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Department of Food Safety and Infection Biology

Phenotypic characterization of extended spectrum cephalosporin resistant *Escherichia coli*

May Linn Buberg

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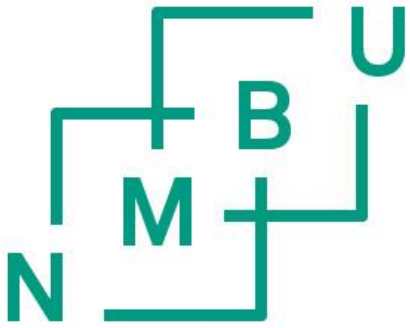
by May Linn Buberg

Supervisors: Ingun Lund Witsø, Yngvild Wasteson



Department of Food Safety and Infection Biology

Faculty of Veterinary Medicine

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Acknowledgements

The work presented in this thesis was carried out at the Norwegian University of Life Sciences at the Department for Food Safety and Infection Biology in 2019. This year has been very rewarding, providing both professional experience and personal growth. I have had the joy of learning a broad range of laboratory protocols, lots of lab-experience, and great learned how to think and evaluate methods and literature critically.

I wish to express my sincere gratitude to my supervisors Ingun and Yngvild for creative ideas, patience and always being available to answer questions and providing their endless knowledge at my disposal. Furthermore, I would like to thank Tina, Toril, Helge, Anki and Solveig for excellent technical assistance.

Finally, I would like to express my sincere gratitude to my family and friends. To my hero, my wonderful and patient husband Edvind and our loving friend Marie.

Oslo, November 2019

May Linn Buberg

List of abbreviations

APEC: Avian pathogenic *E. coli*

AMR: Antimicrobial Resistance

ESBL: Extended spectrum beta-lactamase

ESC: Extended spectrum cephalosporin

ExPEC: Extraintestinal pathogenic *E. coli*

HIS: Heat inactivated serum

HS: Human serum

LB broth: Luria-Bertani broth

MLST: Multi locus sequence typing

MOI: Multiplicity of infection

NMBU: Norwegian University of Life Sciences

NORM-VET: Norsk overvåkningssystem for antibiotikaresistens hos mikrober i veterinærmedisin

PBS: Peptone buffered saline

UPEC: Uropathogenic *E. coli*

UTI: Urinary tract infection

VF: Virulence factors

WHO: World health organization

WP: Work package

Sammendrag Norsk

Mat-produserende dyr og produktene fra disse har blitt foreslått som mulige kilder for human kolonisering av utvidet spektrum cefalosporin resistente (ESC-resistente) *E. coli*, og kylling er regnet som den mest sannsynlige kilden til ESC-resistent ekstraintestinal patogen *E. coli* (ExPEC) isolert fra humane infeksjoner. Kyllingkjøtt har også blitt ansett som en kilde for uropatogene *E. coli* (UPEC) i stand til å forårsake urinveisinfeksjoner (UVI) hos mennesker. Infeksjoner med antibiotikaresistente bakterier har begrensede behandlingsmuligheter og har derfor alvorlige konsekvenser både for dyre- og humanhelse. Målet med denne prosjektoppgaven var å karakterisere ESC-resistente *E. coli* fra kyllingkjøtt med tanke på utrykk av fenotypiske virulensegenskaper. Atten isolater ble valgt ut blant 141 ESC-resistente *E. coli* samlet inn gjennom NORM-VET-programmet mellom 2012 og 2016. Disse isolatene ble testet i ulike fenotypiske virulenstester; motilitet, biofilmdannelse, overlevelse i humant serum, vekst i urin, produksjon av coliciner og evne til å invadere og feste seg til eukaryote celler. Resultatene bekrefter at *E. coli* isolater forekommer med en høy diversitet. Selv om alle isolatene har den samme resistensprofilen utrykker de en stor grad av varierende fenotypiske virulensfaktorer. Basert på testene i denne studien kan vi konkludere med at *E. coli* fra kyllingkjøtt i Norge er en lite sannsynlig kilde for UPEC med potensiale for å forårsake UVI.

Introduction

The following gives an introduction to and a background for the topics covered by my thesis.

Background

The work presented in this thesis has been a part of the project NoResist at the Norwegian University of Life Sciences, in cooperation with the Norwegian Veterinary Institute, Nofima and Norwegian Institute of Public Health. In addition to myself, two postdoc students have been assigned to the main project. The aim of NoResist is to "Obtain knowledge on persistence and spread of antimicrobial resistance in the Norwegian food production chain which can be used to prevent, reduce or inhibit such resistance", and is divided into 5 work packages, where NMBU is responsible for WP2. Objectives are given under "Aims".

Norwegian chicken production chain

Broiler production amounted 28% of total amount of meat produced in Norway in 2017 (1). Norwegian chickens live inside insulated and heated houses. They move around freely on bedding consisting of wood shavings and have free access to food and water. The lightening is adapted to the animal`s needs, assuring that they have adequate amounts of light during the day. Normally they are slaughtered between 28-32 days of age and have a living weight of approximately 1.2 kg. They are not fed any growth promotors or antimicrobials. In 2014, the industry decided to stop using the coccidiostatic Narasin, and replaced it with a vaccine by the end of 2016. Chicken are now only fed commercially produced pelleted feed consisting only of essential nutrients (2).

Antimicrobial resistance

Antimicrobial resistance (AMR) is defined as "the ability of a microorganism (like bacteria, viruses, and some parasites) to stop an antimicrobial agent (such as antibiotics, antivirals and antimalarials) from working against it. As a result, standard treatment become ineffective, infections persist and may spread to others." (3). The spread of AMR has become a threat against human and animal health (4, 5). Part of this resistance has its origin within the agriculture sector, and dissemination of resistant bacteria from the food production chains may be one out of several routes in which consumers can be exposed to AMR bacteria (6-10). Several risk factors for the

emergence of AMR have been discovered, such as overuse and misuse, but there are still a lot of unanswered questions that needs to be addressed.

NORM-VET detected in 2012 cephalosporin resistant *Escherichia coli* in 43% of the Norwegian broiler flocks. In addition, 32.2% of *E. coli* from retail chicken meat were categorized as cephalosporin resistant (11), The NORM-VET findings were surprising as the use of antimicrobial agents in the poultry production in Norway is limited, and among the lowest in Europe (11, 12).

The most important mediator of betalactam resistance is production of betalactamases, which are enzymes that inactivate betalactam antibiotics (13). These are commonly referred to as extended spectrum betalacatamases (ESBLs). A common group of antibiotics among the betalactams are the cephalosporins, and resistance against these, a subgroup under the ESBL-resistance, are extended spectrum cephalosporin (ESC) resistance. Third and fourth generation cephalosporin have been defined as critically important antimicrobials by the WHO (14), however, extended spectrum beta-lactamase (ESBL) producing bacteria have previously been isolated from a variety of animal species in different European countries (15-19). In 2015, the Norwegian Scientific Committee for Food and Environment concluded in their "Assessment of antimicrobial resistance in the food chains in Norway" that the probability of human exposure of ESBL-producing *Enterobacteriaceae*, and their respective corresponding genes, from live poultry and poultry meat was considered non-negligible (20). Such exposure of resistant bacteria may result in consumers becoming carriers of resistant strains of *E. coli,* if these bacteria colonize the human gut (7, 21-23). In the case that they at later occasions cause disease, their resistance characteristics may lead to failure of treatment and increased mortality (24, 25).

