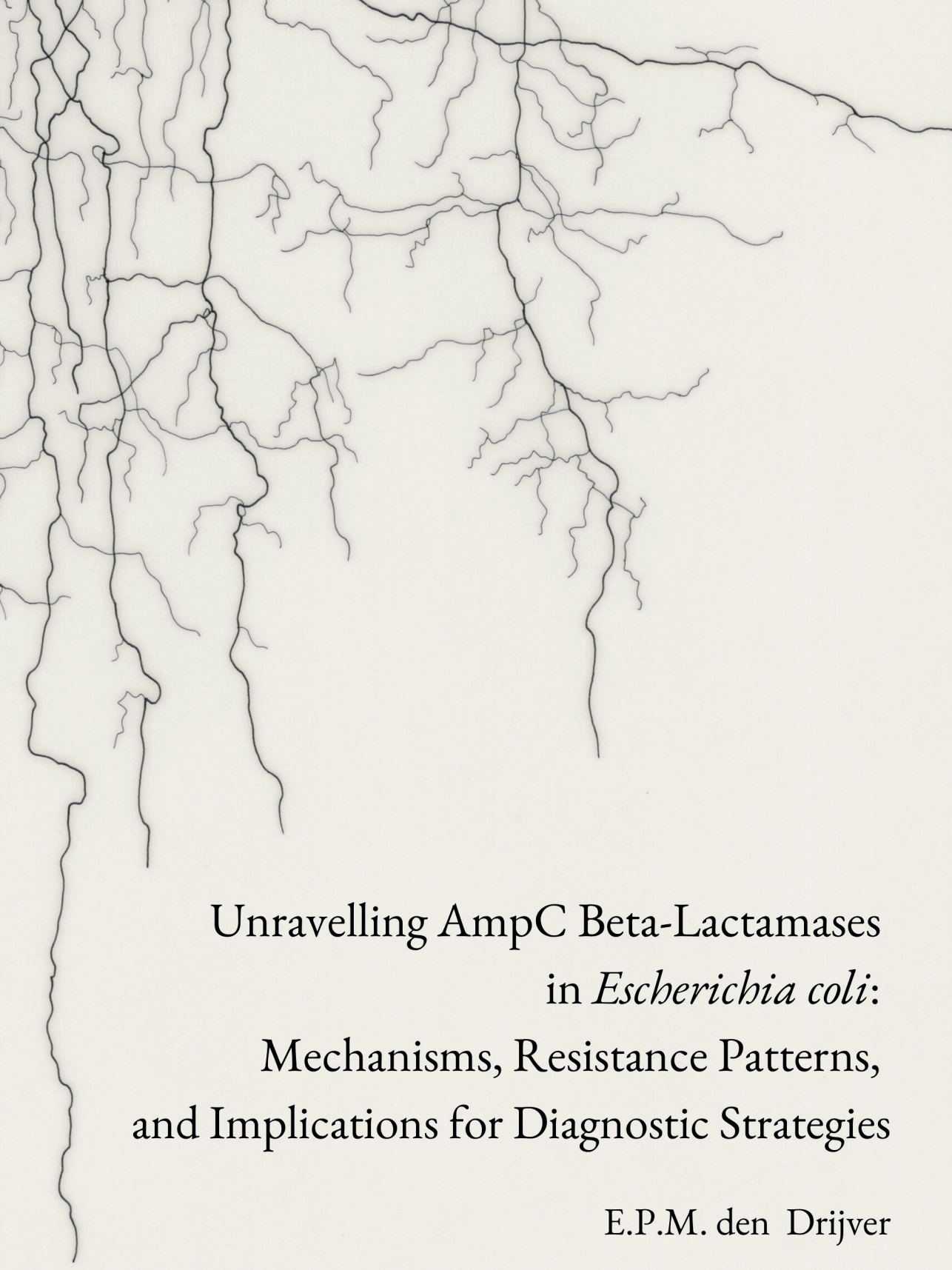

Unravelling AmpC Beta-Lactamases in *Escherichia coli*: Mechanisms, Resistance Patterns, and Implications for Diagnostic Strategies

E.P.M. den Drijver

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Unravelling AmpC Beta-Lactamases in *Escherichia coli*: Mechanisms, Resistance Patterns, and Implications for Diagnostic Strategies

Analyseren van AmpC Beta-Lactamasen in *Escherichia coli*: Mechanismen, Resistentiepatronen en Implicaties voor Diagnostische Strategieën
(met een samenvatting in het Nederlands)

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Jordy Coolen

“Gras groeit niet sneller door aan de sprietjes te trekken.”

Marcel van Roosmalen, 2012

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Chapter 1

General introduction

Antibiotic resistance, what is it and why is it a global problem?

Infectious diseases have plagued humanity since ancient times, presenting a significant challenge. For centuries, little was understood about their causes, let alone finding cures. Among the microorganisms responsible for these diseases, bacteria have played a crucial role in mortality and morbidity. From the devastating plagues of *Yersinia pestis* in the Middle Ages to the widespread cholera outbreaks in the 19th century, bacterial infections have been a leading cause of death and despair throughout history (Glatter and Finkelman 2021; Waldman, Ronald and Claeson, Mariam 2023).

A pivotal moment in the fight against infectious diseases occurred in the 19th century with the discovery that bacteria were the root cause of many common infections. This breakthrough was made possible by the pioneering work of Robert Hermann Koch, one of the foremost scientists in the field of microbiology (Münch 2003). Using Koch's postulates, which provided a framework for establishing the pathogenicity of bacteria, scientists were able to confirm their role as disease-causing agents. This understanding laid the foundation for the development of antimicrobial therapeutics to combat infectious diseases.

The first breakthrough in this regard came in 1910 with the discovery of arsphenamine, also known as salvarsan, as a cure for syphilis (Hutchings, Truman, and Wilkinson 2019; Christensen 2021). Shortly thereafter, the antibiotic effect of sulphonamides was uncovered, leading to their widespread use as antibiotics. One of the most well-known discoveries in the field was made by Alexander Fleming in 1928 when he observed the inhibitory effect of penicillin, produced by the fungus *Penicillium chrysogenum*, on the growth of *Staphylococcus aureus* bacteria he was studying (Lobanovska and Pilla 2017). Although the antimicrobial properties of many soil microorganisms, such as actinomycetales, were known before Fleming's discovery, the identification, purification, and stabilization of penicillin marked a monumental scientific breakthrough in the medical and pharmaceutical realms (Clardy, Fischbach, and Currie 2009).

Many widely used antibiotics, including cephalosporins and carbapenems, belong to the same class as penicillin—beta-lactams. These beta-lactam antibiotics play a crucial role in the treatment of bacterial infections. For instance, in the Netherlands, cephalosporins and carbapenems are the preferred antimicrobial agents for septicaemia (Sieswerda *et al*. 2020).

The discovery of penicillin, a significant antibiotic, has been accompanied by a drawback—the emergence of antibiotic resistance (Lobanovska and Pilla 2017; Zaman

et al. 2017). Even before its clinical usage began in the 1940s, resistance to penicillin was observed. This phenomenon was initially detected in bacteria that had been exposed to sublethal doses of penicillin over time. In 1940, Abraham *et al*. discovered that certain bacteria, like *Escherichia coli*, produce an enzyme called penicillinase that can destroy penicillin (Abraham and Chain 1940). The clinical impact of resistance became evident as the rate of penicillin resistance in *S. aureus* rose rapidly, reaching over 80% by the late 1960s (Lowy 2003). This meant that nearly 4 out of 5 patients with *S. aureus* infections were no longer responsive to the first-choice antibiotic (Lobanovska and Pilla 2017; Lowy 2003). Fortunately, new broad-spectrum antibiotics and resistance inhibitors (e.g., clavulanic acid) were subsequently discovered (Docquier and Mangani 2018). Currently, we have a range of antimicrobial agents from various groups, such as quinolones and lipopeptides, although the development of new antibiotic groups has slowed down (Garcia-Bustos, Cabañero-Navalón, and Salavert Lletí 2022; Ventola 2015).

The slow pace of antibiotic development raises concerns, particularly in light of the rapid increase in antibiotic resistance rates over the past two decades, particularly among gram-negative bacteria (Ventola 2015; Plackett 2020; Paterson 2006). Among these, the Enterobacterales order holds significant clinical importance (Paterson and Bonomo 2005). Comprising a large group of gram-negative rod-shaped bacteria, Enterobacterales are commonly found in the human gut and are associated with prevalent bacterial infections, such as biliary and urinary tract infections (Janda and Abbott 2021). Septicaemia caused by Enterobacterales is a frequently encountered and potentially fatal complication if not effectively treated (Bone 1993). While many beta-lactam antibiotics, such as cephalosporins and carbapenems, traditionally exhibit susceptibility against most Enterobacterales, the rising rates of beta-lactam resistance within this bacterial order pose a challenge to treatment and have led to decreasing cure rates (Kang *et al*. 2005; Pop-Vicas and Opal 2014).

A major cause of beta-lactam resistance is the presence of enzymes called beta-lactamases (D M Livermore 1995). These enzymes diminish the effectiveness of beta-lactam antibiotics by hydrolysing their molecular structure. Specifically, they break open the beta-lactam ring of the antibiotic, rendering its antimicrobial activity inactive. One example of a beta-lactamase is penicillinase. There exists a wide array of different beta-lactamases, some exerting a greater impact on antibiotic resistance than others. Certain beta-lactamases are capable of hydrolysing only narrow-spectrum antibiotics like penicillin, while others can destroy broad-spectrum antibiotics like cephalosporins and carbapenems.

Among the frequently encountered beta-lactamases are the extended spectrum beta-lactamases (ESBLs). This group comprises numerous variants (e.g., TEM, SHV, CTX-M, etc.), all of which possess the ability to hydrolyse the broader-spectrum cephalosporin antibiotics (David M. Livermore 2008). The escalating prevalence of ESBLs in the past two decades has significantly influenced the clinical use of cephalosporins (David M. Livermore *et al*. 2007). In certain countries, resistance due to ESBLs has reached alarming levels, necessitating the restriction of cephalosporins as the first-line treatment for common infections. Consequently, more broad-spectrum antibiotics like carbapenems and quinolones are employed. However, the efficacy of these broad-spectrum antibiotics may be limited as resistance to these agents rapidly emerges due to their widespread use.

What is an "AmpC beta-lactamase"?

The AmpC beta-lactamase is a bacterial enzyme that primarily targets specific beta-lactam antibiotics, especially cephalosporins (Jacoby 2009). It is prevalent among gram-negative bacteria, particularly in the Enterobacterales order. AmpC beta-lactamase production leads to resistance against commonly used beta-lactam antibiotics, and it is often unaffected by beta-lactamase inhibitors, distinguishing it from other ESBLs.

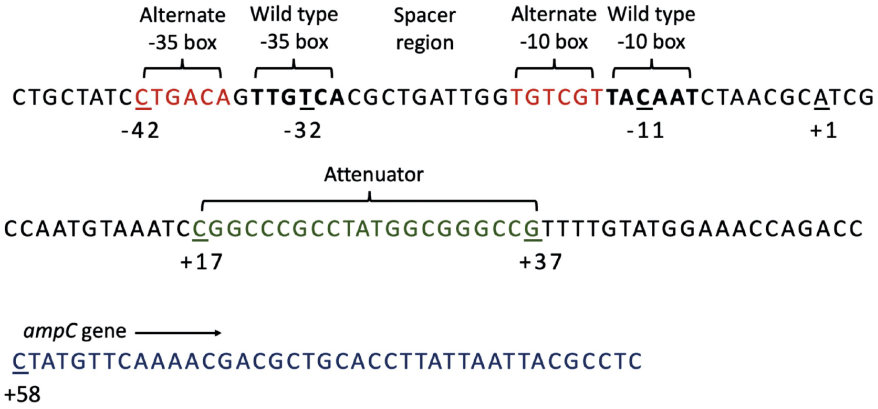
The AmpC beta-lactamase belongs to molecular class C and is regulated by pathways involved in cell-wall degradation. Mutations in the regulatory gene *ampR* result in overexpression of *ampC*, causing broader resistance to cephalosporins, including third-generation cephalosporins like ceftriaxone, cefotaxime, and ceftazidime. Additionally, mutations primarily in the *ampD* gene can lead to constitutive overexpression of *ampC*, resulting in long-lasting antibiotic resistance, particularly in species like *Enterobacter cloacae* complex and *Citrobacter freundii* (Kohlmann, Bähr, and Gatermann 2018).

In most Enterobacterales, AmpC enzymes are inducible, but certain species like *E. coli* and *Shigella* spp. express *ampC* at low levels due to the absence of the *ampR* gene. However, mutations in the promoter/attenuator region of the *ampC* gene can lead to hyperproduction of the AmpC beta-lactamase (Caroff N, Espaze E, Gautreau D, Richet H 2000; Tracz *et al*. 2007). Figure 1 shows the sequence of the *E. coli* ATCC 25922 *ampC* promoter/attenuator region. Although most AmpC hyperproducers show relatively low resistance to third-generation cephalosporins, alterations in the AmpC beta-lactamase or changes in membrane permeability can contribute to increased resistance (Nordmann and Mammeri 2007; Martínez-Martínez 2008).

Various types of plasmid-encoded *ampC* genes have been identified, with bla_{CMY-2} (beta-lactamase type CMY-2) being the most common in the Netherlands (E. Ascelijn Reuland *et al*. 2015; E. Den Drijver *et al*. 2018). These genes vary in their hydrolysing capability and are associated with specific bacterial species, such as bla_{CMY-2} in *E. coli* and *Salmonella* spp. and bla_{DHA} in *Klebsiella* spp (Philippon, Arlet, and Jacoby 2002).

Multiple studies have investigated the epidemiology of plasmid-encoded *ampC* genes in the Netherlands, but information on trends remains limited.

Figure 1. Sequence of the *E. coli* ATCC 25922 *ampC* promoter/attenuator region. Sequence regions based on Tracz *et al*. with numbering according to Jaurin *et al*. and (Jaurin *et al*. 1981; Tracz *et al*. 2007).



How to detect AmpC beta-lactamases

Detection and differentiation of plasmid-encoded AmpC and chromosomal-encoded AmpC genes pose challenges due to their coexistence. This difficulty is amplified in the presence of ESBL. The Dutch guideline for detection of highly-resistant micro-organisms recommends initial screening for plasmid AmpC by assessing resistance to cephamycins, using a cefoxitin minimal inhibitory concentrations (MICs) of 8 mg/L or higher and elevated MICs for cefotaxime, ceftriaxone, or ceftazidime (MIC >1 mg/L), as indicators of AmpC production (J.A.J.W Kluytmans *et al*. 2021). Confirmation tests involve inhibitory tests using cloxacillin or boric acid and various disc diffusion or gradient strip methods that compare zone differences between third-generation cephalosporins with or without an inhibitor.

