L., Omer, M.K., Alvseike, O., Høy, M., Måge, I., Axelsson, L., 2010. Reduction of verotoxigenic *Escherichia coli* by process and recipe optimisation in dry-fermented sausages. Int. J. Food Microbiol. 141, 195-202.

Händel, N., Otte, S., Jonker, M., Brul, S., ter Kuile, B.H., 2015. Factors that affect transfer of the IncI1 beta-lactam resistance plasmid pESBL-283 between *E. coli* strains. PLoS One 10, e0123039.

Jefferson, K.K., 2004. What drives bacteria to produce a biofilm? FEMS Microbiol. Lett. 236, 163-173.

Król, J.E., Nguyen, H.D., Rogers, L.M., Beyenal, H., Krone, S.M., Top, E.M., 2011. Increased transfer of a multidrug resistance plasmid in *Escherichia coli* biofilms at the air-liquid interface. Appl. Environ. Microbiol. 77, 5079-5088.

Król, J.E., Wojtowicz, A.J., Rogers, L.M., Heuer, H., Smalla, K., Krone, S.M., Top, E.M., 2013. Invasion of *E. coli* biofilms by antibiotic resistance plasmids. Plasmid 70, 110-119.

Kumar, C.G., Anand, S.K., 1998. Significance of microbial biofilms in food industry: a review. Int. J. Food Microbiol. 42, 9-27.

Langsrud, S., Møretrø, T., Sundheim, G., 2003. Characterization of *Serratia marcescens* surviving in disinfecting footbaths. J. Appl. Microbiol. 95, 186-195.

Mo, S.S., Kristoffersen, A.B., Sunde, M., Nødtvedt, A., Norström, M., 2016a. Risk factors for occurrence of cephalosporin-resistant *Escherichia coli* in Norwegian broiler flocks. Prev. Vet. Med., Submitted after revision.

Mo, S.S., Norström, M., Slettemeås, J.S., Løvland, A., Urdahl, A.M., Sunde, M., 2014. Emergence of AmpC-producing *Escherichia coli* in the broiler production chain in a country with a low antimicrobial usage profile. Vet. Microbiol. 171, 315-320.

Mo, S.S., Slettemeås, J.S., Berg, E.S., Norström, M., Sunde, M., 2016b. Plasmid and Host Strain Characteristics of *Escherichia coli* Resistant to Extended-Spectrum Cephalosporins in the Norwegian Broiler Production. PLoS One 11, e0154019.

Nesse, L.L., Sekse, C., Berg, K., Johannesen, K.C., Solheim, H., Vestby, L.K., Urdahl, A.M., 2014. Potentially pathogenic *Escherichia coli* can form a biofilm under

conditions relevant to the food production chain. Appl. Environ. Microbiol. 80, 2042-2049.

NORM/NORM-VET, 2007. NORM/NORM-VET 2006. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo. ISSN:1502-2307.

NORM/NORM-VET, 2012. NORM/NORM-VET 2011. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo. ISSN:1502-2307 (print)/1890-9965 (electronic).

NORM/NORM-VET, 2013. NORM/NORM-VET 2012. Usage of Antimicrobial Agents and Occurrence of Antimicrobial Resistance in Norway. Tromsø/Oslo. ISSN:1502-2307 (print)/1890-9965 (electronic).

NORM/NORM-VET, 2015. NORM/NORM-VET 2014. Usage of antimicrobial agents and occurrence of antimicrobial resistance in Norway. In: Simonsen, G.S., Urdahl, A.M. (Eds.), Tromsø/Oslo. ISSN:1502-2307 (print)/1890-9965 (electronic).

Overdevest, I., Willemsen, I., Rijnsburger, M., Eustace, A., Xu, L., Hawkey, P., Heck, M., Savelkoul, P., Vandenbroucke-Grauls, C., van der Zwaluw, K., Huijsdens, X., Kluytmans, J., 2011. Extended-spectrum beta-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. Emerg. Infect. Dis. 17, 1216-1222.

Pitout, J.D., Laupland, K.B., 2008. Extended-spectrum beta-lactamase-producing *Enterobacteriaceae*: an emerging public-health concern. Lancet Infect. Dis. 8, 159-166.

Refsum, T., 2015. Antimicrobial use in the Norwegian poultry production (Antibiotikabehandling i norsk fjørfeproduksjon; in Norwegian). Go' mørning Animalia, http://www.animalia.no/upload/FIler%20til%20nedlasting/Go%C2%B4m%C3%B8rning/2015/GM%204-15%20web.pdf (last accessed 23.05.2016).