Virulence factors

Virulence factors are essential *in vivo* for the invading organism to colonize, invade host tissue, adapt to various environments in the host, subvert host functions and overcome the defences of the host (26). Both structural and secretory features are included in the UPEC virulence potential (27). Examples of structural virulence factors include adhesins, pili, flagella and other surface components, while the secreted components responsible for cell-damage are toxins and enzymes, which often are associated with outer-membrane vesicles (28). UPEC surface factors ensure colonization to the urethra and invasion of the host cells in the bladder to avoid the immune system

(29, 30). Another important feature to survive in the urinary tract is the production of biofilm. Biofilms are communities of bacteria adherent to each other and/or to a surface, this provides protection from shear forces in the urinary tract (31). Colicins are examples of secretory components that are toxic to other coliform bacteria providing competitive advantages (32). As the environment in the urinary tract offers limited accessibility to iron, iron acquisition is also an important feature to become a successful pathogen (27). Virulence factors investigated specific in this work include ability to invade and adhere to eukaryote cells, biofilm formation, motility, production of fimbriae and the ability to resist serum killing.

E. coli and urinary tract infections

Among the *Enterobacteriaceae, E. coli* is a common commensal, opportunist and pathogen and a common cause of a variety of diseases inside and outside the gastrointestinal tract (33). *E. coli* that cause infections outside of the intestinal tract is commonly referred to as ExPEC (Extra intestinal pathogenic *E. coli*), and incorporates variants such as avian pathogenic *E. coli* (APEC) and uropathogenic *E. coli* (UPEC) (34). To be considered an ExPEC a substantial amount of virulence factors has to be present, and to cause disease a compromised host immune system is required in addition (35). During a urinary tract infection the bacteria start by colonizing the distal parts of the urethra, thereby ascending into the bladder, adhere to the surface of the bladder, forming biofilm and then invade and replicate within the hosts cells (27). Gram-negative *Enterobacteriaceae* are the most common pathogens isolated from UTI, between 70-95% of isolated UTI agents has been shown to be *E. coli,* predominately UPEC (36, 37). Phylogenetic analyses have divided *E. coli* into four different phylogenetic groups; A, B1, B2 and D, respectively (38). UPEC strains which routinely causes infections belong mainly to phylogroups B2 and D (39). Clinical symptoms vary from a harmless bacteriuria to serious urosepsis (40). The human intestinal tract has been believed to be the reservoir of UPEC (41). However, there is no consensus on how these bacteria end up colonizing the host's intestinal flora. Wiles et al. described in 2008 community-onset clonal outbreaks of UTIs, possibly due to consumption of food contaminated with UPEC, in addition to sexual transmission (42). Other work highlights the genetic similarities between APEC and UPEC and suggests poultry meat as a possible source of origin (43-45).

Knowledge gaps

Jakobsen et al. called in 2010 for investigation of potential reservoirs of UPEC. Recent reports have suggested that poultry is a likely source of UPEC isolates, however, the likelihood of these isolates to cause disease has not been studied in detail. Other studies have focused on the genetic linkage between human and avian *E. coli* isolates, but little information is provided regarding the transmission route, and the actual expressed virulence potential (28).

We wanted to investigate the potential of *E. coli* with origin from the Norwegian poultry population to cause urinary tract infections in humans, with transmission through the food production chain.

Hypothesis

We hypothesized that ESC-resistant *E. coli* from retail chicken meat have the potential to become urinary tract pathogens in humans. We therefore performed phenotypic virulence testing on 18 ESC-resistant *E. coli* collected from retail chicken meat to evaluate virulence related characteristics that increase the potential of these strains to cause UTIs in humans.

My contributions

My contributions included discussing ideas and plans for the study, performing the phenotypical testing in the laboratory, analysis and presentation of results, in addition to writing.

Other contributors

Samples were collected through NORM-VET. DNA extraction, whole genome sequencing and *in silico* analysis of sequences was performed by our collaborators at the Veterinary Institute in Norway; Solveig Sølverød Mo, Camilla Sekse and Marianne Sunde. Ingun Lund Witsø has contributed in the establishment of protocols for phenotypic testing, included writing together with Yngvild Wasteson.

Aims

The work performed in this thesis has been part of a larger study, where a manuscript is currently in preparation. This has been performed under the project «NoResist – Combating antimicrobial resistance in the Norwegian food production chain». NoResist address the need for more knowledge based countermeasures against the development and dissemination of antimicrobial resistance in Norwegian food production chain (46). The focus has been on the poultry production chain, which internationally has been associated with a challenge of AMR-bacteria.

The aim of our study was to characterize ESC-resistant *E. coli* from retail chicken meat regarding occurrence of UPEC-associated virulence genes. This was accomplished by phenotypic virulence testing on a selection of isolates to evaluate possible potential of these strains to cause UTIs in humans.

List of publications

Population structure and zoonotic potential of extended-spectrum cephalosporin-resistant *Escherichia coli* from retail chicken meat

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Manuscript in preparation – November 2019

# These authors contributed equally to this work

Summary of the paper

Food-producing animals and their products have been suggested as possible sources for human acquisition of ESC-resistant *E. coli* (47-49), and poultry are considered the most probable source of ESC-resistant extra-intestinal pathogenic *E. coli* (ExPEC) obtained from food (50). Chicken meat has also been regarded as a source of uropathogenic *E. coli* (UPEC) capable of causing urinary tract infections (UTIs) in humans (51-53). Infections caused by AMR bacteria have limited treatment options and can therefore have severe consequences for both human and animal health (54, 55). The aim of our study was to characterize ESC-resistant *E. coli* from retail chicken meat regarding occurrence of UPEC-associated virulence genes. Also, phenotypic virulence testing was performed on a selection of 18 isolates to evaluate possible potential of these strains to cause UTIs in humans. Methods included, *in silico* typing, evaluation of motility, biofilm formation, ability to resist serum killing, growth in urine, colicin production and cell-adhesion, and -invasion. Furthermore, the population structure of all ESC-resistant *E. coli* isolated from retail chicken meat from 2012-2016 in the Norwegian monitoring programme on AMR in the veterinary sector (NORM-VET) was explored.

Materials and methods

This section gives a summary of the sample materials and methods used in the study. Methodological considerations will be addressed in the discussion.

Materials

Bacterial isolates

All ESC-resistant *E. coli* isolated from retail chicken meat in Norway in 2012 (n=66), 2014 (n=58) and 2016 (n=17) were included in the selection population (total n=141). The isolates were collected in the Norwegian surveillance programme for antimicrobial resistance in the veterinary sector (NORM/NORM-VET), and were known to carry the pAmpC gene *bla*$_{\text{CMY-2}}$ (56-58). Eighteen of these isolates were selected for phenotypical virulence testing and are presented in Table 1. The selection was made on the background of genetic information about the isolates provided by our collaborators at the Norwegian Veterinary Institute (Table 1). Our goal was to select a collection of isolates that to a high degree represents the genetic diversity in the population. A phylogenetic tree was computed in PHYLOViZ© to confirm the representability of our sample (Figure 5). *E. coli* CFT073, a well characterized UPEC was used as a positive control in some of the tests performed, while *E. coli* DH5α, and *E. coli* E377 were used as negative controls, respectively.

Table 1: Sample isolates and their respective phylogroup and sequence type.

ID	Phylogroup	MLST
2012-01-3586	B2	131
2014-01-3678	D	117
2016-22-0832	B1	442
2014-01-5656	A	10
2014-01-7037	B2	355
2016-22-0220	B2	429
2014-01-1336	A	1594
2012-01-1295	D	38
2012-01-0707	D	38
2014-01-3680	D	1158
2014-01-4991	D	57
2014-01-5104	D	115
2012-01-0771	D	69
2014-01-7011	D	1944
2014-01-4267	A	191
2012-01-1292	D	38
2012-01-2798	A	3249
2016-22-1061	A	2040

Laboratory methods

Agglutination *FimH* / Type 1 fimbriae detection

The ability to express a D-mannose-binding phenotype, characteristic for functional Type 1-fimbriae, was assayed by the ability to agglutinate yeast cells (*Saccharomyces cerevisiae)*. Each isolate was inoculated in LB broth and incubated over night at 37 °C. 1mL overnight culture was centrifuged (3000 x g, 5min) and the pellet was resuspended in 100 µL PBS. 10 µL of the bacteria suspension was mixed with 10 µL yeast cells (5mg/mL, dissolved in PBS) with and without 1% D-mannose solution on a microscopy slide and agglutination was investigated.