Molecular confirmation tests, such as multiplex PCRs, microarrays, and whole genome sequencing (WGS), are often required to specifically identify plasmid-encoded *ampC* genes and differentiate them from chromosomal *ampC*. WGS allows for the detection of promoter/attenuator mutations in *E. coli* and the examination of different plasmid families. WGS facilitates cluster analysis and confirmation of outbreaks by integrating cluster analysis results with epidemiological data (Quainoo *et al*. 2017).

In settings where molecular diagnostics may not be accessible, the implementation of a practical algorithm for distinguishing *ampC* genotypes in *E. coli* through phenotypic susceptibility testing can be valuable. Notably, utilizing cefotaxime minimal inhibitory concentrations obtained from gradient test results demonstrated a high level of accuracy in predicting the *ampC* genotype.

The utilization of diverse screening media in prevalence studies introduces variability in the interpretation of prevalence data. While specific media for screening ESBL are available, the options for screening AmpC-producing Enterobacterales are limited. Although there are media with increased cephamycin concentrations, the effectiveness of these media has been insufficiently studied. The effectiveness of media with increased cephamycin concentrations requires further study, and standardization of screening strategies using antibiotic enrichment broth is yet to be established.

What are the sources of AmpC-producing Enterobacterales?

Plasmids are extrachromosomal DNA elements that can be transferred between bacteria, often carrying antibiotic resistance genes. Plasmid-based resistance poses a significant challenge to infection control, with a particular focus on Enterobacterales carrying plasmid-mediated AmpC beta-lactamases (pAmpC-E). Certain plasmid families, such as IncA/C, IncB/O/K, and IncI, are commonly associated with $bla_{\text{CMY-2}}$, the prevalent AmpC resistance gene (Accogli *et al*. 2013; Alessandra Carattoli *et al*. 2018). Notably, IncI plasmids, including the prevalent IncI-ST12 sequence type, have been detected in diverse sources, including human clinical samples, traveller rectal carriage samples, livestock samples, and dog samples (Lorme *et al*. 2018; Hansen *et al*. 2016). The transmission mechanisms of these $bla_{\text{CMY-2}}$-carrying IncI plasmids remain to be fully understood.

The origin of the promoter/attenuator mutations that lead to *ampC* hyperexpression in *E. coli* is difficult to confirm due to the phenomenon of convergent evolution, where mutations can independently reoccur in multiple isolates and separate lineages. This process, known as homoplasy, is potentially influenced by selective pressure from the

use of antibiotics or antiseptics. Homoplasy is a biological phenomenon characterized by the independent occurrence or recurrence of similar traits or genetic changes in different organisms or lineages, irrespective of their genetic relatedness (Wake, Wake, and Specht 2011). It refers to the parallel evolution of similar features, mutations, or genetic variations across diverse populations. This phenomenon poses challenges in determining the true origin or evolutionary history of a particular trait or mutation, as it can arise through convergent evolution rather than through shared ancestry.

Is AmpC-mediated resistance a significant concern?

Antibiotic resistance resulting from the production of broad-spectrum beta-lactamases in Gram-negative bacteria presents a formidable challenge in both clinical and community settings (Murray *et al*. 2022). The global dissemination of ESBL-producing variants of *E. coli* has severely restricted treatment options, and the emergence of AmpC beta-lactamases is also causing a notable impact, albeit to a lesser extent, by compromising the effectiveness of broad-spectrum penicillins and third-generation cephalosporins. The resistance exhibited by AmpC beta-lactamases to beta-lactamase inhibitor combinations, such as clavulanic acid and tazobactam is of particular concern. Various hypotheses have been proposed to explain this phenomenon, one of which suggests that an elevation in the plasmid copy number of $bla_{\text{CMY-2}}$ containing plasmids could be a contributing factor (Kurpiel and Hanson 2012).

Aims of this thesis

The main objectives of this thesis are to delve into various aspects of AmpC-related antimicrobial resistance in Enterobacterales. Firstly, the thesis aims to assess the prevalence of rectal colonization by AmpC-producing Enterobacterales in the Netherlands and identify potential trends in colonization rates over time.

Secondly, the thesis seeks to optimize the detection of AmpC genes in *E. coli* through the use of selective media and phenotypic characterization techniques, such as determining minimal inhibitory concentrations of specific antibiotics. The obtained results contribute to the development of a screening strategy that utilizes the phenotype of *E. coli* to predict the underlying genotype.

The third objective focuses on investigating the relatedness between plasmids containing the bla_{CMY-2} gene in epidemiologically linked and unrelated Enterobacterales isolates from humans and livestock. The aim is to explore the feasibility of accurately distinguishing related samples from unrelated ones based solely on plasmid sequencing data.

The fourth aim of this thesis is to examine whether mutations occurring in the *ampC* promoter/attenuator region of *E. coli* are homoplastic and whether these homoplastic mutations are associated with cefotaxime resistance. This investigation sheds light on the relationship between specific mutations and antibiotic resistance.

The fifth aim of this thesis was to compare the sequencing depth between chromosomal household genes and plasmid-encoded scaffolds containing the bla_{CMY-2} gene, as well as to utilize the ratio as an estimated plasmid copy number. This analysis aimed to provide insights into the abundance of plasmids carrying the bla_{CMY-2} gene within the studied isolates. Furthermore, the relationship between the estimated plasmid copy number and the minimal inhibitory concentrations of cefotaxime, ceftazidime, and piperacillin-tazobactam was investigated. This examination allowed for a deeper understanding of the association between plasmid presence and the level of resistance to these specific antibiotics.

Chapter 2

AmpC beta-lactamases: epidemiology, infection control and treatment

Evert den Drijver, Jaco J. Verweij, Jan A.J.W. Kluytmans

Adapted from: Tijdschrift voor Infectieziekten, 18 (1), March 2023

Summary

Antibiotic resistance is an increasing problem. Particularly in gram-negative bacteria, there is a wide variety of resistance mechanisms affecting different antibiotics, e.g. the beta-lactam group. Besides the already common extended spectrum beta-lactamases (ESBL), the AmpC beta-lactamases make a significant contribution to resistance in gram-negative bacteria. Some of these resistance mechanisms are already intrinsically present on the bacterium's chromosome, but can also be transferred from bacteria to bacteria on plasmids. The latter may be a significant factor in the spread and increase of antibiotic resistance in healthcare. This article has been written to provide more insight into the background of AmpC beta-lactamases, as well as epidemiology and diagnostics. Hopefully, it can provide tools for microbiologists or clinical infectious disease specialists for the diagnosis, treatment and prevention of further transmission of this resistance mechanism.

Introduction

A well-known healthcare-related problem among patients in hospitals and nursing homes is the increasing antibiotic resistance in gram-negative bacteria (David M. Livermore *et al*. 2007). Extended spectrum beta-lactamase (ESBL)-producing gram-negative bacteria within the order Enterobacterales, such as *Escherichia coli* and *Klebsiella pneumoniae,* are increasingly found worldwide. Due to the resistance caused by the beta-lactamases, frequently used beta-lactam antibiotics are no longer effective. This limits the options for adequate treatment for the patient, because empirical therapy for bloodstream infections in most Dutch hospitals is based on the use of beta-lactam antibiotics. For example, second- and third-generation cephalosporins such as cefuroxime, ceftriaxone or ceftazidime occupy an important place in the Netherlands as the first-choice treatment for sepsis, as described in the SWAB guideline Sepsis 2020 (Sieswerda *et al*. 2020).

Although to a lesser extent than ESBL, acquired AmpC beta-lactamases have emerged as a potential threat to the utility of broad-spectrum penicillins and third-generation cephalosporins (Jacoby 2009). Acquired AmpC beta-lactamases are encoded on plasmids and are transferable between different bacteria species. Detecting AmpC production in Enterobacterales can be challenging and guidelines and protocols on the detection and infection prevention are still scarce. In this review, the background of the resistance mechanism and possible detection methods of plasmid-encoded AmpC are described. A summarized overview of the epidemiology of plasmid-encoded AmpC in the Netherlands is provided as well. Furthermore, some descriptions of outbreaks with plasmid AmpC are given with possible guidance on infection prevention regarding the spread of AmpC producing Enterobacterales. Finally, the treatment options for infections with AmpC producing Enterobacterales are summarized.

Background of AmpC

The existence of the AmpC beta-lactamase in *E. coli* has been known since the 1940s of the twentieth century (Jacoby 2009). In the 1960s, the name AmpC was first used in scientific literature for a specific mutant of a penicillinase regulated by the *ampA* (Jacoby 2009). This type of penicillinase was later found to be different from other known penicillinases such as *bla*TEM-1. In the Ambler classification, the AmpC beta-lactamases are classified in a separate class C (Bush and Jacoby 2010). The most notable feature of the AmpC beta-lactamases is that they can hydrolyse cephalosporins and cephamycins, such as cefoxitin and cefotetan. This last type of beta-lactam antibiotic cannot be hydrolysed by ESBLs, such as the SHV or CTX-M beta-lactamases (Jacoby

2009). Fourth generation cephalosporins such as cefepime are an exception, as they are not hydrolysed by AmpC beta-lactamases. A second difference to most ESBLs is that commonly used beta-lactamase inhibitors such as clavulanic acid and tazobactam have very little or no effect on the AmpC activity. Cloxacillin and avibactam do have a good inhibitory effect on AmpC beta-lactamases. This characteristic of AmpC can be used in diagnostics (see paragraph *Detection of plasmid AmpC*) (Jacoby 2009; J.A.J.W Kluytmans *et al*. 2021).

Ever since the first studies on AmpC beta-lactamase in *E. coli,* this class of beta-lactamases has been found in a multitude of Enterobacterales and other Gram-negative bacteria over the years. For example, AmpC enzymes have been detected in *Pseudomonas aeruginosa* and *Acinetobacter baumannii complex* (Jacoby 2009). Although different AmpC beta-lactamases are very similar in enzyme structure, the genetic sequence differs per variant and species. The phenotype can also be very diverse. This is partly related to the phenomenon of induction and derepression of the AmpC beta-lactamase. AmpC production is related to cell wall degradation in gram-negative bacteria. As soon as more degradation products, such as 1,6-anhydromuropeptides, are released during the degradation of the cell wall, the expression of the *ampC* gene increases via a transcription regulator (AmpR). A second enzyme, called AmpD amidase, can initiate an alternative pathway in the cell wall degradation cycle that can inhibit *ampC* expression. Normally this phenomenon is balanced, but the administration of beta-lactam antibiotics increases the cell wall degradation and therefore causes an elevated *ampC* expression and subsequently the AmpC beta-lactamase production. This phenomenon is called induction, resulting in the typical resistant phenotype. Once the supply of cell wall degradation products decreases again, the expression of the *ampC* gene and the beta-lactamase production will return to the original level (Jacoby 2009). However, mutations can occur in the *ampR* or *ampD* gene, resulting in an adaptation in the enzyme structure. The cycle remains in a continuously increased status and in such cases, the beta-lactam resistance will be maintained without an increased supply of cell wall degradation products. This phenomenon is known as derepression and is mainly seen in *Enterobacter spp, Klebsiella aerogenes* and *Citrobacter freundii* (Jacoby 2009; Kohlmann, Bähr, and Gatermann 2018). This group of Enterobacterales is often referred to as "group II Enterobacterales". When these mutants are selected out, for example under antibiotic pressure, the beta-lactam resistance remains constant and the micro-organism can no longer be adequately treated with third-generation cephalosporins.

In recent decades it has been discovered that *ampC* genes can also be encoded on plasmids (Jacoby 2009; Philippon, Arlet, and Jacoby 2002). Plasmids are circular

strands of DNA apart from the chromosomal DNA of the bacterium. They can be transferred from bacterium to bacterium by horizontal transfer. Several types of plasmid-encoded *ampC* (*pampC*) genes have been detected in Enterobacterales species often referred to as "group I Enterobacterales" (including *E. coli, K. pneumonia, P. mirabilis, Salmonella enteritidis*), with bla_{CMY-2} being the most common *pampC* resistance gene in the Netherlands (E. Ascelijn Reuland *et al*. 2015). Other less commonly isolated *pampC* genes are other variants of bla_{CMY}, as well as variants that differ more from bla_{CMY}, e.g. bla_{DHA}, bla_{ACC}, bla_{ACT}, bla_{MIR}, bla_{MOX} and bla_{FOX} (Philippon, Arlet, and Jacoby 2002) (see Table 1.). All of these different *pampC* genes are originally derived from chromosomal *ampC* genes. For example, the bla_{CMY} originates from the *C. freundii* chromosome, but several mutations have resulted in a great diversity of variants of the bla_{CMY} gene (Jacoby 2009). Depending on the variant of AmpC beta-lactamase the hydrolysing capacity varies (Philippon, Arlet, and Jacoby 2002). This leads to different phenotypes per variant of *ampC* gene (see Table 1). The bla_{CMY-2} gene most common in the Netherlands generally leads to increased minimum inhibitory concentrations (MICs) for ceftriaxone, ceftazidime and cefoxitin (Philippon, Arlet, and Jacoby 2002; Coolen *et al*. 2019). However, in isolates with bla_{DHA} this hydrolysing activity is less prominent, so that the effect on ceftriaxone or ceftazidime MICs may be less pronounced (Coolen *et al*. 2019). Moreover, the bla_{DHA} gene is inducible when exposed to different antibiotics, e.g., imipenem. The hydrolysing effect can increase under the influence of these antibiotics (Jacoby 2009). Another example is the bla_{ACC} gene, which has the specific effect that it cannot hydrolyse cephamycins (e.g., cefoxitin) (Jacoby 2009; Philippon, Arlet, and Jacoby 2002). This is unique, as the hydrolysis of this antibiotic group is considered typical for AmpC beta-lactamases and the increased cephamycin MICs are used in many diagnostic algorithms (J.A.J.W Kluytmans *et al*. 2021; Martinez and Simonsen 2017).

It seems that certain *pampC* genes are more common in certain species of the group 1 Enterobacterales. For example, bla_{CMY-2} is more commonly detected in *E. coli* and *Salmonella* spp and bla_{DHA} in *Klebsiella* spp (Rodríguez-Guerrero *et al*. 2022). Furthermore, which variant is most prevalent can differ regionally. For example, in North-western Europe, the bla_{CMY-2} gene is most commonly detected in human and veterinary samples, while the bla_{DHA} gene is more often found in equivalent samples in East Asia (e.g., South Korea and Japan) (Rodríguez-Guerrero *et al*. 2022).