Schmidt, G.V., Mellerup, A., Christiansen, L.E., Ståhl, M., Olsen, J.E., Angen, O., 2015. Sampling and Pooling Methods for Capturing Herd Level Antibiotic Resistance in Swine Feces using qPCR and CFU Approaches. PLoS One 10, e0131672.

Schwaiger, K., Huther, S., Hölzel, C., Kämpf, P., Bauer, J., 2012. Prevalence of antibiotic-resistant enterobacteriaceae isolated from chicken and pork meat purchased at the slaughterhouse and at retail in Bavaria, Germany. Int. J. Food Microbiol. 154, 206-211.

Shi, X.M., Zhu, X.N., 2009. Biofilm formation and food safety in food industries. Trends Food Sci. Tech. 20, 407-413.

Smith, H., Bossers, A., Harders, F., Wu, G., Woodford, N., Schwarz, S., Guerra, B., Rodríguez, I., van Essen-Zandbergen, A., Brouwer, M., Mevius, D., 2015. Characterization of epidemic IncI1-Igamma plasmids harboring ambler class A and C genes in *Escherichia coli* and *Salmonella enterica* from animals and humans. Antimicrob. Agents Chemother. 59, 5357-5365.

Solheim, H.T., Sekse, C., Urdahl, A.M., Wasteson, Y., Nesse, L.L., 2013. Biofilm as an environment for dissemination of *stx* genes by transduction. Appl. Environ. Microbiol. 79, 896-900.

Srey, S., Jahid, I.K., Ha, S.D., 2013. Biofilm formation in food industries: A food safety concern. Food Control 31, 572-585.

Sunde, M., Sørum, H., 2001. Self-transmissible multidrug resistance plasmids in *Escherichia coli* of the normal intestinal flora of healthy swine. Microb. Drug Resist. 7, 191-196.

Sunde, M., Tharaldsen, H., Slettemeås, J.S., Norström, M., Carattoli, A., Bjorland, J., 2009. *Escherichia coli* of animal origin in Norway contains a blaTEM-20-carrying plasmid closely related to blaTEM-20 and blaTEM-52 plasmids from other European countries. J. Antimicrob. Chemother. 63, 215-216.

Säde, E., Murros, A., Björkroth, J., 2013. Predominant enterobacteria on modified-atmosphere packaged meat and poultry. Food Microbiol. 34, 252-258.

Sørensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., Wuertz, S., 2005. Studying plasmid horizontal transfer in situ: A critical review. Nat. Rev. Microbiol. 3, 700-710.

Turtura, G.C., Massa, S., Ghazvinizadeh, H., 1990. Antibiotic resistance among coliform bacteria isolated from carcasses of commercially slaughtered chickens. Int. J. Food Microbiol. 11, 351-354.

Voets, G.M., Fluit, A.C., Scharringa, J., Schapendonk, C., van den Munckhof, T., Leverstein-van Hall, M.A., Stuart, J.C., 2013. Identical plasmid AmpC beta-lactamase genes and plasmid types in *E. coli* isolates from patients and poultry meat in the Netherlands. Int. J. Food Microbiol. 167, 359-362.

Willinghan, E.M., Sander, J.E., Thayer, S.G., Wilson, J.L., 1996. Investigation of bacterial resistance to hatchery disinfectants. Avian Dis. 40, 510-515.

Table 1. All isolates included in the study. Source of isolates and resistance profiles used to distinguish between donor (D) and recipient strains (R) in the conjugation experiments are provided.

Isolate ID	Origin	Resistance profile
E. coli 1292 (IncK) (D)	Retail chicken meat	Ctx^R
E. coli 2798 (IncI1) (D)	Retail chicken meat	Ctx^R
E. coli 1553 (R)	Retail chicken meat	Nal^R
E. coli 6154 (R)	Retail chicken meat	Nal^R
E. coli 706 (R)	Retail chicken meat	Nal^R
E. coli 7079 (R)	Retail chicken meat	Nal^R
E. coli 3064-5 (R)	Faecal flora of healthy broiler	Nal^R
E. coli 6927-5 (R)	Faecal flora of healthy broiler	Nal^R
E. coli 1268 (R)	Faecal flora of healthy broiler	Nal^R
E. coli 1450 (R)	Faecal flora of healthy broiler	Nal^R
E. coli 1667 (R)	Faecal flora of healthy broiler	Nal^R
E. coli 2362 (R)	Faecal flora of healthy broiler	Nal^R
E. coli 4922 (R)	Faecal flora of healthy broiler	Nal^R
E. coli 5792 (R)	Faecal flora of healthy broiler	Nal^R
E. coli 3064-2 (R)	Faecal flora of healthy broiler	Nal^R
E. coli 4064-1 (R)	Faecal flora of healthy broiler	Nal^R
Serratia marcescens 2336 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3297 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3298 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3299 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3300 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3301 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3302 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3303 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3304 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3305 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3306 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3307 (R)	Disinfecting footbath	Rif^R
Serratia marcescens 3309 (R)	Disinfecting footbath	Rif^R