Motility

One colony from fresh blood agar plate was perpendicularly inoculated into a tube containing 5 mL semi-solid LB agar, 0.03%, 0.2% and 0.7% respectively, and incubated for 24 hours at 37 °C. Motile bacteria appear as a "cloud" of bacterial growth in the agar around the inoculation spot (Figure 1).

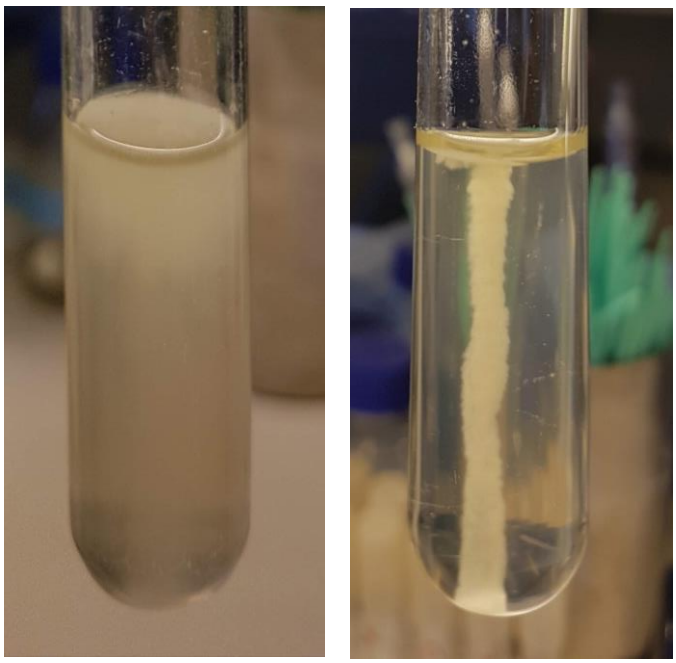

Figure 1: Ability to move in 0.2% agar (left), and inability to move in 0.2% agar (right).

Biofilm

Overnight culture in LB broth was diluted 1:200 in LB without NaCl and 200 µL was added to a 96 well microtiter plate (Greiner, Sigma-Aldrich, Germany). Wells containing uninoculated media were used as negative control. The plates were incubated at 37 ºC for 24 hours and at 20 ºC for 48 hours. After incubation, the plates were washed three times with PBS to remove planktonic cells, adhered bacteria were stained with 0.1% crystal violet for 15 min followed by washing three times with PBS. 200 µL ethanol was added to each well and OD_{600} was measured using Tecan platereader. The assay was performed in three parallels for each isolate, and each experiment was repeated three times.

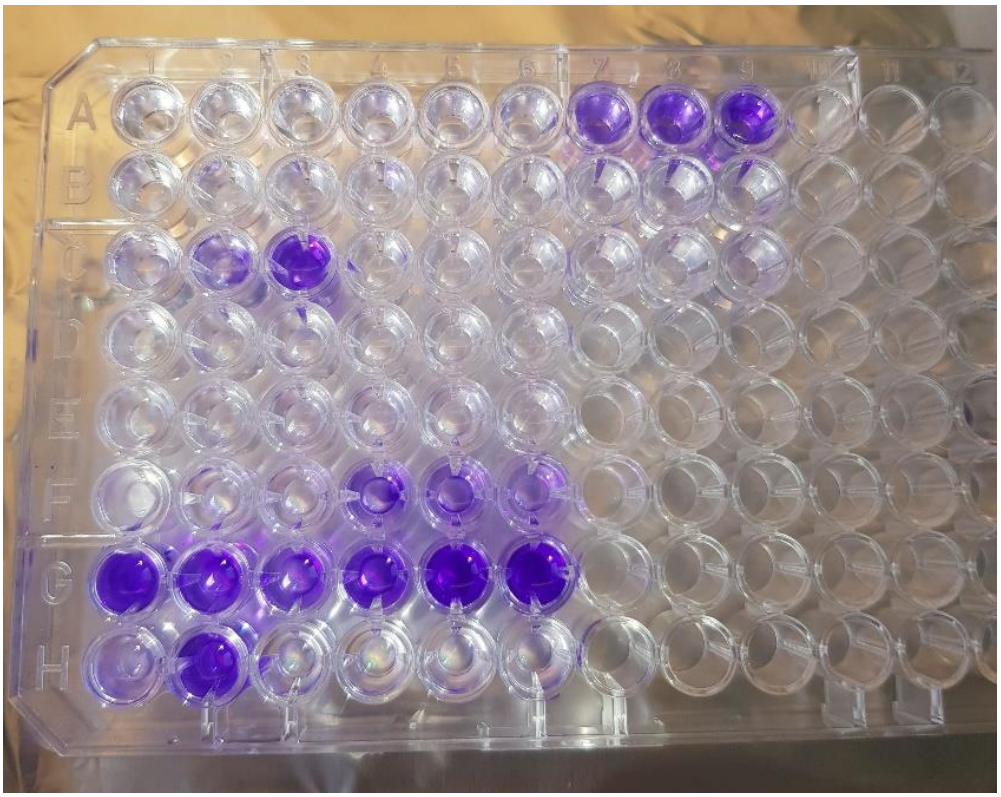

Figure 2: Stained biofilm dissolved in ethanol before OD_{600} measurement.

Growth curves

Overnight cultures of each isolate were diluted 1:1000 in fresh LB broth. 200 µL of each isolate was transferred to a 96 well microtiter plate (Greiner, Sigma-Aldrich, Germany) and incubated at 37 °C in Tecan platereader. OD_{600} was measured every 10 min for 24 hours. Growth curves were repeated three times for each isolate. In addition, the same procedure was performed in sterile-filtered human urine collected from healthy female volunteers (pH = 6.5).

Figure 3: Microtiterplate (left) after 24h incubation in Tecan platereader for growth curves in sterile-filtered urine (right).

Serum resistance

To investigate the ability to resist serum-killing, 250 µL overnight cultures were added to 750 µL 20% human serum (HS, diluted in PBS) or heat inactivated serum (HIS, control for comparison). Serum was inactivated by heating (56 ºC for 60 min). Bacteria with serum was incubated in room-temperature, and samples were taken every hour for three hours. Samples were diluted and plated on LB agar plates. The plates were incubated for 24 hours at 37 ºC and colonies counted. The colonies from HS samples were calculated as a percentage of the HIS samples. The results were categorized as followed: < 1% - serum sensitive, > 90% - serum resistant, and all in between was considered as intermediate.

Cell adhesion- and invasion

Adhesion to and invasion of cells were tested in Vero monkey kidney epithelial cells grown at 37 ºC. The cells were grown to 80% confluency. 200 µL of cells in fresh minimal essential medium (DMEM (Gibco™ 11568876) with added 10% Fetal Bovine serum (Gibco™ 10270106) and 1 mL Penicillin/Streptomycin solution to 100 mL DMEM) was transferred to a microtiter plate (Greiner, Sigma-Aldrich, Germany) in duplicate at a concentration of approximately $5*10^4$ cells per mL (counted in Countess), and grown to 80% confluence. Overnight culture of bacteria was diluted 1:100 in fresh LB broth to OD = 0.1. 1 mL was centrifuged (500 x g, 5 min) and the pellet was resuspended in 500 µL fresh DMEM cell-medium without antibiotics. The bacterial suspension was diluted 1:100 and 50 µL was added to the confluent Vero cells with fresh cell medium, equivalent to approximately 30 bacteria per cell (MOI 30:1). The plates were centrifuged (100 x g, 2 min) to increase contact between bacteria and cells and incubated for 2 hours at 37 ºC.