Table 1. Different beta- lactamase families with possible original species, number of variants and expected phenotype (↑↑ = strongly increased MIC, ↑ = moderately increased MIC, ↓ = low MIC) [3,4]

	AmpC beta-lactamase family	Probably from chromosome of species	Genetic similarity chromosomal gene	Number of variants	Phenotype
1	*bla*$_{CMY\ -2}$ family	*Citrobacter freundii*	*96%*	*n =171*	*Third-generation cephalosporins* ↑↑, *cephamycins* (*bla*$_{CMY\ -13}$ inducible)
2	*bla*$_{DHA}$ family	*Morganella morgagnii*	*99%*	*n =29*	*Third-generation cephalosporins* ↑, *cephamycins* (inducible)
3	*bla*$_{ACC}$ family	*Hafnia alvei*	*99%*	*n = 7*	*Third-generation cephalosporins* ↑, *cephamycins*
4	*bla*$_{ACT\ /MIR}$ family	*Enterobacter cloacal complex*	*98-99%*	*n = 83* (*bla*$_{ACT}$), *n = 22* (*bla*$_{MIR}$)	*Third-generation cephalosporins* ↑, *cephamycins* (*bla*$_{ACT}$ inducible)
5	*bla*$_{FOX}$ family	*Aeromonas caviae*	*99%*	*n=16*	*Third-generation cephalosporins* ↓, *cephamycins* ↑↑
6	*bla*$_{MOX/CMY-1}$ family	*Aeromonas hydrophila*	*80-82%*	*n=14* (*bla*$_{MOX}$), n=6 (*bla*$_{CMY-1}$)	*Third-generation cephalosporins* ↑, *cephamycins*

In certain species within the group 1 Enterobacterales, e.g., *E. coli* and *Shigella* spp, the production of AmpC beta-lactamase is not only encoded on plasmids, but can also be mediated by hyperexpression of a chromosome-encoded *ampC* gene (*campC*) (Jacoby 2009; Tracz *et al*. 2007). Normally, *campC is* only expressed at a low level in *E. coli*, but mutations in the promoter/attenuator region of the *campC* gene lead to hyperproduction of the chromosome-encoded AmpC beta-lactamase. The presence of these "chromosomal AmpC hyperproducers" complicates the detection of *pampC* genes in *E. coli* when

using only phenotypic assays (Coolen *et al*. 2019). Although most chromosomal AmpC hyperproducing *E. coli* appear to have lower MICs for third-generation cephalosporins, there is evidence that changes in the AmpC beta-lactamase or changes in membrane permeability can lead to an increased cephalosporin resistance (Coolen *et al*. 2019; Nordmann, Poirel, and Nordmann 2007). Co-expression of *ESBL* and *pampC* genes in the same isolate can be even more challenging to detect using phenotypic assays because this detection is dependent on genotypic confirmation (J.A.J.W Kluytmans *et al*. 2021; Martinez and Simonsen 2017).

Plasmid-based resistance is considered a greater threat regarding infection control than clonal transmission of chromosome-encoded resistance genes. Therefore, the focus within infection control is mainly on pAmpC producing Enterobacterales and less on the AmpC hyperproducing Enterobacterales. Certain types of plasmids are associated with specific *pampC* genes. Common plasmid families related to bla_{CMY-2} are IncA /C-, IncB /O/K and IncI (Alessandra Carattoli *et al*. 2018; Pietsch *et al*. 2018). In particular, IncI plasmid families are increasingly found in combination with bla_{CMY-2}. IncI-ST12 is one of the most common plasmid sequence types (Pietsch *et al*. 2018; Roer *et al*. 2019). Strikingly, the IncI plasmids harbouring bla_{CMY-2} are found in several domains, such as human clinical samples, rectal carrier samples from travellers, veterinary samples and pet samples (Lorme *et al*. 2018; Hansen *et al*. 2016; Roer *et al*. 2019; E. P. M. Den Drijver *et al*. 2020). It is not yet known whether the elevated prevalence of these IncI plasmids is due to the presence of a common clone or more efficient transfer mechanisms among bacteria.

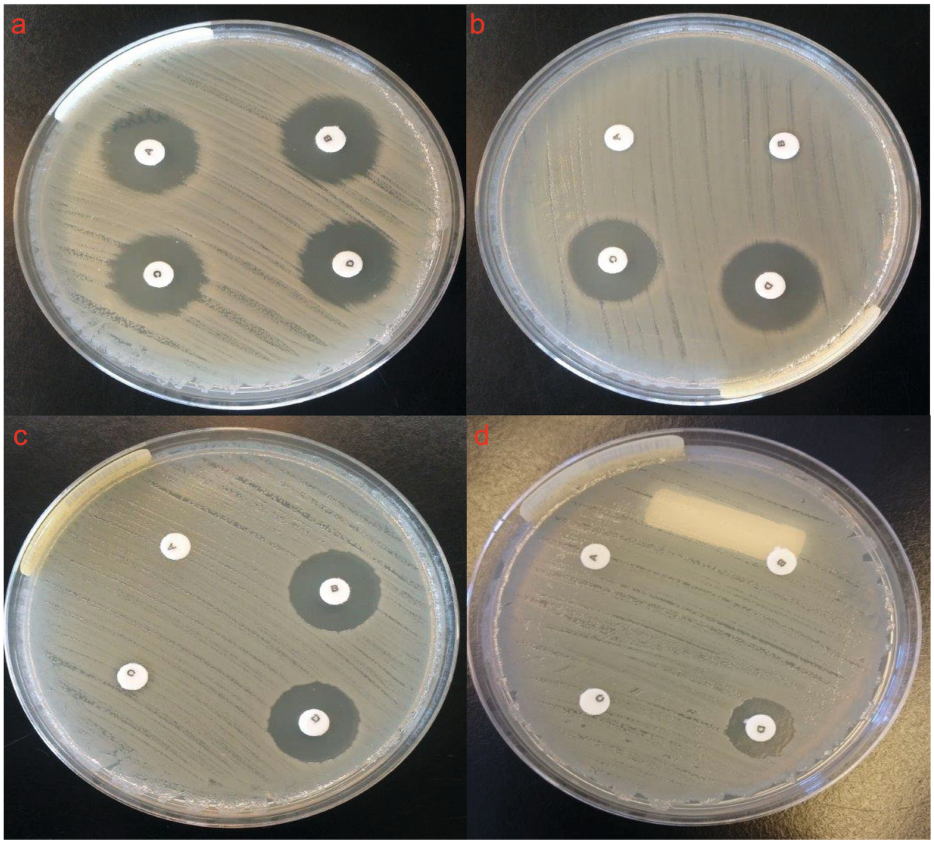
Detection of Plasmid AmpC

Detection of plasmid encoded AmpC is based on phenotypic and genotypic testing. Due to the coexistence of both chromosome and plasmid encoded *ampC* genes, it can be difficult to distinguish them. The presence of ESBL can make detection even more difficult. The recently updated NVMM guideline for BRMO detection recommends screening and confirmation of plasmid AmpC at Enterobacterales (J.A.J.W Kluytmans *et al*. 2021). The high pAmpC resistance phenotype to cephamycins is often used as a first screening criterion. A cefoxitin MIC >8 mg/L in combination with elevated MICs for cefotaxime, ceftriaxone or ceftazidime (MIC >1 mg/L) may indicate the presence of AmpC production (J.A.J.W Kluytmans *et al*. 2021).

The confirmation tests are based on the aforementioned inhibitory capacity of cloxacillin or boric acid. Various disc diffusion tests and gradient strips are available, which confirm the presence of AmpC production based on zone difference between e.g., a third-generation cephalosporin with or without an inhibitor (J.A.J.W Kluytmans

et al. 2021). Figure 1. shows an example of an AmpC and ESBL confirmation test based on disk diffusion.

Figure 1. Example of a disk confirmation test for AmpC and ESBL (Mast® D68C, Mast Group Ltd., Bootle, United Kingdom). Disk A: Cefpodoxime 10µg, Disk B Cefpodoxime 10µg + ESBL Inhibitor, Disk C Cefpodoxime 10µg + AmpC Inhibitor, Disk D Cefpodoxime 10µg + ESBL Inhibitor + AmpC Inhibitor. Figure 1a. no AmpC if no ESBL, Figure 1b. AmpC positive, Figure 1c. ESBL positive, 1d AmpC and ESBL positive. (photo C. Verhulst, Microvida, Breda, Netherlands)



The confirmation of AmpC production in various species within the Enterobacterales cannot differentiate the expression of a plasmid and a chromosome encoded *ampC* gene. In many cases, it will be necessary to perform a molecular confirmation test, which specifically determines the presence of plasmid encoded *ampC* genes. Various multiplex PCRs and microarrays have been developed (J.A.J.W Kluytmans *et al*. 2021). In recent years, whole genome sequencing has taken flight, making it possible to analyse the entire chromosome and plasmids of the bacterium. This has the additional advantage

that promoter/attenuator mutations in *E. coli* can be confirmed and the presence of the different plasmid families can be examined. Moreover, whole genome sequencing allows is that cluster analysis can be performed and outbreaks can be confirmed based on the combination of this cluster analysis and epidemiological data (Quainoo *et al*. 2017). However, the reconstruction of full plasmid sequences may necessitate the utilization of multiple sequencing methods, resulting in a time-consuming and labour-intensive analysis.

Screening with specific media for AmpC producing Enterobacterales, a common method for other resistance presences such as ESBL, is currently still limited. Although there are producers of media that contain elevated cephamycin concentrations, the number of comparative studies on the effectiveness is limited. The use of a specific antibiotic enrichment broth as a screening strategy has not yet been standardized. The lack of guidelines on screening strategies makes the diversity of screening methods in prevalence studies wide, which influences their heterogeneity. When interpreting prevalence data, it is therefore important to take into account the possible differences in screening methods.

Epidemiology of plasmid encoded AmpC in the Netherlands

Within the Netherlands, various studies have been conducted into the prevalence of plasmid encoded AmpC producing Enterobacterales (see Table 2). Most studies have focused on rectal or perineal carriage in humans. In general, the prevalence of AmpC producing Enterobacterales rectal carriage in the Netherlands is considered low. In the general population, the prevalence of plasmid AmpC producing *E. coli* varies between 0.2% and 1.3% (E. Ascelijn Reuland *et al*. 2015; Van Hoek *et al*. 2015; van den Bunt *et al*. 2017). In the two studies of carrier status in hospital patients within the Netherlands, the prevalence was not significantly higher (0.7% to 0.9%) (E. Den Drijver *et al*. 2018; X. Zhou *et al*. 2017).

Few studies have looked at trends over time. A 2013-2016 study did not find a significant increase in plasmid levels AmpC producing *E. coli* (E. Den Drijver *et al*. 2018). Prevalence studies of plasmid AmpC-producing Enterobacterales in clinical isolates, for example from blood cultures, are scarcely performed in the Netherlands. Voets *et al*. stated that in a collection of isolates from urine cultures and blood cultures with 3rd generation cephalosporin resistance, plasmid AmpC was the cause of the resistance in 5% of *E. coli* and 4% of *K. pneumoniae* (Voets *et al*. 2013). More recent prevalence data of plasmidal AmpC-producing Enterobacterales in clinical isolates are absent in the Netherlands. Outside of the Netherlands, the prevalence of plasmid encoded AmpC producing Enterobacterales is significantly higher (Rodríguez-Guerrero *et al*. 2022).

Table 2. Prevalence studies of AmpC producing Enterobacterales based on rectal carriage

	Author	Year	Study population	Source	Screened species	Prevalence (pAmpC / cAmpC)	Prevalence per pAmpC gene
1	Hoek *et al.* 2015 (21)	2011	General population (*n*=1033)	Straighten out the rectum	*Enterobacterales*	*E. coli* pAmpC 0.39% *P. agglomerans* 0.10%	bla_{CMY-2} 0.29% bla_{DHA-1} 0.10% bla_{CMY-48} 0.10%
2	Reuland *et al.* 2015 (22)	2011	General population (*n*=550)	Faeces	*Enterobacterales*	*E. coli* pAmpC 1.27%	bla_{CMY-2} 1.09% bla_{DHA-1} 0.18%
3	Zhou *et al.*, 2017 (24)	2012-2013	Hospital (*n*=445)	rectal smear	*E. coli*	*E. coli* pAmpC 0.67% *P. mirabilis* pAmpC 0.22%	bla_{CMY} 0.67% bla_{DHA} 0.22%
		2012-2013	General population (*n*=400)	rectal smear	*E. coli*	*E. coli* pAmpC 0.25%	bla_{CMY} 0.25%
4	Van den Bunt *et al.* 2016 (23)	2013-2015	General population (*n*=1004 children)	faeces	*Enterobacterales*	*E. coli* pAmpC 0.40% *K. pneumonia* pAmpC 0.10%	bla_{CMY-2} 0.40% bla_{DHA-1} 0.10%

Table 2. Continued.

	Author	Year	Study population	Source	Screened species	Prevalence (pAmpC / cAmpC)	Prevalence per pAmpC gene
			General population (*n*=995 adults)	faeces	*Enterobacterales*	*E. coli* pAmpC 0.20% *K. pneumonia* pAmpC 0.10%	$bla_{CMY\ -2}$ 0.20% $bla_{DHA\ -1}$ 0.10%
5	Den Drijver *et al.* 2018 (25)	2013-2016	Hospital (*n*=2126)	rectal smear	*E. coli*, *K. pneumoniae*	*E. coli* pAmpC 0.90% cAmpC 1.46%	bla_{CMY} 0.85% $bla_{DHA\ 0.05}$ % cAmpC 1.46%

Outbreaks with Plasmid AmpC

Within the Netherlands outbreaks with plasmid encoded AmpC producing Enterobacterales have not yet been described. However, transmission can occur within hospitals which has been described in other countries as summarized below.

One of the first reports of an outbreak was made by Papanicolaou *et al.* in 1990 of a bla_{MIR} producing *K. pneumoniae* in Rhode Island, USA (*n*=11 patients) (Papanicolaou, Medeiros, and Jacoby 1990). A larger outbreak with a bla_{ACC} producing *K. pneumoniae* was later described in France, where a cluster of 57 patients was detected between 1999 and 2003 (Ohana *et al*. 2005). Clusters of both bla_{DHA} and bla_{CMY-2} producing *K. pneumoniae* were identified at a liver transplant facility in Japan (Matsumura *et al*. 2015). Interestingly, eight of the bla_{DHA} positive and one of the bla_{CMY-2} positive isolates showed carbapenem resistance, probably due to a combination of AmpC production and changes in membrane permeability.