Serratia sp. 3612 (R)	Slaughterhouse	Rif^R
Serratia liquefaciens 5676 (R)	Retail chicken meat	Rif^R
Serratia proteamaculans 5682 (R)	Retail chicken meat	Rif^R
Serratia proteamaculans 5685 (R)	Retail chicken meat	Rif^R

MIC=minimum inhibitory concentration, Ctx^R=cefotaxime resistant, Nal^R= nalidixic acid resistant, Rif^R=rifampicin resistant

Table 2. Isolates included in mating pairs selected for conjugation experiments on agar, in broth and in biofilm. Source of isolates and resistance profiles used to distinguish between donor (D) and recipient strains (R) in the conjugation experiments are provided.

Isolate ID	Origin	Resistance profile
E. coli 1292 (IncK) (D)	Retail chicken meat	Ctx^R (MIC>2 mg/L)
E. coli 2798 (IncI1) (D)	Retail chicken meat	Ctx^R (MIC>2 mg/L)
E. coli 6927-5 (R)	Faecal flora of healthy broiler	Nal^R (MIC>128 mg/L)
Serratia marcescens 3306 (D and R)	Disinfecting footbath	Rif^R (MIC=16 mg/L)
Serratia marcescens 3307 (D and R)	Disinfecting footbath	Rif^R (MIC=16 mg/L)
Serratia proteamaculans 5685 (R)	Retail chicken meat	Rif^R (MIC=100 mg/L)

MIC=minimum inhibitory concentration, Ctx^R=cefotaxime resistant, Nal^R= nalidixic acid resistant, Rif^R=rifampicin resistant

Table 3. Overview of results from conjugation experiments performed by colony mating and broth mating. Confirmed transfer of plasmid carrying *bla*$_{CMY-2}$ is indicated by "+", while "-" indicates no observed transfer of *bla*$_{CMY-2}$-carrying plasmid.

Mating pair (donor → recipient)	Time	25°C		30/37°C[a]	
		Colony mating	Broth mating	Colony mating	Broth mating
E. coli 1292 (IncK)→	4 h			+	+
E. coli 6927-5	24 h	+	+	+	+
	48 h	+	+	+	+
E. coli 2798 (IncI1)→	4 h			+	+
E. coli 6927-5	24 h	-	+	+	+
	48 h	-	+	+	+
E. coli 1292 (IncK)→	4 h			-	+
S. marcescens 3306	24 h	+	-	-	+
	48 h	-	-	-	+
E. coli 1292 (IncK)→	4 h			+	+
S. marcescens 3307	24 h	+	+	+	+
	48 h	+	+	+	+
S. marcescens 3306	4 h			+	+
transconjugant (IncK)→	24 h	+	+	+	+
E. coli 6927-5	48 h	+	+	+	+
S. marcescens 3307	4 h			+	+
transconjugant (IncK) →	24 h	+	+	+	+
E. coli 6927-5	48 h	+	+	+	+
S. marcescens 3307	4 h			+	+
transconjugant (IncK)→	24 h	-	-	+	+
S. proteamaculans 5685	48 h	-	+	+	+

[a]30°C was applied for all matings involving *Serratia* spp, while 37°C was applied for matings involving *E. coli* only.

Table 4. Overview of maximum transfer frequencies for different mating pairs and incubation times for conjugation experiments in biofilm. The transfer frequencies are calculated as total number of transconjugants (T) divided by total number of recipients (R).