To address adhesion to cells, the cells were washed three times with PBS to remove non-adherent bacteria and lysed with 30 µL 1% Triton X for 10 min. The lysates were diluted in PBS and plated on LB agar (10^0 and 10^{-1}, respectively). Bacterial invasion was assessed as described for the adhesion assay, however 200 µL of fresh medium with antibiotics (0.1 mg/mL Gentamicin, and 20 mg/mL Nalidixic acid) was added to the cells and incubated for 2 hours to kill adherent bacteria. The cells were lysed and plated as described before.

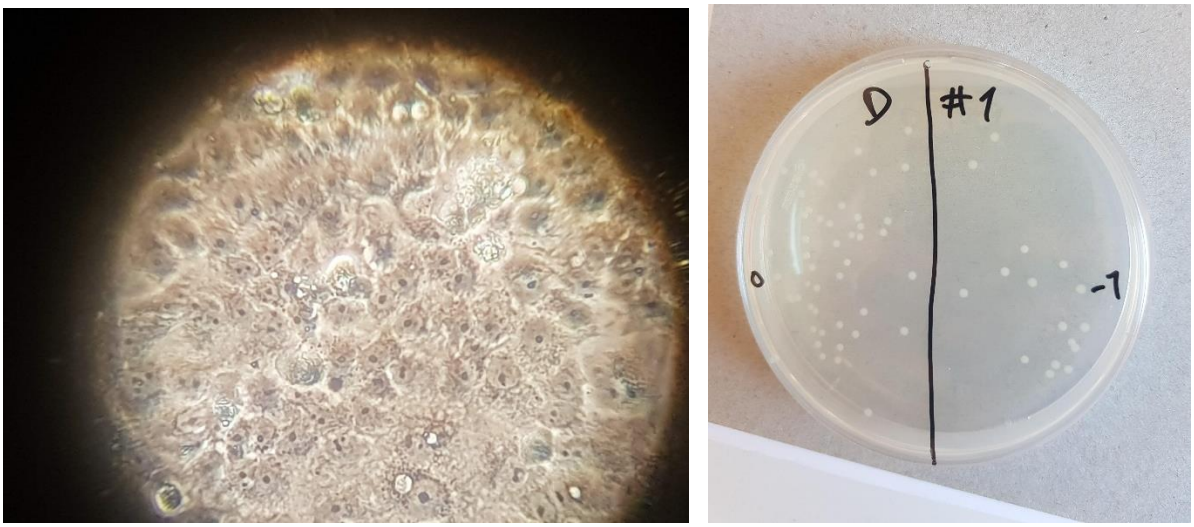

Figure 4: Vero monkey kidney cells in the microscope (left), and plated cell associated bacteria (right) on LB-agar plate at dilutions $10^0$and 10^{-1}.

Colicin production

Colicin production as described by Calcuttawala et al. (32). Briefly, 100 µL of *E. coli* DH5α overnight culture in LB broth was spread on LB agar plates and left to dry for approximately 10 min in room temperature. 1 mL overnight culture of the respective isolates were centrifuged at 13 000 x g for 10 min and the supernatants were sterile filtered through a 0.22-µm Minisart® syringe filter (Sartorius Stedim Biotech GmbH, Germany). 10 µL of the filtrate was spot-inoculated on the dried LB agar plates with *E. coli* DH5α and incubated in 37 °C for 24 hours. Production of colicin was determined by the presence of an inhibition zone around the place of inoculation.

Statistical methods

As this was considered a descriptive study, extensive statistics were not performed. Standard deviations (SD) are provided in the figures for quantitative data. All experiments were performed in triplicate with three biological replicates.

Results

This section presents the results from each of the phenotypical tests.

Confirmation in PHYLOViZ© confirmed the representability of the selected isolates. Figure 5 demonstrates that the selected isolates are spread evenly throughout the population, belonging to different phylogroups and different sequence types, as presented in Table 1.

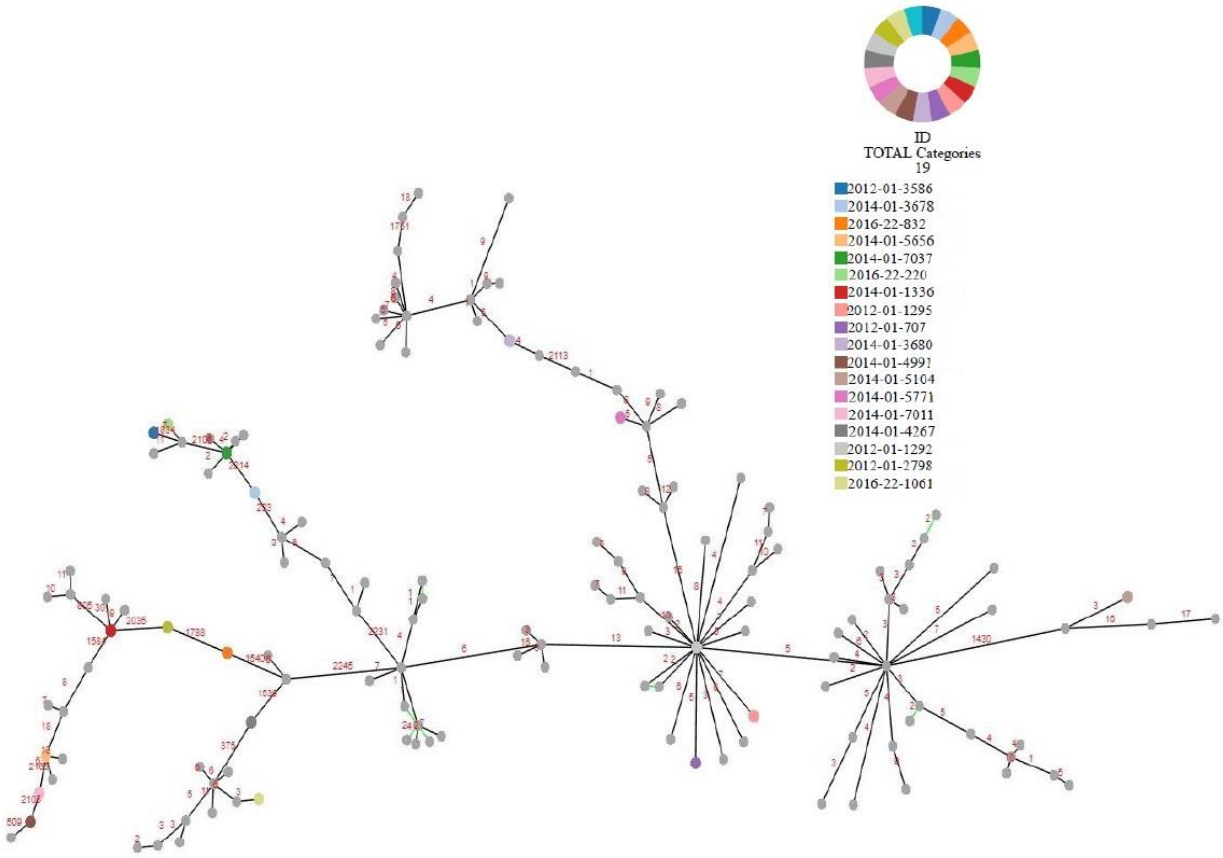

Figure 5: Sample selection. The chosen 18 isolates were selected from a total of 141 ESC-resistant *E. coli* collected from retail chicken meat in Norway between 2012 and 2016. This figure illustrates the genetical relationship of the population. Each dot represents an isolate, the sample of 18 isolates are marked with colors, red numbers demonstrates the number of alleles difference between each isolate.