In 2013, Wendorf *et al.* reported a small outbreak (*n*=7) of *E. coli* carrying a bla_{CMY-2} variant gene in the United States. It concerned patients who had undergone endoscopic retrograde cholangiopancreatography, which suggested an association with contaminated endoscopes (Wendorf *et al*. 2015).

AmpC and Infection Prevention

Guidelines on infection prevention and plasmid AmpC-producing Enterobacterales are scarce. Currently, plasmid encoded AmpC is not yet specified in the WIP guidelines 'High resistant micro-organisms in hospitals" and "'High resistant micro-organisms in nursing homes, residential care centres and facilities for small-scale living for the elderly'' (Werkgroep Infectiepreventie *et al*. 2018; 2014). However, given the similarity to ESBL-producing Enterobacterales, it is expected that comparable measures will be effective against the spread of AmpC producing Enterobacterales. A revision of this guideline is currently under review by the Partnership for Infection Prevention Guidelines. In the revised version, plasmid AmpC-producing Enterobacterales are not classified as highly resistant microorganisms in routine care settings. However, it is recommended to monitor nosocomial transmission, and in the event of an outbreak of plasmid AmpC-producing Enterobacterales, additional interventions are advised to prevent further spread (Severin, J.A. *et al*. 2023).

Treatment recommendations for AmpC- producing Enterobacterales

Treating infections of AmpC producing Enterobacterales can be complicated due to their resistance to many of the first-choice beta-lactam antibiotics. Both penicillins

with beta-lactamase inhibitor combinations (such as amoxicillin-clavulanic acid) and third-generation cephalosporins are in most cases ineffective. Often it will be necessary to switch to a carbapenem or another group of antibiotics, such as quinolones. In some cases, the MIC values of third-generation cephalosporins are still below the resistance breakpoint in Enterobacterales with *campC* genes. Nonetheless, resistance to these agents can still occur due to derepression during treatment. Based on the study by Kohlmann *et al*, the risk of derepression appears to be different per species. *Enterobacter cloacae complex* isolates have a greater chance of developing resistance than *Morganella morgagnii* (Kohlmann, Bähr, and Gatermann 2018). That is why a distinction can be made per *campC* expressing species as to whether or not third-generation cephalosporins can be safely used when treating infections with AmpC producing Enterobacterales with low cephalosporin MIC values. A fourth-generation cephalosporin such as cefepime could be an alternative, but is currently used only to a limited extent in the Netherlands (Tamma *et al*. 2019). Beta-lactamase inhibitors such as tazobactam and avibactam may also be an alternative therapy. In the Merino-II study, no significant difference was seen in clinical outcomes between the treatment with carbapenems and piperacillin-tazobactam, although microbiological failure occurred significantly more often in the piperacillin-tazobactam group (Stewart *et al*. 2021). Clinical comparative studies have mainly been performed with therapeutics that need parenteral administration. Data on oral treatment with 3rd generation cephalosporins (e.g., ceftibuten) of infections with chromosomal AmpC-producing Enterobacterales are scarce, although a recent study of a new oral combination preparation (e.g. ceftibuten/VNRX-7145) shows effectiveness in urinary tract infections (Karlowsky, Hackel, and Sahm 2022). As this new combination drug is not yet available in the Netherlands, oral treatment with, for example, quinolones or trimethoprim-sulfamethoxazole will depend on the resistance pattern and (local) antibiotic guidelines.

It is unknown if Enterobacterales containing *pampC* genes can be treated similarly, when measured MICs are below breakpoint level. Comparative clinical studies such as with chromosomal AmpC are lacking. The NVMM guideline "Laboratory detection of high resistant microorganisms (BRMO)" recommends blocking the result for the antibiotic in question, reporting it as resistant, warning of unclear therapeutic effect, or prescribing only in consultation with a clinical microbiologist or infectious disease specialist (J.A.J.W Kluytmans *et al*. 2021). Future studies on the optimal treatment of Enterobacterales containing different plasmids AmpC variants are needed.

Conclusion

The presence of plasmid AmpC in Enterobacterales may affect the patient's treatment options. Although the prevalence of plasmid AmpC producing Enterobacterales in the Netherlands is lower than the prevalence of ESBL-producing Enterobacterales, it is important to be aware of this resistance mechanism. Since empirical treatment of infections in the Netherlands is currently based on the 2nd and 3rd generation cephalosporins, detection of AmpC- beta-lactamase producing Enterobacterales is critical, so that any increase in this resistance mechanism can be halted at an early stage. Attention to the detection of AmpC producing Enterobacterales and limiting the spread of this resistance mechanism can ensure that antibiotic resistance within the Netherlands remains limited in the future.

Directions for practice

- The presence of plasmid AmpC- producing Enterobacterales may affect a patient's treatment due to related resistance to commonly used beta-lactam antibiotics.
- Diagnostics for the presence of plasmid AmpC deviates from the diagnosis for the presence of ESBL in Enterobacterales, but can be confirmed with specific phenotypic and genotypic tests in most laboratories
- Although plasmid AmpC- producing Enterobacterales are currently still limited in the Netherlands, attention to prevalence and transmission is desirable to prevent further spread

Chapter 3

Decline in AmpC beta-lactamase-producing *Escherichia coli* in a Dutch teaching hospital (2013-2016)

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Abstract

Objective

The objective of this study is to determine the prevalence of rectal carriage of plasmid- and chromosome-encoded AmpC beta-lactamase-producing *Escherichia coli* and *Klebsiella* spp. in patients in a Dutch teaching hospital between 2013 and 2016.

Methods

Between 2013 and 2016, hospital-wide yearly prevalence surveys were performed to determine the prevalence of AmpC beta-lactamase-producing *E. coli* and *Klebsiella* spp. rectal carriage. Rectal swabs were taken and cultured using an enrichment broth and selective agar plates. All *E. coli* and *Klebsiella* spp. isolates were screened for production of AmpC beta-lactamase using phenotypic confirmation tests and for the presence of plasmid-encoded AmpC (pAmpC) genes. *E. coli* isolates were screened for chromosome-encoded AmpC (cAmpC) promoter/attenuator alterations.

Results

Fifty (2.4%) of 2,126 evaluable patients were identified as rectal carrier of AmpC beta-lactamase-producing *E. coli*. No carriage of AmpC beta-lactamase producing *Klebsiella* spp. was found. Nineteen (0.9%) patients harboured isolates with pAmpC genes and 30 (1,4%) patients harboured isolates with cAmpC promoter/attenuator alterations associated with AmpC beta-lactamase overproduction. For one isolate, no pAmpC genes or cAmpC promotor/attenuator alterations could be identified. During the study period, a statistically significant decline in the prevalence of rectal carriage with *E. coli* with cAmpC promotor/attenuator alterations was found ($p = 0.012$). The prevalence of pAmpC remained stable over the years.

Conclusions

The prevalence of rectal carriage of AmpC-producing *E. coli* and *Klebsiella* spp. in patients in Dutch hospitals is low and a declining trend was observed for *E. coli* with cAmpC promotor/attenuator alterations.

Introduction

Antibiotic resistance caused by broad-spectrum beta-lactamase production in Gram-negative bacteria is a well-known problem in clinical settings and in the community. Extended-spectrum beta-lactamases (ESBL) in Enterobacteriaceae are generally accepted as a major cause of beta-lactam resistance (David M. Livermore *et al*. 2007; Jan A. J. W. Kluytmans *et al*. 2013; Willemsen *et al*. 2015). Willemsen *et al* studied the epidemiology of ESBL rectal carriage between 2010 and 2014 in the same teaching hospital. Although the annual prevalence of ESBL was stable, a decline was seen in the proportion of certain ESBL groups, mainly CTX-M-1-1. In addition to ESBL, AmpC beta-lactamases are increasingly recognized as a growing and clinically relevant problem (Jacoby 2009; Rodríguez-Baño *et al*. 2012; Park *et al*. 2013; Vanesa Pascual *et al*. 2015; V Pascual *et al*. 2016). Most studies have focused on the dissemination of mobile genetic elements encoding these beta-lactamases (Rodríguez-Baño *et al*. 2012; Park *et al*. 2013; V Pascual *et al*. 2016). However, in certain species (e.g. *Escherichia coli* and *Shigella* spp.), AmpC beta-lactamase production is not only plasmid-encoded, but can also be caused by chromosomal hyperproduction due to mutations within the promoter/attenuator region (Jacoby 2009; Jørgensen *et al*. 2010; Alonso *et al*. 2016). However, little is known on the carriage of either plasmid-encoded (pAmpC) or chromosome-encoded AmpC (cAmpC) Enterobacteriales in hospitalised patients in the North-Western European region and no studies have been performed over a multiple year period. Moreover, screening methods for ESBL, such as ESBL selective media, may not always be optimal to screen for AmpC-producing Enterobacteriales. The present study describes the prevalence of rectal carriage with AmpC beta-lactamase-producing *E. coli* and *Klebsiella* spp. in patients in Dutch hospitals during a four-year period.

Materials and methods

Sample collection and phenotypical AmpC testing

Four yearly point prevalence surveys (PPS) were performed in the Amphia Hospital from 2013 to 2016 in the months October or November. All hospitalised patients, including patients on dialysis and day-care, were screened for AmpC carriage using rectal swabs (Eswab, Copan, Italy). After vortexing, the swab was plated on Blood Agar plate (growth control, performed since 2011) and the liquid Amies eluent was inoculated in selective tryptic soy broth, containing cefotaxime (0.25 mg/L) and vancomycin (8 mg/L) (TSB-

VC) and incubated for 18–24 hours (35–37°C). In 2013, broths were subcultured on a MacConkey agar containing cefotaxime 1 mg/L (Mediaproducts, Groningen, The Netherlands). In 2014, a switch to a more selective double MacConkey agar plate (containing on one side cefotaxime 1 mg/L, cefoxitin 8 mg/L and on the other side ceftazidime 1 mg/L, cefoxitin 8 mg/L, Mediaproducts, Groningen, the Netherlands) was made to improve sensitivity and specificity of the screening. Broths were simultaneously subcultured on both sides of an EbSA agar plate (AlphaOmega, 's-Gravenhage, Netherlands). The Extended Beta-Lactamase Screening Agar (EbSA) plate consists of a split MacConkey agar plate containing ceftazidime (1.0 mg/L) on one side and cefotaxime (1.0 mg/L) on the other side. Both sides contain cloxacillin (400 mg/L) and vancomycin (64 mg/L) for inhibition of AmpC beta-lactamase-producing bacteria and Gram-positive bacteria, respectively. Subsequently, the plates were incubated for 18–24 hours (35–37°C). AmpC producing isolates found in 2013, were retrospectively cultured on the new selective AmpC agar to confirm if they would have been detected using the new agar plate.

For all oxidase-negative isolates that grew on either side of the selective agar plates, species identification was performed by MALDI-TOF (bioMérieux, Marcy l'Etoile, France). The presence of AmpC in all *E. coli* and *Klebsiella* spp. isolates was phenotypically confirmed using the D68C AmpC & ESBL Detection Set (Mastdiscs, Mastgroup Ltd, Bootle, United Kingdom) and interpreted according to manufacturer's instructions (Ingram *et al*. 2011; Nourrisson *et al*. 2015). The presence of ESBL in isolates with a MIC of > 1 mg/L for ceftazidime and/or cefotaxime was phenotypically confirmed with the combination disk diffusion method for cefotaxime, ceftazidime, and cefepime with and without clavulanic acid (Rosco, Taastrup, Denmark)) and interpreted according to manufacturer's instructions. Minimal inhibitory concentration (MIC) values for cefotaxime (CTX), ceftazidime (CAZ) and cefoxitin (FOX) were measured using the gradient on a strip method (E-test, bioMérieux, Marcy l'Etoile, France).

Genetic confirmation of phenotypically confirmed isolates

All phenotypically confirmed *E. coli* and *Klebsiella* spp. isolates were screened for the presence of *pampC* genes using the microarray check MDR CT103 according to the manufacturer's instructions (Check-Points, Wageningen, the Netherlands) (Cuzon *et al*. 2012). In addition, all phenotypically confirmed *E. coli* isolates were subjected to Sanger sequencing of the promoter/attenuator region of the cAmpC gene using M-13 tailed primers as described by Corvec *et al.* (Stephane Corvec *et al*. 2002). The obtained sequences of each isolate were assembled and aligned against the promoter/attenuator

region of the *campC* gene of the *E. coli* K-12 strain MG1655 (GenBank database accession number U00096) using Vector NTI Advance 11 software (ThermoFisher Scientific, Waltham, USA) or CLC Genomic Workbench version 8.5 (CLC Bio, Qiagen, Hilden, Germany). Hyperproduction of chromosome-encoded AmpC was assumed when similar alterations in the promoter/attenuator region were found as described by Tracz *et al.* (Tracz *et al*. 2007).

Amplified Fragment Length Polymorphism

Isolates from patients who were admitted to the same ward and revealed the same AmpC mechanism were selected for Amplified Fragment Length Polymorphism (AFLP) to determine clonal relatedness. If a patient harboured more than one AmpC-producing isolate from different genus or species, or with different resistance mechanisms or genes, all isolates were typed. AFLP typing was performed and interpreted as described by Savelkoul *et al* (Savelkoul *et al*. 1999).

Statistical methods

All data were pseudonymized and subsequently analysed with Statistical Package for Social Science software (SPSS; IBM Corp., Armonk, New York, US; version 19). The 95% confidence intervals of proportions were calculated using the modified Wald method. A trend analysis for the prevalence of AmpC genes was performed using the Mantel-Haenzsel test for linear association. The trend result was adjusted for gender using logistic regression. Statistical significance was accepted at $p < 0.05$.

Ethical considerations

The yearly PPS for AmpC-producing *E. coli* and *Klebsiella* spp. rectal carriage is part of the routine hospital infection control policy and is approved by the management of the hospital. This is in accordance with the current regulations in the Netherlands and requires verbal consent from participating patients. According to the Dutch regulation for research with human subjects, neither medical nor ethical approval, was required to conduct the surveillance since it was part of the local hospital policy, and all data were processed anonymously. Patients who indicated that they did not want to participate in the survey were excluded (opt-out) (Willemsen *et al*. 2015).