Mating pair (donor → recipient)	Time	Transfer frequency (T/R), 25°C	Transfer frequency (T/R), 30/37°C [a]
E. coli 1292 (IncK)→	4 h		NTD
E. coli 6927-5	24 h	$3x10^{-6}$	$5x10^{-4}$
	48 h	$3x10^{-6}$	$8x10^{-2}$
E. coli 2798 (IncI1)→	4 h		$4x10^{-5}$
E. coli 6927-5	24 h	NTD	$4x10^{-3}$
	48 h	NTD	$2x10^{-2}$
E. coli 1292 (IncK)→	4 h		NTD
S. marcescens 3306	24 h	NTD	NTD
	48 h	NTD	NTD
E. coli 1292 (IncK)→	4 h		NTD
S. marcescens 3307	24 h	NTD	NTD
	48 h	NTD	$7x10^{-6}$
S. marcescens 3306	4 h		$9x10^{-6}$
transconjugant (IncK)→	24 h	$2x10^{-6}$	$2x10^{-5}$
E. coli 6927-5	48 h	NTD	$3x10^{-4}$
S. marcescens 3307	4 h		$1x10^{-5}$
transconjugant (IncK) →	24 h	$2x10^{-5}$	$6x10^{-4}$
E. coli 6927-5	48 h	$9x10^{-5}$	$2x10^{-3}$
S. marcescens 3307	4 h		NTD
transconjugant (IncK)→	24 h	NTD	NTD
S. proteamaculans 5685	48 h	NTD	$5x10^{-7}$

[a]30°C was applied for all matings involving *Serratia* spp, while 37°C was applied for matings involving *E. coli* only. NTD: No transfer detected.

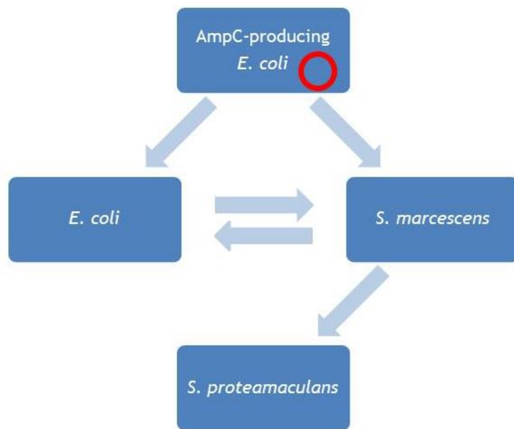


Figure 1. Schematic overview of confirmed routes of conjugative transfer for the pNVI1292/IncK plasmid harbouring *bla*$_{CMY-2}$ commonly found in *E. coli* resistant to extended-spectrum cephalosporins isolated from retail chicken meat in Norway in 2012 and 2014. The red circle symbolizes the IncK plasmid in the initial *E. coli* host. The arrows indicate the routes of transfer confirmed in this study.

1 **Table 5.** Minimum inhibitory concentrations (MICs) of beta-lactam antimicrobials for recipient strains before and after acquisition of
2 pNVI1292/IncK or pNVI2798/IncI1 plasmids.

Recipient strain (plasmid)	Antimicrobial (epidemiological cut-off value)									
	FOX (8 mg/L)	ETP (0.06 mg/L)	IMI (0.5 mg/L)	MERO (0.12 mg/L)	TAZ (0.5 mg/L)	FEP (0.12 mg/L)	F/C (0.25/4 mg/L)	T/C (0.5/4 mg/L)	FOT (0.25 mg/L)	TRM (NA)
E. coli 6927-5	4	≤0.015	≤0.12	≤0.03	≤0.25	≤0.06	≤0.06/4	≤0.012/4	≤0.25	4
E. coli 6927-5 (IncK)	32	0.03	≤ 0.12	≤ 0.03	8	0.12	4/4	4/4	4	4
E. coli 6927-5 (IncI1)	64	0.03	≤ 0.12	≤ 0.03	16	0.25	4/4	8/4	8	4
S. marcescens 3306	16	≤0.015	0.5	0.06	≤0.25	≤0.06	≤0.06/4	0.5/4	≤0.25	8
S. marcescens 3306 (IncK)	32	0.03	0.5	0.06	8	0.25	4/4	8/4	4	8
S. marcescens 3307	8	≤0.015	0.5	≤0.03	≤0.25	≤0.06	≤0.06/4	≤0.12/4	≤0.25	8
S. marcescens 3307 (IncK)	32	0.06	0.5	0.6	8	0.25	8/4	8/4	4	8
S. proteamaculans 5685	4	≤0.015	0.25	≤0.03	≤0.25	≤0.06	≤0.06/4	≤0.12/4	≤0.25	4
S. proteamaculans 5685 (IncK)	64	≤ 0.015	0.25	≤ 0.03	2	0.12	4/4	1/4	8	4

3 FOX: cefoxitin, ETP: ertapenem, IMI: imipenem, MERO: meropenem, TAZ: ceftazidime, FEP: cefepim, F/C: cefotaxime/clavulanic acid, T/C: ceftazidime/
4 clavulanic acid, FOT: cefotaxime, TRM: temocilin