Agglutination

Agglutination demonstrate expression of the Type 1-fimbriae and was evaluated as positive or negative. Seven of the 18 isolates tested were not able to agglutinate yeast and reported as negative. Results are presented in Table 1.

Table 2: Agglutination. Expression of Type 1-fimbriae. (+) indicates ability to agglutinate, while (-) indicates inability to agglutinate.

ID	Fimbriae
2012-01-3586	+
2014-01-3678	-
2016-22-0832	+
2014-01-5656	-
2014-01-7037	+
2016-22-0220	+
2014-01-1336	-
2012-01-1295	+
2012-01-0707	-
2014-01-3680	-
2014-01-4991	-
2014-01-5104	+
2012-01-0771	+
2014-01-7011	-
2014-01-4267	+
2012-01-1292	+
2012-01-2798	+
2016-22-1061	+

Biofilm

We observed that the formation of biofilm varied between the isolates and with varying temperatures and incubation periods. Figure 6 reports the mean OD_{600nm} for all replicates and demonstrate the different degrees of biofilm formation. 2012-01-0707 produced the strongest biofilm at 37 ºC, while 2016-22-1061 was the strongest at 20 ºC. Interestingly half of the sample population reported as poor biofilm formers under the given experimental conditions.

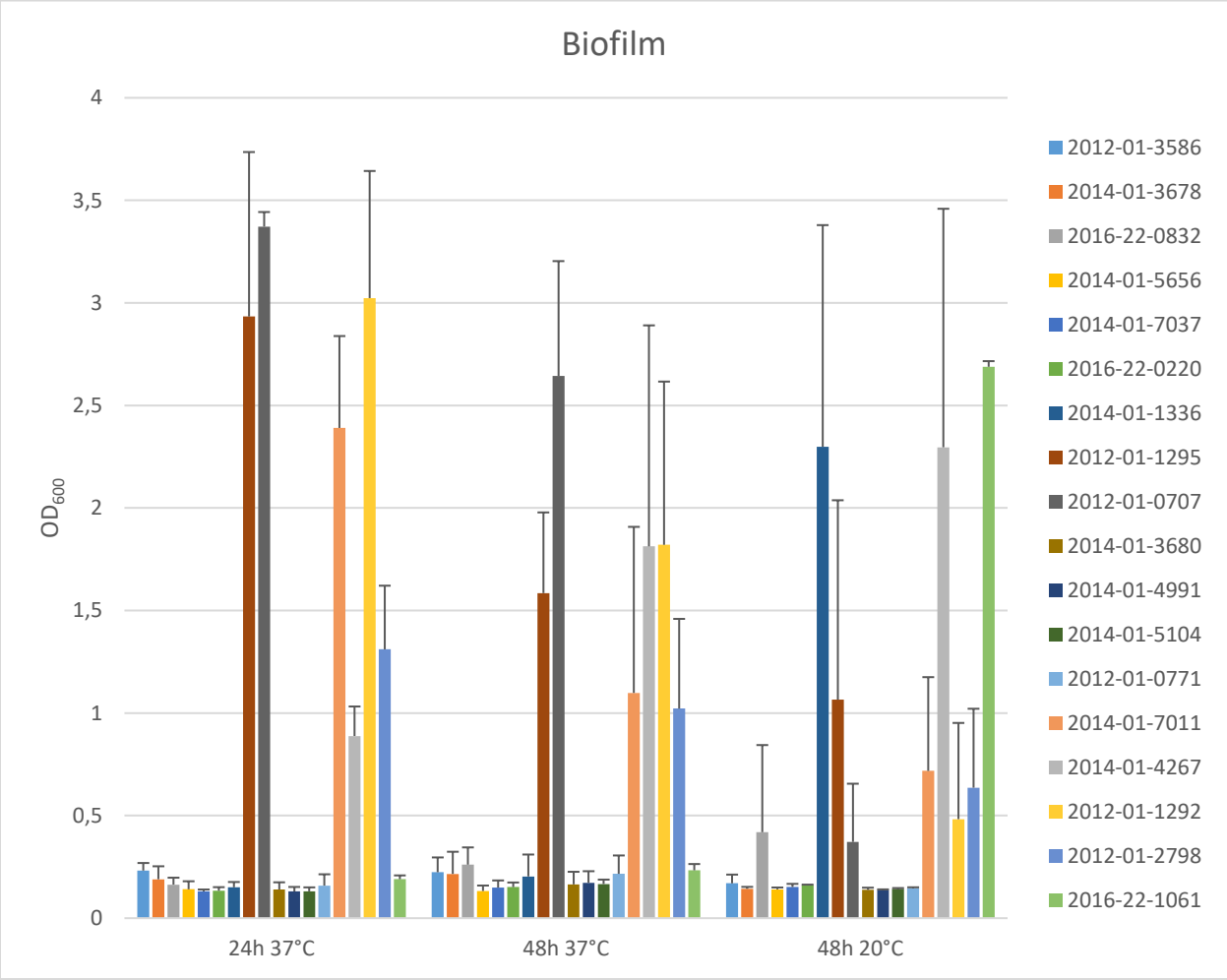

Figure 6: Biofilm formation. Ability to form biofilm after 24 h and 48 h at 37 ºC and 20 ºC. Errorbars indicate +SD.

Motility

Motility was evaluated as ability to move through semi-liquid LB agar. None of the isolates were able to move in 0.7% agar, while 8 of the isolates were non-motile in 0.2% agar, only one isolate was non-motile in 0.03% agar. Results are presented in Figure 7.

Figure 7: Motility in semi-liquid agar. Colored bars indicate ability to move in the given agar concentration. None of the isolates were able to move in 0.7% agar and is therefore not included in the figure.

Growth curves

All isolates grew better in regular LB broth than in urine. Curves for both growth in urine and in broth are reported for each isolate in Figure 8 and 9. Isolate 2016-22-1061 had the most rapid growth of the 18 isolates, both in urine and in LB.