According to the patient's preferences, samples were taken by nursing staff or by patients themselves. The Infection Control Practitioner collected information on the patient's characteristics from the medical record.

Results

A total of 2,527 patients were eligible for AmpC screening. Response rates over the four-year period are shown in Table 1. A total of 2,126 evaluable cultures (positive growth control) were collected and screened for the presence of AmpC rectal carriage. The median age of screened patients was 66 years (range 0–100 years) and 52% of the patients were female. The median length of hospital stay was 2 (range 0–79) days. In total 1,783 (84%) patients were admitted to a clinical ward, 343 (16%) were considered as day-care. The distribution of patients across various medical specialities is shown in Table 1. Over the four-year period this distribution remained stable, with the majority of patients in internal medicine (25%) and general surgery (17%) wards.

Table 1. Overview of the hospitalized patients on the days of survey, cultured patients, negative growth control, response rate, evaluable cultures, as well as baseline characteristics of screened patients in point prevalence surveys over the years (2013–2016).

	2013	2014	2015	2016	Total
Hospitalized patients on day of survey (no.)	601	652	654	620	2,527
Response rate (%)	85.9%	87.3%	86.2%	83.1%	85.6%
No rectal swab taken	85	83	90	105	363
Negative growth control	8	12	4	15	39
Patients with evaluable cultures (%)	508 (84.5%)	557 (85.4%)	560 (85.6%)	501 (80.8%)	2,126 (84.1%)
Age in years, median (range)*	65(0-93)	65(0-99)	67(0-100)	67(0-97)	66 (0-100)
Male, no. (%)*	259(51%)	268(48%)	269(48%)	231(46%)	1,027 (48%)
Hospitalization>2days, no. (%)*	231(46%)	271(49%)	222(40%)	269(54%)	993 (39%)
Length of stay on day of culture, median in days, (range)*	2(0-51)	2(0-70)	2(0-47)	3(0-79)	2 (0-79)

Table 1. Continued.

	2013	2014	2015	2016	Total
Patients in day-care, no. (%)*	66(13%)	101(18%)	114(20%)	62(12%)	343 (16%)
Medical specialty, no. (%)*					
Anesthesiology (non-ICU)	10 (2%)	9(2%)	23 (4%)	14(3%)	56 (3%)
Cardiology	46(9%)	50(9%)	47(8%)	51(10%)	194 (9%)
Geriatrics	11(2%)	11(2%)	9(2%)	12(2%)	43 (2%)
Intensive Care Unit (ICU)	17(3%)	14(3%)	14(3%)	20(4%)	65 (3%)
Internal medicine	132(26%)	134(24%)	131(23%)	126(25%)	523 (25%)
Neurology	31(6%)	35(6%)	29(5%)	32(6%)	127 (6%)
Obstetrics and gynaecology	43(8%)	21(4%)	40(7%)	20(4%)	124 (6%)
Orthopedic surgery	34(7%)	42(8%)	37(7%)	40(8%)	153 (7%)
Otorhinolaryngology	8(2%)	20(4%)	14(3%)	10(2%)	52 (2%)
Pediatrics	24(5%)	31(6%)	18(3%)	27(5%)	100 (5%)
Pulmonary diseases	32(6%)	41(7%)	43(8%)	33(7%)	149 (7%)
Surgery, cardiothoracic	17(3%)	17(3%)	24(4%)	17(3%)	75 (4%)
Surgery, general	86(17%)	94(17%)	93(17%)	89(18%)	362 (17%)
Urology	17(3%)	21(4%)	18(3%)	8(2%)	64 (3%)
Other speciality	0(0%)	17(3%)	20(4%)	2(0%)	39 (2%)

**Data and percentages based upon evaluable cultures.*

The prevalence of AmpC rectal carriage over the four-year period is shown in Table 2. All AmpC-producing isolates were identified as *E. coli*. No AmpC producing *Klebsiella* spp were found. Eighteen of 19 (94.7%) of the pAmpC-producing isolates harboured $bla_{\text{CMY-2-}}$like, one isolate bla_{DHA}-like. The prevalence of 3rd generation cephalosporine resistant *E. coli*, based on growth on selective media in the evaluable cultures, and the amount of ESBL confirmed isolates are shown in Table 2 as well.

Table 2. Prevalence of patients with AmpC-producing *E. coli* and distribution of pAmpC genes and cAmpC hyperproducers over the years.

	2013	2014	2015	2016	Overall
Total *E.coli* cultured on selective media (no. and %)[1]	43 (8.5%)	48 (8.6%)	41 (7.3%)	20 (3.9%)	152 (7.1%)
Total AmpC isolates (no. and %)[2]	19(3.7%)	16(2.9%)	9(1.6%)	6(1.2%)	50(2.4%)
Primary (no.)	18	14	9	6	47
Secondary (no.)[2]	1	2	0	0	3
AmpC genes (incl. secondary cases)					
$bla_{\text{CMY-2-like}}$	7	6	2	3	18
$bla_{\text{DHA-like}}$	0	0	0	1	1
cAmpC hyperproducers	11	10	7	2	31
Inconclusive[4]	1				
Total ESBL isolates (no. and %)[5]	25 (4.9%)	29 (5.2%)	32 (5.7%)	13 (2.6%)	99 (4.7%)

1= Number of patients with E. coli rectal carriage based on selective media divided by the number of patients with evaluable cultures
2 = Number of patients with AmpC-producing E. coli rectal carriage divided by the number of patients with evaluable cultures
3 = Number of patients with isolates that were clonally related based on AFLP
4 = negative microarray results for pAmpC genes, but AmpC amplicon couldn't be amplified
5 = Number of patients with ESBL-producing E. coli rectal carriage divided by the number of patients with evaluable cultures

All but one (30 out of 31) of the isolates that were phenotypically AmpC producers and negative for pAmpC in the microarray, showed alterations in the promoter/attenuator

region associated with cAmpC hyperproduction (Tracz *et al*. 2007). Moreover, none of the pAmpC producing isolates (n = 19) showed mutations that are associated with cAmpC hyperproduction.

One isolate could not be amplified using Sanger sequencing technique and was negative in Micro-array which was therefore considered as inconclusive. An overview of all alterations in the promoter/attenuator region as well as MIC values for cefotaxime, ceftazidime and cefoxitin can be found in the additional S1 Table.

In 2013, two bla_{CMY-2}-like producing *E. coli* isolates from two patients were confirmed to have the same AFLP pattern. Three cAmpC producing *E. coli* (all Mulvey Type 3) from three different patients in 2014 showed a similar AFLP pattern as well. AFLP patterns can be found in the additional data S1 Fig.

A declining trend was seen in the overall prevalence of AmpC in *E. coli* isolates using Mantel-Haenzsel test for linear association (p = 0.006). When probable genetically related AmpC isolates based upon AFLP were excluded the decline was still significant. The decrease was only significant for cAmpC hyperproducers (p = 0.012) and not for pAmpC (p = 0.287) (Figs 1 and 2). A univariable and multivariable logistic regression analysis was performed on the cAmpC rectal carriage prevalence over the four-year period (2013–2016) adjusted for gender (univariate analysis, p = 0.15) (see supplementary data, S2 Table).

Figure 1. Prevalence of cAmpC hyperproducing *E. coli* from 2013 to 2016 with 95% confidence interval.

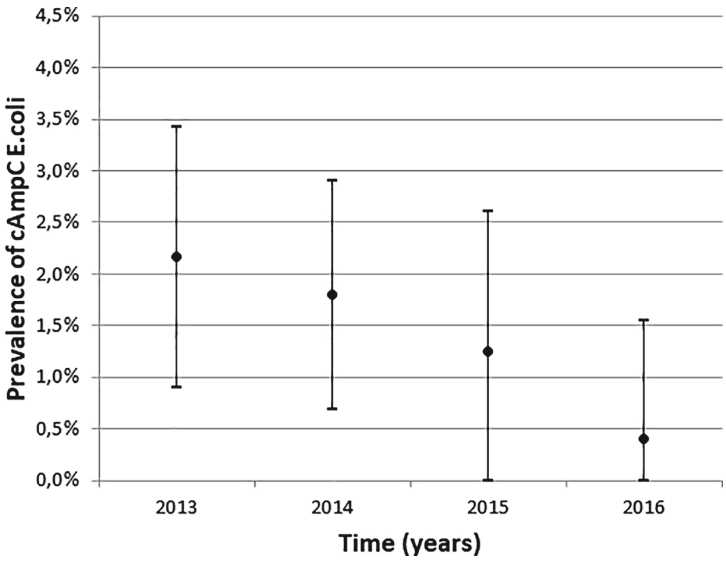
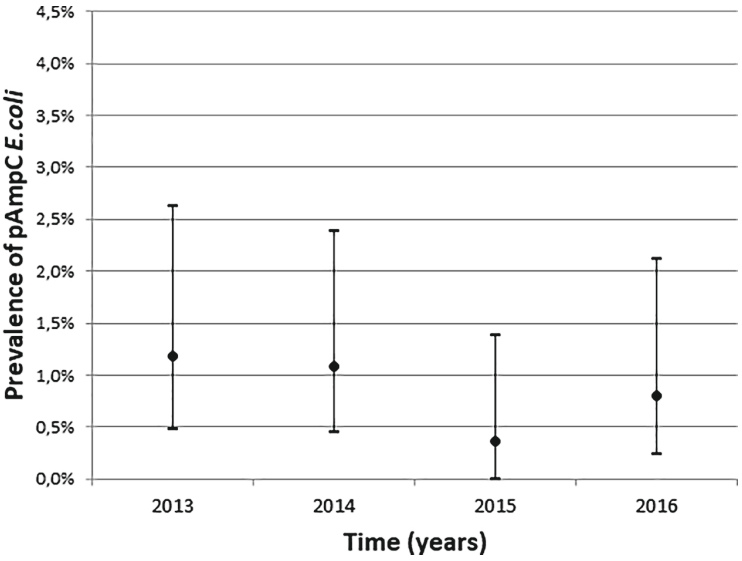


Figure 2. Prevalence of pAmpC producing *E. coli* from 2013 to 2016 with 95% confidence interval.



Discussion

The present study shows a significant decline in the prevalence of cAmpC producing *E. coli* measured in four yearly point prevalence surveys. The pAmpC prevalence remained stable over time. To the best of our knowledge, there are no other studies published on trends in rectal carriage of both cAmpC and pAmpC positive isolates in hospitalised patients, which prohibits a comparison of trends with other studies.

Few studies have looked at pAmpC carriage in hospitalised patients. Garrido *et al* found a low prevalence of 0.59% pAmpC in faecal samples of a Spanish hospitalised population (Garrido *et al*. 2014). In the same year Husickova *et al.* published a survey on rectal carriage of pAmpC producing isolates and found a prevalence of 0.3% (Husickova *et al*. 2012). These results are slightly lower in comparison to our study. However, both studies did not use a (selective) pre-enrichment broth, which may explain the lower pAmpC prevalence (M. F.Q. Kluytmans-Van Den Bergh *et al*. 2015). No studies were performed on the occurrence of rectal carriage of cAmpC isolates in hospitalised patients. Data on the prevalence of AmpC rectal carriage in the community within the North Western European region is more extensively described, although not all studies screened or confirmed cAmpC production in *E. coli* isolates. In Norway, Ulstad *et al.*

found a carriage rate of 3.2% of phenotypically confirmed AmpC-producing E.coli and 0.7% pAmpC producers based on PCR and WGS (Ulstad *et al*. 2016). In the Netherlands three studies on AmpC prevalence were performed in the community. A random sample of adult volunteers (n = 1033) by van Hoek *et al.* found an overall AmpC prevalence of 1.4%, consisting of 0.6% pAmpC and 0.8% cAmpC. Koningstein *et al.* screened faecal samples of day-care centre attending children and found an AmpC rectal carriage rate of 2.4%, with 1.2% bla_{CMY-2} producing *E.coli* and 1.1% cAmpC hyperproducers (Van Hoek *et al*. 2015; Koningstein *et al*. 2015). Finally, a study by Reuland *et al*., that screened adult volunteers in the region of Amsterdam found 1.3% carriage of pAmpC producing *E. coli* (E. Ascelijn Reuland *et al*. 2015). These results correspond with the prevalence rates in the present study for 2013 and 2014 for pAmpC and cAmpC. The three Dutch studies used pre-enrichment broths, but applied different screening tests. No studies have looked at trends over time in rectal carriage of AmpC. To our knowledge, this study is the first to show a declining trend of cAmpC within the North-Western European region.

Based on the mentioned criteria in combination with AFLP, few cases of probable genetic clonality were detected. As not all isolated were subjected to strain typing a conclusion on epidemiological relationship cannot be made. Although we noticed heterogeneity of cAmpC alterations over the years, we cannot fully rule out the possibility of clustering of cAmpC isolates in the strains that were not subjected to AFLP during the first years. Future analysis is needed to clarify the situation of clonal spread and the possibility of horizontal spread of resistance genes.

Trend analysis was adjusted for gender using logistic regression analysis. Data on other risk factors known for ESBL-E carriage, which might be applicable to AmpC carriage as well, like travel history or antibiotic use before admission were not available.

A limitation of this PPS is the lack of evidence on sensitivity and specificity of screening agars used to detect AmpC producing isolates. No commercial AmpC specific screening agars are available. During the 4-year period, a change in our used screening agar was made to improve the sensitivity and specificity. The used MacConkey agar, containing cefotaxime 1 mg/L (Mediaproducts, Groningen, The Netherlands) was accepted to be sensitive for AmpC producing isolates, but not very specific, as other cephalosporin resistant Enterobacteriaceae (e.g. ESBL or K1-betalactamase producing isolates) were cultured as well. This could lead to overgrowth of isolates with other resistance mechanisms. To improve specificity, new AmpC agars were used, containing cefoxitin and cefotaxime as well as a combination of cefoxitin and ceftazidime. Retrospective screening of the 2013 isolates on the new screenings agar showed growth of all AmpC producing isolates. Unfortunately, we were not able to use the original rectal

swabs to study the new screening agar. Loss of sensitivity because of the addition of cefoxitin was expected to be negligible, as most AmpC producing isolates described in literature have increased MIC's for cefoxitin (Jacoby 2009; Tracz *et al*. 2007; Philippon, Arlet, and Jacoby 2002). The switch in media is unlikely to be the reason for the decline in AmpC. Using both cefotaxime and ceftazidime might even increase the sensitivity, using two different cephalosporin antibiotics. Only the bla_{ACC}-like producing isolates could be of concern, which are known to have lower MIC's for cefoxitin (Philippon, Arlet, and Jacoby 2002). However, we did not find any bla_{ACC}-like producing isolates with either of the used agars. Still, a comparative study on the diagnostic performance of screening agars for AmpC producing isolates, might provide more insight into this matter.