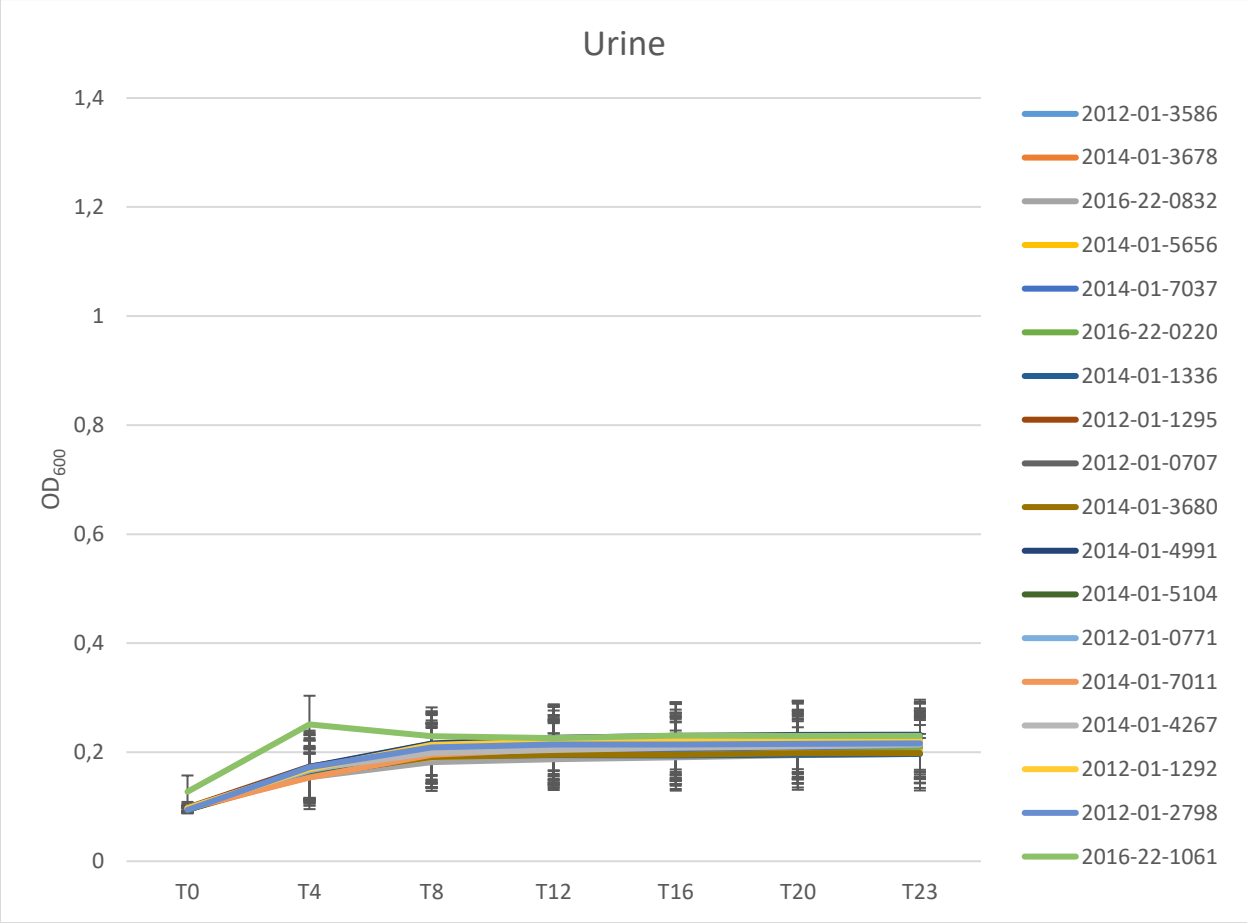

Figure 8. Growth curves in urine. Errorbars indicate +/- SD.

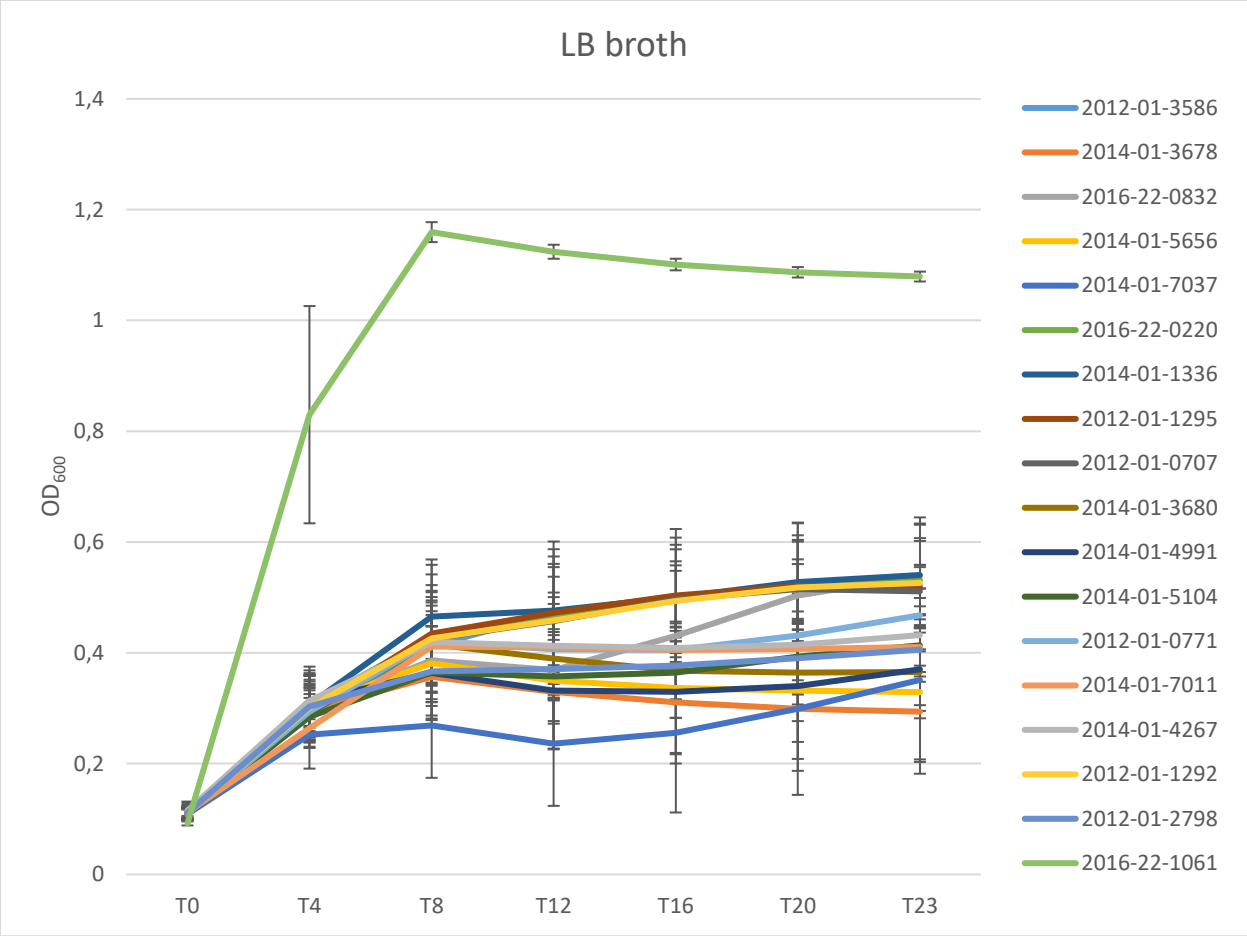

Figure 9: Growth curves in LB broth. Errorbars indicate +/- SD.

Invasion and adhesion

Overall the isolates showed a low capability of intracellular invasion, but better capabilities of cell adhesion. Results are presented in Figure 10. Data for isolate 2016-22-0220 is not included in the results. Concentrations of antibiotics used to kill adherent bacteria to evaluate number of invading bacteria were not sufficient for this isolate, even though the concentrations used exceeded the MIC values.