Another limitation of our study is the use of a micro-array system. Although we assume that the AmpC phenotype can be related to the microarray- and Sanger sequencing data, we cannot discard that micro-array system only recognize the six main plasmid AmpC-like groups (bla_{CMY-2}-like, bla_{DHA}-like, bla_{ACC}-like, $bla_{ACT/MIR}$-like, bla_{FOX}-like, $bla_{CMY-1/MOX}$-like). Although former studies showed robust sensitivity and specificity of the microarray check MDR CT103, it is limited to these specific targets (Cuzon *et al*. 2012; Cunningham SA ; Vasoo S ; Patel R 2016; Pierre Bogaerts *et al*. 2016).

We did not perform reverse-transcriptase PCR, as described by Trazc *et al.* (Tracz *et al*. 2007). Nevertheless, we expect that hyperproduction of AmpC is likely when known alterations in promoter and attenuator region were found as described in this paper.

It is difficult to ascertain the cause of the decrease in cAmpC during the four-year period. The link between trends in e.g. ESBL and pAmpC and the dissemination of these beta-lactamases in the environment (e.g. food products, livestock, companion animals), as well as the influence of antibiotic usage in both humans and livestock, is under debate. Carriage of cAmpC has been described in studies on veal calves, broilers and companion animals within the Netherlands (Hordijk, Wagenaar, van de Giessen, et al. 2013; Huijbers et al. 2014; C. M. Dierikx et al. 2012; Hordijk, Schoormans, et al. 2013). The study by Hordijk *et al.* showed an increase of cAmpC in veal cattle in the period 1997 to 2010 (Hordijk, Wagenaar, van de Giessen, et al. 2013). Between 2009 to 2015 a decrease of 58.4% of antibiotic sales in the livestock sector was achieved (Veldman, K.T. , Mevius, D. J. 2016). However, to our knowledge, no new data on cAmpC prevalence in livestock in the Netherlands has been published since the decrease in antibiotic sales. Therefore, we can only speculate on the possible impact of the antibiotic decrease in the veterinary sector and the decreasing trend we found.

Conclusions

Four yearly point prevalence surveys show that rectal carriage of both pAmpC and cAmpC beta-lactamase producing *E. coli* and *Klebsiella* spp. was low. This study shows a significant decline in rectal carriage of *E. coli* with cAmpC promotor/attenuator alterations in hospitalised patients in a Dutch teaching hospital during this four-year period. The underlying reasons are unclear and deserve further investigations as they may provide new insights to further control antimicrobial resistance.

Supplementary data

Supplementary table S1 for this chapter is available online via:

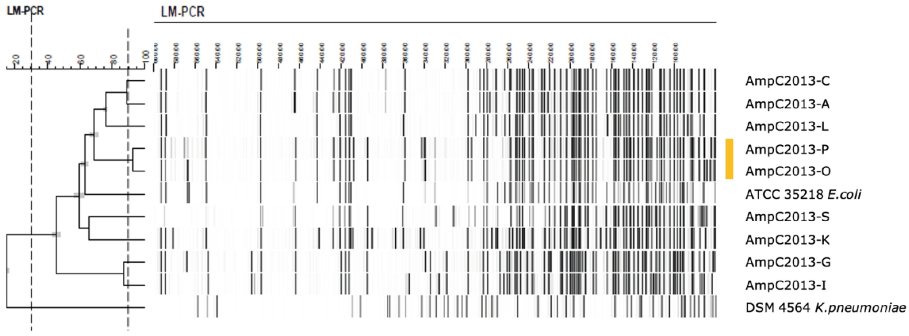
https://figshare.com/articles/dataset/Decline_in_AmpC_-lactamase-producing_i_Escherichia_coli_i_in_a_Dutch_teaching_hospital_2013-2016_/7154066

Table S2. Univariable and multivariable logistic regression analysis of cAmpC carriage during a four-year period (2013-2016) adjusted for gender. CI confidence interval.

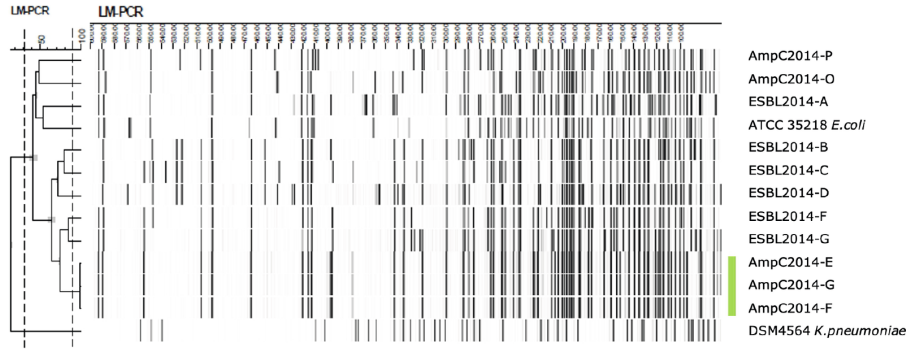
	Univariable logistic regression analysis		**Multivariable logistic regression analysis**	
	Odds Ratio	**95% CI**	**Odds Ratio**	**95% CI**
2013	reference		reference	
2014	0.826	0.348-1.962	0.813	0.342-1.932
2015	0.572	0.220-1.487	0.563	0.238-1.463
2016	0.181	0.040-0.821	0.176	0.039-0.800
Gender (male)	1.628	0.771-3.438	1.681	0.795-3.555
Short stay	1.037	0.394-2.727		
Age, median	1.004	0.988-1.021		

Figure S1. AFLP patterns from all AmpC producing *E. coli* eligible for cluster analyses (2013 & 2014)

A. *E.coli* 2013



B. *E.coli* 2014



AFLP patterns from all AmpC producing E. coli eligible for cluster analyses. Strains clustering with a similarity between 90 and 100% were defined as identical strains. Strains clustering with a similarity above 35% were defined as different strains of the same species and strains clustering with a similarity below 35% were defined as different species. Identical strains are indicated in colour. Each strain was coded with type of resistance mechanism, the number of the year in combination with a letter. ATCC 35218 E.coli and DSM 4564 K.pneumoniae were used as reference strains. In 2014 a number of E.coli ESBL strains were selected for AFLP typing as well (further data on ESBL strains not shown is this publication).

Chapter 4

Development of an algorithm to discriminate between plasmid- and chromosomal-mediated AmpC beta-lactamase production in *Escherichia coli* by elaborate phenotypic and genotypic characterization

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Abstract

Objectives

AmpC-beta-lactamase production is an under-recognized antibiotic resistance mechanism that renders Gram-negative bacteria resistant to common beta-lactam antibiotics, similar to the well-known ESBLs. For infection control purposes, it is important to be able to discriminate between plasmid-mediated AmpC (pAmpC) production and chromosomal-mediated AmpC (cAmpC) hyperproduction in Gram-negative bacteria as pAmpC requires isolation precautions to minimize the risk of horizontal gene transmission. Detecting pAmpC in *Escherichia coli* is challenging, as both pAmpC production and cAmpC hyperproduction may lead to third-generation cephalosporin resistance.

Methods

We tested a collection of *E. coli* strains suspected to produce AmpC. Elaborate susceptibility testing for third-generation cephalosporins, WGS and machine learning were used to develop an algorithm to determine *ampC* genotypes in *E. coli*. WGS was applied to detect *pampC* genes, cAmpC hyperproducers and STs.

Results

In total, 172 *E. coli* strains (n=75 ST) were divided into a training set and two validation sets. Ninety strains were pampC positive, the predominant gene being $bla_{\text{CMY-2}}$ (86.7%), followed by $bla_{\text{DHA-1}}$ (7.8%), and 59 strains were cAmpC hyperproducers. The algorithm used a cefotaxime MIC value above 6 mg/L to identify pampC-positive *E. coli* and an MIC value of 0.5 mg/L to discriminate between cAmpC-hyperproducing and non-cAmpC-hyperproducing *E. coli* strains. Accuracy was 0.88 (95% CI=0.79–0.94) on the training set, 0.79 (95% CI=0.64–0.89) on validation set 1 and 0.85 (95% CI=0.71–0.94) on validation set 2.

Conclusions

This approach resulted in a pragmatic algorithm for differentiating *ampC* genotypes in *E. coli* based on phenotypic susceptibility testing.

Introduction

Escherichia coli is an important pathogen in both community and healthcare-associated infections (Weinstein, Gaynes, and Edwards 2005; J. D. D. Pitout 2012). ESBL-producing *E. coli* have spread worldwide, restricting available treatment options. Although to a lesser degree, acquired AmpC beta-lactamases in *E. coli* are also emerging as a potential threat to the activity of broad-spectrum penicillins and third-generation cephalosporins (3GCs). Acquired AmpC beta-lactamases are encoded on plasmids and hence transferable between species. The prevalence of plasmid-mediated AmpC (pAmpC) beta-lactamases in *E. coli* clinical isolates reported in the literature varies between 0.06% and 10.1% (Jørgensen *et al*. 2010; Ding *et al*. 2008); however, variance in prevalence is likely to be influenced by diagnostic strategies used in these studies, and there are also regional differences in prevalence. In the Netherlands, a country with low levels of antimicrobial resistance, a pAmpC prevalence between 0.6% and 1.3% was found in *E. coli* isolates recovered from faecal samples in the community (E. Ascelijn Reuland *et al*. 2015; Van Hoek *et al*. 2015). Recently, Harris *et al*.[7] described pAmpC as the second most common group (17.1%) of 3GC-hydrolysing beta-lactamases in *E. coli* bloodstream infections in Australia, New Zealand and Singapore. Different types of plasmid-mediated *ampC* (*pampC*) genes have been detected in Enterobacterales, with $bla_{\text{CMY-2}}$ as the most common AmpC-encoding resistance gene (Harris *et al*. 2018). Other, less frequently isolated AmpC beta-lactamase genes are other varieties of bla_{CMY}, as well as bla_{DHA}, bla_{ACT}, bla_{ACC}, bla_{MIR}, bla_{MOX}, bla_{FOX} and bla_{CFE}. Depending on the type of pAmpC beta-lactamase, the hydrolysing capability might vary (Jacoby 2009; Philippon, Arlet, and Jacoby 2002).

E. coli naturally carries a chromosomal-mediated *ampC* (*campC*) gene, but unlike in other Enterobacterales this gene is non-inducible (Jacoby 2009). In *E. coli* AmpC production is regulated by promoter and attenuator mechanisms resulting in constitutive low-level *ampC* expression and hence allows the use of beta-lactam antibiotics to treat these *E. coli* infections in the absence of other resistance mechanisms. Various mutations in the promoter/attenuator region of *E. coli* may cause constitutive hyperexpression of *campC*. These *E. coli* strains may then become resistant to cephamycins, broad-spectrum penicillins or even 3GCs, making it difficult to differentiate these strains phenotypically from pAmpC enzyme production.

In contrast to hyperexpressed *campC* genes, *pampC* genes are capable of spreading this resistance mechanism to other bacteria within a hospital setting by horizontal gene transfer (San Millan 2018; Rozwandowicz *et al*. 2018). This poses a greater threat to

infection control than pure clonal transfer. Consequently, pAmpC production in *E. coli* requires active detection and contact precautions for colonized or infected patients, as recommended by different guidelines (*Australian Guidelines on Prevention and Control of Infection in Healthcare* 2010; RCPI 2012); however, this is often ignored due to the more cumbersome identification in the microbiological laboratory.

Current commercial phenotypic AmpC confirmation tests fail to reliably discriminate between pAmpC and constitutive hyperproduction of the chromosomal-mediated AmpC (cAmpC) (Ingram *et al*. 2011). In *E. coli*, an approach solely based on phenotypic testing has a high sensitivity to detect pAmpC production, but lacks specificity as it detects a high number of isolates that overproduce cAmpC, resulting in unnecessary patient isolation precautions with increased unnecessary healthcare costs. PCR is capable of detecting various *pampC* genes (Javier Pérez-Pérez and Hanson 2002). The recommended method for detection of pAmpC production in Enterobacterales according to the EUCAST guidelines is to screen isolates for cefoxitin MICs >8 mg/L combined with phenotypic resistance to cefotaxime and/or ceftazidime (Martinez and Simonsen 2017). Confirmation is advised in a two-step algorithm using cloxacillin synergy detection and PCR to discriminate *pampC* from hyperexpressed *campC* in *E. coli*. Several studies suggest the screening of isolates in a similar fashion (Polsfuss *et al*. 2011; Edquist *et al*. 2013). However, molecular tests are not always available in laboratories and are relatively expensive and often time-consuming.

The aim of this present study was to evaluate various diagnostic approaches through determining the MICs of specific cephalosporins, two commercial AmpC disc-diffusion confirmation tests and WGS to develop an algorithm to detect pAmpC production in ESBL-negative and cefoxitin-resistant *E. coli*.

Materials and methods

Overall study design

Three datasets consisting of *E. coli* cefoxitin-resistant and ESBL-negative strains were identified. Most strains were suspected of having either a pAmpC or a cAmpC resistance mechanism. All strains were subjected to WGS to obtain the genotypes [*pampC*, *campC*, promoter mutations (hyperproducer) and absence of both (negative)] and subjected to Etests and two AmpC disc-diffusion confirmation tests. The training set contained a wide variety of phenotypes and was used as input for constructing an algorithm to classify the three genotypes (*pampC*, hyperproducer and negative). The most accurate

algorithm was selected as the final algorithm and validated in two validation sets. Validation set 1 was used to validate the algorithm and represents the epidemiology in a Dutch hospital setting. Due to a low number of *pampC*-positive strains and restricted geographical background we broadened the representation of suspect AmpC-producing isolates in a second validation set (validation set 2). An extensive description of the selection of samples in the training set, validation set 1 and validation set 2 can be found in the Supplementary Materials and methods (available at *JAC* Online).

Etests and AmpC disc-diffusion confirmation tests

Deep-frozen samples of the selected strains were recultured on Columbia III agar (BD Diagnostic Systems, Sparks, MD, USA) or blood agar (Media production, Elizabeth-Tweesteden Hospital, Tilburg, the Netherlands) prior to testing. Strains were tested using Etest (bioMérieux, Marcy-l'Étoile, France) to determine the MICs of cefotaxime, ceftazidime and cefoxitin. Etests were placed on Mueller–Hinton (Oxoid Ltd, Altrincham, Cheshire, England) culture plates, which were placed in the oven within 15 min and incubated for 16–20 h under an O_2 atmosphere at 36°C. Exact MIC values were noted. The presence of AmpC was phenotypically confirmed using the AmpC Confirm Kit (Rosco Diagnostica A/S, Taastrup, Denmark) according to the manufacturer's guidelines. A second phenotypical confirmation with the D68C AMPC + ESBL detection set (MAST Group Ltd, Bootle, UK) was performed according to the manufacturer's guidelines. From both confirmation tests the zone inhibition differences, measured in millimetres, were recorded for further use.

DNA isolation, library preparation and DNA sequencing

For logistical reasons DNA isolation, library preparation and DNA sequencing were performed at two different centres. For training and validation set 2, bacterial DNA was extracted by a CTAB-based method and a paired-end 2 × 150 bp library was sequenced using an Illumina NextSeq500 sequencer (Illumina, San Diego, CA, USA) (see the Supplementary Materials and methods). For validation set 1, bacterial DNA was extracted using the MagNA Pure LC Total Nucleic Acid Kit - High Performance on a MagNA Pure LC instrument (Roche Diagnostics International Ltd, Rotkreuz, Switzerland) according to the manufacturer's protocol. A 2 × 300 bp paired-end library was sequenced on an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA) (Supplementary Materials and methods).

WGS analyses

Sequence reads were demultiplexed and merged to obtain fastq files for each sample. Reads were quality assessed and adapter trimmed by Trim_galore (version 0.4.1)[19] followed by a custom NextSeq read cleaning script to remove reads containing six or more As and Gs introduced by the sequencing chemistry (Martin 2011). Read coverage was calculated by dividing the number of sequence bases for each sample by the length of *E. coli* K-12 strain C3026 (RefSeq: NZ_CP014272.1). Samples not exceeding 30× read coverage were excluded for further analyses and samples containing >120× read coverage were subsampled to 120×. Reads were *de novo* assembled to create contigs by SPAdes (version 3.11.1) using default settings and *k*-mer sizes 21, 41, 61, 81 and 101 (Bankevich *et al*. 2012). MLST STs were derived from the contigs using mlst (version 2.5 pubMLST, 31 October 2017) (Jolley and Maiden 2010; Seemann, T, n.d.).

Plasmid-mediated ampC detection

To detect *pampC* genes, contigs were BLASTed (version 2.2.30+) against the ResFinder database (2018-02-16) using abricate (version 0.5) (Altschul *et al*. 1990; Camacho *et al*. 2009; Seemann, T, n.d.). Genes that had a coverage of ≥90% and a sequence identity >75% were interpreted as present. To circumvent the absence of genes due to wrong assembly, *pampC* genes were validated using KMA (version 0.14.3) with the ResFinder database (2018–02-16), which is a method that uses raw sequences as input (Clausen, Aarestrup, and Lund 2018). Genes were marked present if KMA matched >90% coverage and >90% identity. Finally, *pampC* genes were considered present if both methods reported an identical gene and the strain was labelled *pampC* accordingly.

Detection of ampC hyperproducer genotype

The promoter and attenuator region of *campC* was extracted from all samples to obtain a similar 271 bp fragment, as described by Peter-Getzlaff *et al* (Peter-Getzlaff *et al*. 2011). The sequence of each strain was aligned against the promoter/attenuator region of the *campC* gene of the *E. coli* K-12 strain MG1655 (GenBank accession number U00096.3) using AliView (version 1.23) (Larsson 2014). Strains were labelled cAmpC hyperproducer when promoter mutations were found, as reported by Caroff *et al* and Tracz *et al* (Caroff N, Espaze E, Gautreau D, Richet H 2000; Tracz *et al*. 2007).

Creating an algorithm based on the training set

For the decision tree model, Recursive Partitioning And Regression Trees (RPART), an R package (version 4.1-13), was used; this is an implementation of Classification and Regression Tree (CART), a statistical technique to solve classification problems, developed by Breiman *et al* (Breiman *et al*. 1984). RPART was used to create a decision tree model to classify strains based on Etest MICs, AmpC Confirm Kit or D68C test results into a *pampC*, hyperproducer or negative class. Model optimization and cross-validation were performed within the caret R package (version 6.0-80) in R (version 3.5.1)(R Core Team 2018). The RPART model was trained to optimize for accuracy and by using seed 825 to be able to reproduce model creation. The cross-validation was performed using a 10-fold three-times-repeated cross-validation using the *repeatedcv* parameter. Student's *t*-test was used to compare model performances (P=0.05). A two-class model was derived from the three-class model by combining the negatives with the hyperproducer class and recalculating the statistics.

Results

Training set

Between January 2014 and March 2018, 267 *E. coli* strains that had cefoxitin MICs >8 mg/L and were ESBL negative were found in the laboratory information management system at Radboudumc. Out of these strains, 98 were selected for further testing. Eleven of these strains could not be retrieved from the freezer and three strains were identified as not being *E. coli* by MALDI-TOF MS. This resulted in a training set of 84 *E. coli* strains. MICs determined using the BD Phoenix System indicated that the training set likely consisted of a wide variety of different resistance phenotypes. A substantial proportion of strains were resistant to both ceftazidime and ceftriaxone (42.9%, *n*=36) (Table 1), 20 strains (23.8%) were susceptible to 3GCs and 28 strains (33.3%) were intermediate or resistant to at least one of the 3GCs. WGS results revealed that 32 of 84 *E. coli* strains (38.1%) contained $bla_{\text{CMY-2}}$ and 29.8% (*n*=25) showed known mutations in the *ampC* promoter region and were therefore labelled as hyperproducers, 20.2% (*n*=17) were negative for both *pampC* genes and mutations in the promoter region of *campC* and were classified as negative (Figure S1A, available as Supplementary data at *JAC* Online).

Table 1. BD Phoenix System susceptibility of 84 *E. coli* strains in the training set

Cefoxitin (R>8 mg/L)[a]	**Ceftriaxone (S ≤1 mg/L; R >4 mg/L)**[b]	**Ceftazidime (S ≤1 mg/L; R >4 mg/L)**[b]	***n***	**Percentage**
R	S	S	20	23.81
R	S	I	13	15.48
R	S	R	8	9.52
R	R	S	1	1.19
R	I	I	1	1.19
R	R	I	1	1.19
R	I	R	4	4.76
R	R	R	36	42.86
			total=84	total=100.00

R, resistant; S, susceptible; I, intermediate.
a MIC cut-off adapted from EUCAST guideline on detection of resistance mechanisms v2.0.
b MIC breakpoints according to EUCAST clinical breakpoints for bacteria v.9.0.

Validation sets 1 and 2

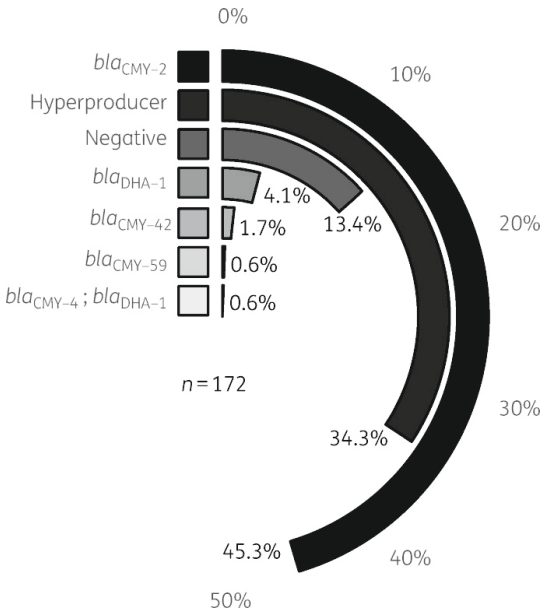
Validation set 1 consisted of 47 clinical *E. coli* strains. WGS results showed that 72.3% (*n*=34) of the strains were hyperproducers and 12.8% (*n*=6) were *pampC* and hyperproduction negative. Two *pampC* variants were found, 12.8% (*n*=6) $bla_{\text{CMY-2}}$ and 2.1% (*n*=1) $bla_{\text{DHA-1}}$ (Figure S1B). To cope with the low number of *pampC*-positive strains in validation set 1, validation set 2 (*n*=41) consisted of *pampC*-positive strains with mainly $bla_{\text{CMY-2}}$ (97.6%) (Figure S1C).

Genomic composition

In total, the 172 *E. coli* strains represented 75 different MLST STs, of which ST131 (8.14%, *n*=14), ST38 (6.98%, *n*=12) and ST73 (6.98%, *n*=12) were the most prevalent. Furthermore, the STs of 13 strains were unknown (see Table S1). For the identification of *pampC* genes we found that there was 100% concordance between the tools abricate and KMA, which supports the accurate detection of *pampC* genes from WGS data.

Overall, in 172 *E. coli* strains, bla_{CMY-2} was the most prevalent (45.3%) resistance mechanism followed by hyperproducers (34.3%) (Figure 1).

Figure 1. Clockplot showing the distribution of ampC genotypes in all 172 *E. coli* strains. The key is sorted in decreasing order of occurrence. Half a circle indicates 50%; each genotype fills part of the circle to indicate the percentage of each genotype.



Etests and AmpC disc diffusion confirmation tests

By combining the WGS results with the Etest results, we found higher median MICs of cefoxitin, ceftazidime and cefotaxime for strains that harbour a *pampC* gene (cefoxitin median=256 mg/L; ceftazidime median=10 mg/L; cefotaxime median=12 mg/L) compared with hyperproducers (cefoxitin median=48 mg/L; ceftazidime median=2 mg/L; cefotaxime median=1.5 mg/L) and negatives (cefoxitin median=32 mg/L; ceftazidime median=0.38 mg/L; cefotaxime median=0.38 mg/L) (Figure S2). Furthermore, zone inhibition differences found with the AmpC Confirm Kit showed higher zone inhibition differences in the *pampC* strains (ceftazidime + cloxacillin versus ceftazidime median=12 mm; cefotaxime + cloxacillin versus cefotaxime median=8 mm) compared with negative strains (ceftazidime + cloxacillin versus ceftazidime median=3 mm; cefotaxime + cloxacillin versus cefotaxime median=1 mm). However, *pampC*-positive strains showed more overlap with the hyperproducer group (ceftazidime + cloxacillin versus ceftazidime median=8 mm; cefotaxime + cloxacillin versus cefotaxime

median=7 mm) as compared with the AmpC-negative group (Figure S3). The boxplots of the D68C test illustrate that there was no clear separation between hyperproducer (D68C C-A median=15 mm; D68C D-B median=14 mm) and *pampC*-positive strains (D68C C-A median=15 mm; D68C D-B median=15 mm) based on zone inhibition differences (Figure S4).

MICs in relation to the presence of different ampC genes

A ridge plot was generated to visualize the Etest MICs for each genotype for all 172 *E. coli* strains (Figure 2). The plot reveals that negative strains showed MICs of ceftazidime of ≤4 mg/L and of cefotaxime of ≤3 mg/L. For hyperproducers, MICs of ceftazidime were predominantly in the range of 0.75–12 mg/L and cefotaxime MICs were in the range of 0.38–4 mg/L. Isolates that harboured bla_{CMY} showed ceftazidime MICs of 1.5–256 mg/L and cefotaxime MICs of 1.5–32 mg/L. In contrast, bla_{DHA-1}-positive strains showed lower MICs of 3GCs (ceftazidime 2–8 mg/L and cefotaxime 1–4 mg/L), which overlapped with MIC ranges for hyperproducing strains.

Performance of susceptibility tests to predict ampC type

Training of the RPART model and the cross-validation on the training set (n=84) were performed to predict whether strains have a negative, hyperproducer or *pampC* genotype. The model indicated that training with Etest MICs performed best (Figure 3). It had the highest average accuracy (0.83) and the performance was significantly better than the AmpC Confirm Kit (0.73) and the D68C test (0.67). Furthermore, cross-validation using Etest MICs resulted in the smallest quartile, implying that the model could be extra stable when applied to other datasets. Therefore, we selected the decision tree trained on Etest MICs as the final decision tree model to test performance on training and validation sets.

Figure 2. Ridge plot of Etest MICs for 172 *E. coli* strains grouped by genotype. The x-axis indicates MICs in mg/L. The left-hand y-axis indicates genotypes of strains. The right-hand y-axis indicates number of counts for each MIC; counts are scaled for each Etest to enhance visibility. R, resistant; S, susceptible. a MIC cut-off adapted from EUCAST guideline on detection of resistance mechanisms v2.0. b MIC breakpoints according to EUCAST clinical breakpoints for bacteria v.9.0.