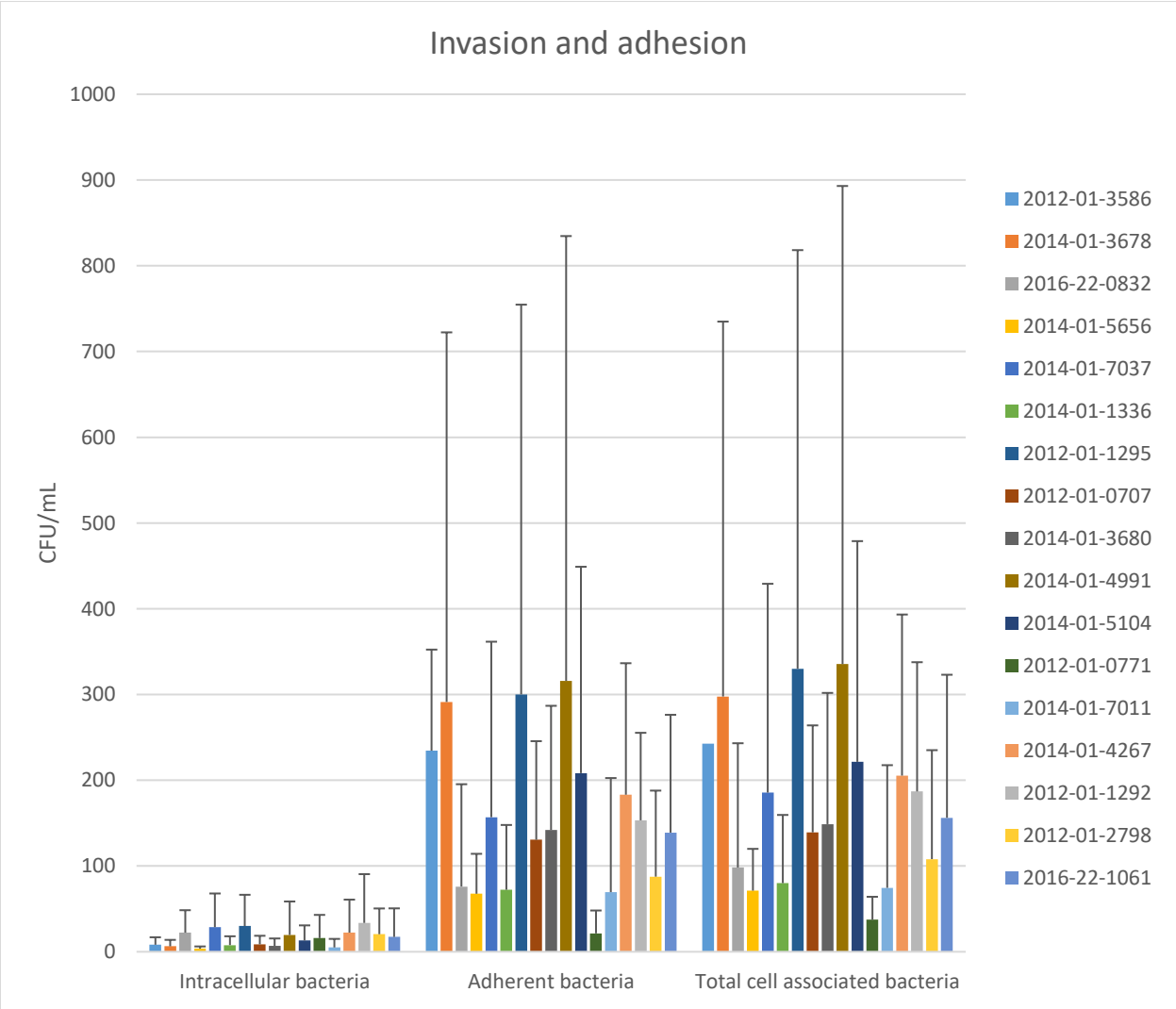

Figure 10: Invasion and adhesion to Vero cells. Errorbars indicate +SD

Colicin production

Among the 18 isolates, 13 produced unspecific colicins. Results are reported in Table 3.

Table 3: Colicin production. (+) indicates ability to produce colicins that inhibits growth of E. coli DH5α.

ID	Colicin
2012-01-3586	+
2014-01-3678	+
2016-22-0832	-
2014-01-5656	+
2014-01-7037	+
2016-22-0220	-
2014-01-1336	+
2012-01-1295	+
2012-01-0707	+
2014-01-3680	+
2014-01-4991	-
2014-01-5104	+
2012-01-0771	-
2014-01-7011	+
2014-01-4267	-
2012-01-1292	+
2012-01-2798	+
2016-22-1061	+

Serum resistance

None of the isolates were sensitive for killing in serum. Eight isolates were highly resistant, while the rest was characterized as intermediate. Results are presented in Figure 7.

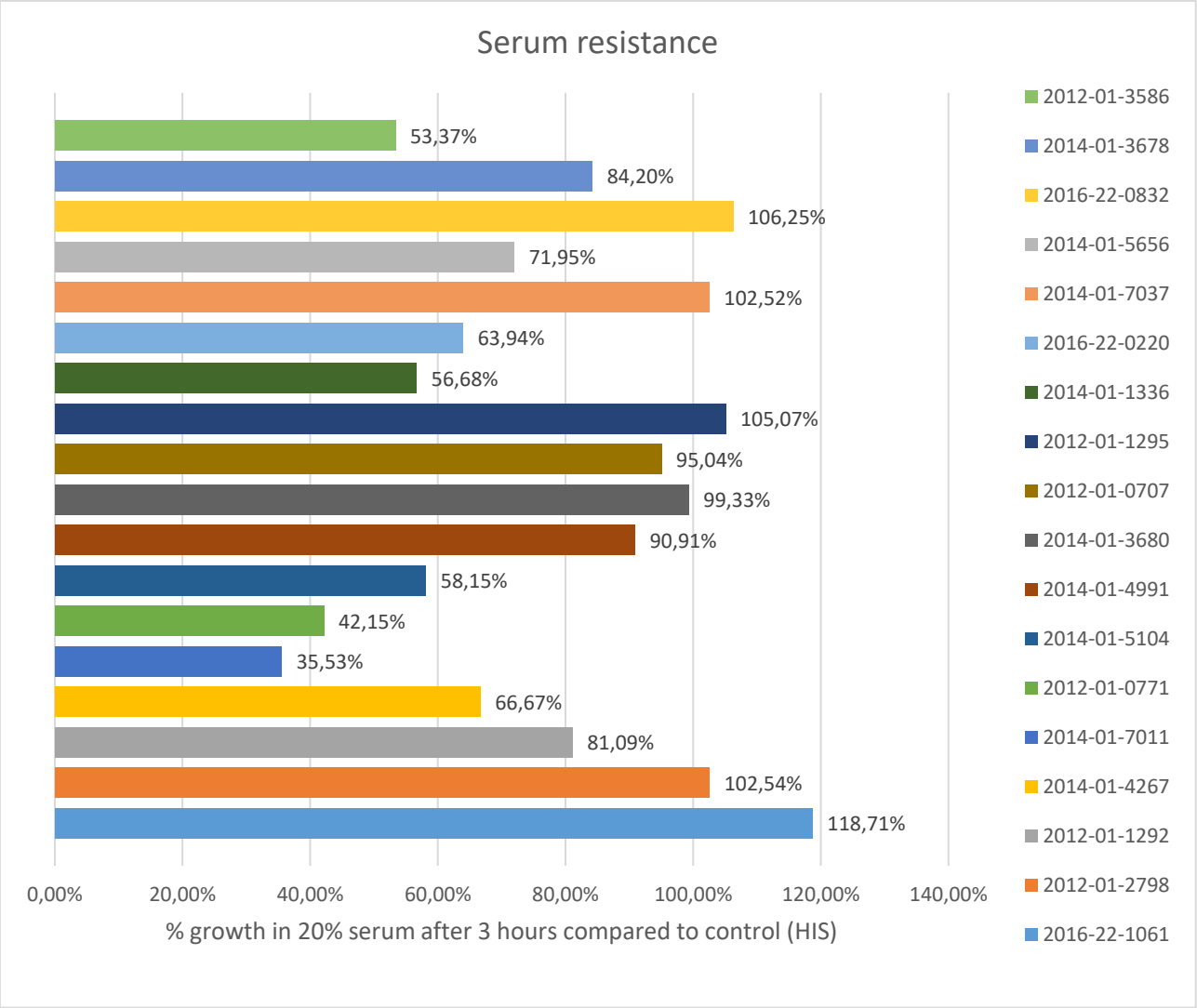

Figure 11: Ability to resist killing in 20% human serum. < 1% = serum sensitive, > 90% = serum resistant, all in between = intermediate.

Discussion

Summary of results

This work included a variety of phenotypical *in vitro* virulence assays, and the results presented display large differences between the respective isolates. All isolates are collected from the same reservoir, belong to the same species, and have the pAmpC gene $bla_{\text{CMY-2}}$, but have varying sequence type (ST) and include representatives from each of the four phylogroups. They had similar growth rates, with exception of isolate 2016-22-1061 that grows faster than the other isolates. Some of the isolates were able to agglutinate yeast, some produced colicins and some were serum resistant. None of the isolates were considered sensitive to serum killing. The ability to form biofilm under the given experimental conditions, in addition to adhesion to and invasion of Vero cells did also vary between the isolates. This high diversity of phenotypical virulence traits demonstrates that the potential for human UTI from colonizing of *E. coli* from poultry meat is highly dependent on the given strain colonizing.

When summarized all the virulence factors isolate 2016-22-1061 came out as the one with the highest potential for causing UTIs, as it expresses the Type 1-fimbriae, produces colicins, was the best survivor in human serum, and has the fastest growth. This isolate is distinctive from the other isolates as it belongs to ST2040, a sequence type that recently has been discovered in both livestock and in food products in Germany (59). Even though it belongs to phylogroup A which rarely has been described to cause extraintestinal infections, its recent broad dissemination is noteworthy.

Isolate 2014-01-7011 is likely the most harmless of the 18 isolates. It produced colicins, but was non-motile, even in 0.03% agar. It was the more sensitive to serum compared to the other isolates, did not express the Type 1- fimbriae, and had low production of biofilm in addition to a modest invasion and adhesion of epithelial cells.

Some of the results were surprising. As all the isolates held the *fimH* gene we expected all the isolates to be positive on the agglutination test. The presence of the *fimH* gene is only considered an indicator for the Type 1-fimbriae, and to be able to express a fully functional fimbriae, the genes encoding all the proteins in the fimbriae must be present. This has not been investigated in detail for these isolates and can explain why only some of the isolates are able to agglutinate.

Another surprising finding was that isolate 2012-01-3586, belonging both to phylogroup B2 and has ST131, which is the most common ExPEC described (60), appeared to be less virulent than expected. It produced both colicin, expressed the Type 1-fimbriae, and adhered to eukaryotic cells. However, it was among the most serum sensitive of the isolates, did not invade the cells and produced less biofilm compared to the other isolates.

One of the most important of the tests performed is the ability to resist serum killing. A complication of UTI is urosepsis, a serious event that requires immediate medical care to avoid a possible life-threatening situation (61). A large proportion of resistant *E. coli* causing blood stream infections in people may be derived from food sources (62), and the fact that none of the isolates tested were considered sensitive to killing in serum is an important finding, especially since these isolates all have been collected from retail chicken meat.

As mentioned earlier, an important prerequisite for causing UTI is colonization. The production of enzymes and toxins, for example colicins as investigated in this work, can provide beneficial colonization conditions as it limits competition from other bacteria. It has also been suggested that growth in urine may be a predictor of the colonization ability of uropathogens (63). However, this is based on a study from 1989. Further work needs to be done to develop good and reliable predicting models for the potential of UTI.

In addition to colicin production, the ability to form biofilm is advantageous to successfully colonize a host. Biofilms are complex structures with several uninvestigated components that provide other growth conditions compared to the commonly studied planktonic growth we face in our laboratory cultures (64, 65). In this work we tested the ability to form biofilm in standard medium without salt at two different temperatures, and at two different timepoints. It is important to note that these are laboratory conditions performed on a single cell culture, and since the production of biofilm varied with the provided conditions even in our experiment, it is expected that this feature is highly dependent on the environment and accessible nutrients. This has also been suggested by others (66). It has even been indicated that biofilm of UPEC may promote virulence under certain growth conditions when formed at the right place (67, 68). As this study provided limited conditions, biofilm formation on various surfaces, in addition to other medium, would be interesting to compare, especially biofilm formation in urine.

Evaluation of materials and methods

The phenotypical assays were selected by the following criteria: 1) Simple enough to perform, making it possible to perform for a sample size of 18 isolates. 2) They could not be too time consuming, given the limited timespan of project thesis. 3) Had to be representative for a feature providing potential to become a uropathogen. The chosen assays are discussed in the Table 4.

Table 4: ***Evaluation of methods used in the project.***

Assay	**Strengths**	**Weaknesses**	**UTI potential**
Agglutination	Confirms the production of a functional Type 1 fimbriae.	No cutoff time, some were slow, some were quick, only quantitative.	Attachment to epithelial cells in the urinary tract.
Motility	Proved ability to move in semi-liquid agar.	Only quantitative, not qualitative results.	Motility for ascending through the urinary tract.
Biofilm	Performed at different timepoints, and for two different temperatures.	Only in LB without salt to force biofilm formation. Unknown urinary tract conditions for biofilm formation. Only biofilm formation on one surface.	Colonization of the urinary tract.
Growth curves	Performed both in LB and in urine. With shaking, representative for the high velocity in the urinary tract.	For 24 hours. Not in biofilm, as it most likely would have been in the urinary tract.	Ability to grow in the non-favorable conditions provided by urine.

Serum resistance	Quick and easy in the laboratory, not necessary to infect humans (or animal models). *E. coli* CFT073 as positive control.	Could not find an isolate as negative control.	Avoid serum killing. Potential for septicemia.
Invasion and adhesion	Important feature to become a successful uropathogen. Performed with kidney cells, likely representative for the epithelium in the rest of the urinary tract.	Time consuming to perform with many isolates. Challenging that isolates display varying MIC for the antibiotics used.	Avoiding the immune system in the bladder.
Colicin production	Quick and easy to perform.	Does not specify which colicin that is being produced.	Advantages during competition with other bacteria

An additional test that would have provided interesting information is the production of siderophores, as the urinary tract is an environment with limited access to iron. This was not performed in this study due to time limitations.

There is a possibility that the *in vitro* models are not able to mimic the urinary tract conditions adequately, and that an *in vivo* model therefore would be beneficial. Since inducing UTI in humans under experimental conditions possesses moral and ethical issues, an animal model, such as the urinary tract infection model described by Jakobsen et al, or a sepsis model in mice as described by Mora et al. would be regarded good alternatives (45, 69).

In this study we focused on resistant *E. coli* isolates. Resistance is important since antimicrobial treatment may be essential to treat a UTI, and in worst case a urosepsis. Johnson et al. suggested that resistant *E. coli* strains are probably more likely to be transmitted from poultry to humans than susceptible variants (70). However, resistance to antimicrobials and virulence genes are not necessarily linked, and the amount of non-resistant *E. coli* from chicken that still are able to

colonize humans and cause UTIs has not been investigated and may still be a risk factor. Another limitation is that Norway is known for having extremely low resistance prevalence in livestock compared to other countries, which limits generalizability. However, in regards of the Norwegian situation, the results presented in this thesis is considered valid and representable. It may also be of value for other countries with a similar resistance burden, and with a similar structure of the food-production chain.

Future efforts to examine further virulence potential of *E. coli* include *in vivo* models, investigation of iron-metabolism and siderophore production. It would also be beneficial to compare the results to non-resistant isolates also collected from chicken meat, in addition to a larger sample size collected over a longer period. The epidemiology of ESC-resistant *E. coli*, in addition to in depth genetic investigation is also important to provide insights in the true virulence potential of these strains.

Conclusion

The results of this study confirm that *E. coli* isolates are extremely diverse. Based on the tests performed in this work, we can conclude that the UTI potential is highly dependent on the given strain, and that ESC-resistant *E. coli* from retail chicken meat in Norway have limited virulence potential and are unlikely to become urinary tract pathogens in humans.

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