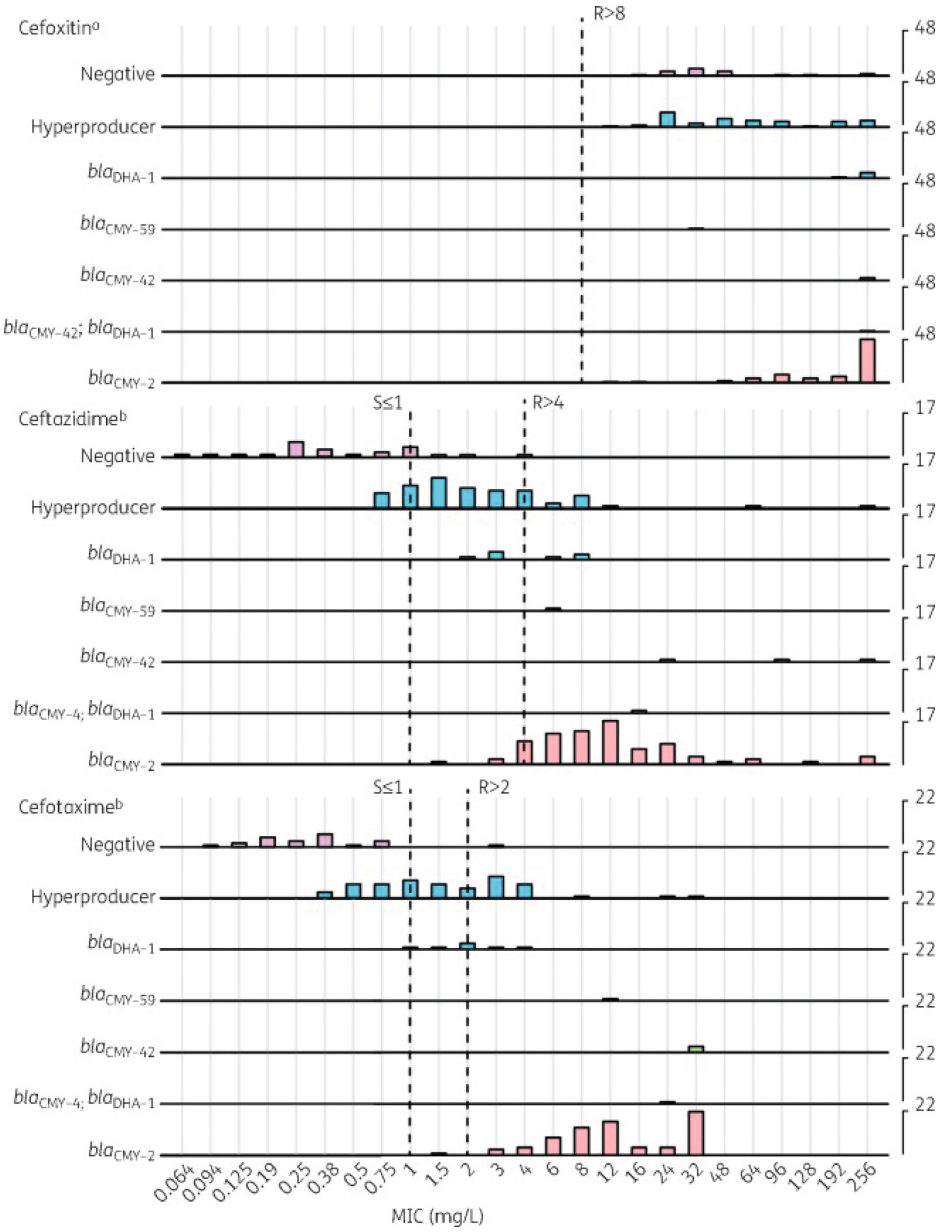
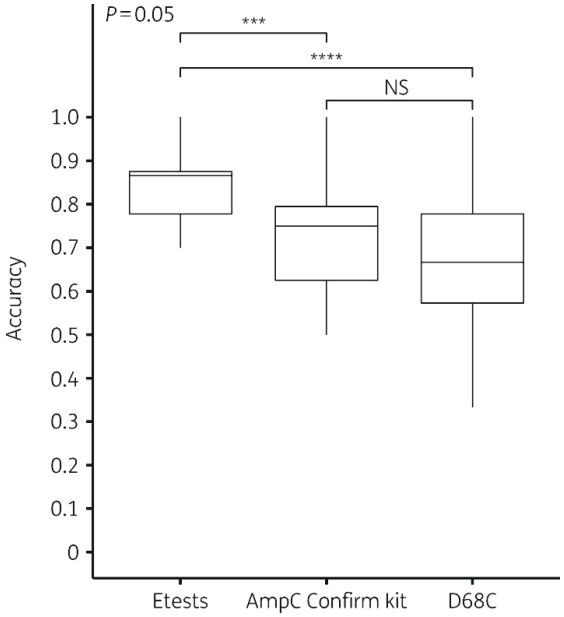


Figure 3. Boxplots of model performance. The x-axis indicates the performance using Etests, AmpC Confirm Kit and MAST D68C. The y-axis indicates accuracy based on 10-fold three-times-repeated cross-validation using all 84 *E. coli* strains of the training set. ***P ≤ 0.001; ****P ≤ 0.0001; NS, not significant.



Model description and performance

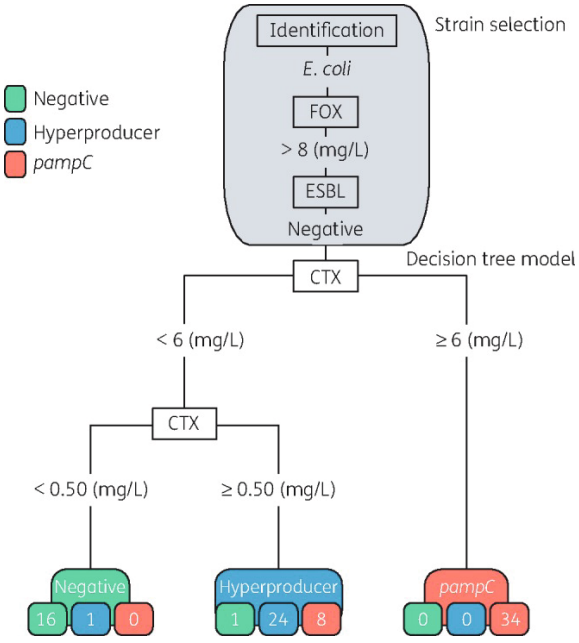
The final RPART model contained two decisions and performance was evaluated on the training set (*n*=84). In the first decision—cefotaxime with an MIC of ≥6 mg/L for all *pampC* strains (*n*=42)—34 were correctly classified as *pampC* positive (*n*=34/42). In the second decision, samples with cefotaxime MIC <6 mg/L were divided by a cefotaxime MIC breakpoint of 0.50 mg/L. With an MIC breakpoint of cefotaxime <0.50 mg/L, 16 strains were correctly classified as negative (*n*=16/17). With a cefotaxime MIC ≥0.50 mg/L, all hyperproducer strains except one were correctly classified as hyperproducers (*n*=24/25). However, nine strains categorized as hyperproducers were either negative (*n*=1/17) or *pampC* positive (*n*=8/42) (Figure 4). This resulted in an overall model accuracy of 0.88 (95% CI=0.79–0.94) (Table 2). From an infection control perspective, it is most important to distinguish *pampC* from non-*pampC*. Therefore, we recalculated the performance from a three-class model to a two-class model. The negative and hyperproducer classes were merged to a non-*pampC* class. The two-class model resulted in an accuracy of 0.90 (95% CI=0.82–0.96) with a sensitivity

and specificity of 0.81 (95% CI=0.66–0.91) and 1.00 (95% CI=0.92–1.00), respectively (Table 2 and Table S2)

Table 2. Accuracy of final decision tree model trained using the 84 *E. coli* strains of the training set on all datasets

Dataset	*n*	Three-class model, percentage accuracy (95% CI)	Two-class model, percentage accuracy (95% CI)
Training set	84	0.88 (0.79–0.94)	0.90 (0.82–0.96)
Validation set 1	47	0.79 (0.64–0.89)	0.91 (0.80–0.98)
Validation set 2	41	0.85 (0.71–0.94)	0.85 (0.71–0.94)

Figure 4. Strain selection and decision tree model based on 84 *E. coli* strains of the training set. The grey area corresponds to strain selection. The numbers in the coloured boxes indicate the numbers of strains classified according to the decision tree. The decision tree cut-off value for pampC is ≥6 mg/L. For the negative strains the cut-off is <0.50 mg/L. For the hyperproducer strains the cut-off is set to <6 mg/L followed by ≥0.50 mg/L. Cut-offs are based on Etest values. CTX, cefotaxime; FOX, cefoxitin.



Model performance on validation sets

To perform a more extensive evaluation of the decision tree, the model was tested by using two validation sets as input, as described in the Materials and methods section. The performance of the algorithm on validation set 1 (*n*=47) resulted in an accuracy of 0.79 (95% CI=0.64–0.89) (Table 2). Using validation set 2 (*n*=41), the decision tree achieved an accuracy of 0.85 (95% CI=0.71–0.94) (Table 2). More details on the performance of the two- and three-class models can be found in the confusion matrix in Table S2 and the confusion matrix in Table S3.

Discussion

To the best of our knowledge, this is the first study that combined susceptibility testing, WGS and simple supervised machine learning to develop a user-friendly algorithm to determine the likelihood of *pampC* in cefoxitin-resistant and ESBL-negative *E. coli* strains (Figure 4). The decision tree requires a single cefotaxime Etest as input, is easy to apply in most laboratory settings and results in an accurate detection of *pampC*-positive strains.

Timely and more accurate identification of *pampC* isolates improves infection control practices and minimizes unnecessary and costly isolation measures. In the current setting a genotypic confirmation is recommended to differentiate between pAmpC and cAmpC production in cefoxitin-resistant *E. coli* as phenotypic confirmation is not reliable (Martinez and Simonsen 2017). Our comparison of the AmpC Confirm Kit, D68C test and Etests shows that disc-diffusion zone differences are useful to detect AmpC production in general, but are inadequate to differentiate between pAmpC and cAmpC production (Figures S2–S4). Therefore, rapid and accurate differentiation is needed to further improve infection control policies. Introducing an Etest in combination with the proposed algorithm illustrates that accurate phenotypic detection and identification of *pampC* harbouring *E. coli* is feasible.

A relationship between 3GC resistance and the presence of pAmpC has been reported in the literature (Polsfuss *et al*. 2011; Edquist *et al*. 2013; Aarestrup *et al*. 2010). Although pAmpC-producing *E. coli* isolates in this present study showed higher MICs of 3GCs than isolates without *pampC* genes, the distributions of MIC between pAmpC and hyperproducing cAmpC isolates overlap. This overlap was mainly caused by the *E. coli* strains that produced DHA-1 enzymes. Edquist *et al* also concluded that clinical resistance to cefotaxime and/or ceftazidime as a screening criterion for

pAmpC might be useful, although discriminatory performance was more prominent when using disc diffusion as compared with MIC testing by Etest strips (Edquist *et al*. 2013). In their study, a multiplex PCR was performed to detect *pampC* genes, but there was no verification for cAmpC hyperproducers in the strain collection used. Our WGS results reliably show that phenotypic hyperproduction of cAmpC beta-lactamase can be caused by mutations in the *ampC* promoter region (Edquist *et al*. 2013). No conclusions can be drawn about mutations resulting in elevated AmpC production, in addition to previously mentioned mutations. However, there is evidence that alterations of the AmpC beta-lactamase[34,35] or changes in membrane permeability may lead to differences in cephalosporin resistance (Martínez-Martínez 2008; Nordmann, Poirel, and Nordmann 2007; Mammeri, Galleni, and Nordmann 2009). Further analysis on the incorrectly classified *campC* isolates is needed to exclude causes of cephalosporin resistance, other than the known promoter mutations.

ACT-1, DHA-1, DHA-2 and CMY-13 are inducible while other pAmpC beta-lactamases (such as CMY-2) are constitutively expressed (Jacoby 2009; Philippon, Arlet, and Jacoby 2002; Reisbig, Hossain, and Hanson 2003). Reisbig *et al* previously reported that absence of the *ampD* gene in combination with the inducible ACT-1 *pampC* gene increased MICs of 3GCs (Reisbig, Hossain, and Hanson 2003). If we assume that the inducible bla_{DHA} group might have a similar mechanism, we would expect higher ceftazidime and cefotaxime MICs in the absence of *ampD*; our strains with bla_{DHA} showed lower MICs of 3GCs compared with the non-inducible bla_{CMY-2} genes, so we can infer that *ampD* might be present; however, further analysis on the influence of *ampD* on bla_{DHA} expression is needed.

Additionally, Reisbig *et al* described the contribution of plasmid copy number of ACT-1 and MIR-1 *pampC* genes to 3GC resistance (Reisbig, Hossain, and Hanson 2003). They concluded that plasmid copy number probably impacts MIC values for *pampC*-positive strains; however, this was not substantiated (Hanson 2003). We were able to accurately detect *pampC*-positive strains even without measuring plasmid copy numbers.

A strength of the present study is that ST information on *E. coli* in our datasets was provided, which made it possible to exclude clonal origin, in contrast to other studies (Polsfuss *et al*. 2011; Edquist *et al*. 2013; Aarestrup *et al*. 2010). Though ST131, ST73 and ST38 predominated, a wide variety of STs was represented in our collection (Table S2). This is in line with other studies that report higher prevalence of ST131 and ST73 in human samples and ST38 in animal samples (Pietsch *et al*. 2018; Doumith *et al*. 2012; Miajlovic *et al*. 2016).

bla$_{CMY-2}$ is the predominant *pampC* gene in Enterobacterales in the Netherlands, which is consistent with the number of *bla*$_{CMY-2}$-positive strains in our datasets (E. Ascelijn Reuland *et al*. 2015; E. Den Drijver *et al*. 2018). The CMY group, including *bla*$_{CMY-2}$, is the most prevalent *pampC* gene (Jacoby 2009). It should be noted that a higher prevalence of other *pampC* genes could influence algorithm outcomes. For example, *bla*$_{ACC}$ will be omitted because it has a cefoxitin-susceptible phenotype (Philippon, Arlet, and Jacoby 2002). Moreover, *bla*$_{DHA-1}$ was included in our panels and use of the decision tree model resulted in a lower discriminatory value for this *pampC* variant. So, our decision tree is probably most optimal in settings with relatively high amounts of *bla*$_{CMY}$.

Strains from validation set 1 were only sequenced when D68C was positive for AmpC. Analyses of the MICs for D68C-negative samples illustrate that MICs of cefotaxime are <6 mg/L (Figure S5). Moreover, it seems unlikely that these strains would have contained *pampC*, as previous studies have shown high sensitivity and specificity with the D68C test for the detection of AmpC production (Ingram *et al*. 2011; Nourrisson *et al*. 2015).

This present study focused on *E. coli*, being the most common and well-studied pathogen (Weinstein, Gaynes, and Edwards 2005). Nonetheless, there are other species with inducible expression of *campC*, such as *Enterobacter* spp., *Citrobacter freundii* and *Pseudomonas aeruginosa* (Jacoby 2009). Our study outcomes may not be extrapolated to these other species.

In conclusion, the use of a cefotaxime MIC test is an inexpensive and relatively quick method to detect pAmpC-producing *E. coli*. Therefore, the proposed decision tree could serve as a good alternative to EUCAST guidelines, which include cloxacillin synergy testing in combination with PCR. A comparison between the two algorithms in a clinical setting may be of interest for future studies.

WGS combined with machine learning algorithms is useful to improve laboratory and infection control methods (Quainoo *et al*. 2017; Nguyen *et al*. 2019). We used a simplified version of machine learning, which is directly applicable in current settings. Results show great potential for further optimization of present microbiological methods. Future work may use an extensive amount of data and state-of-the-art machine learning to improve accuracy of beta-lactamase detection.

Acknowledgements

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Transparency declarations

None to declare.

Supplementary data

Supplementary Materials and methods

Selection of training set strains

A collection of deep-frozen *E. coli* strains was used that were cultured from routinely tested human specimens between January 2014 – March 2018 in the Radboudumc (Nijmegen, The Netherlands). Strains were routinely identified using the MALDI-TOF MS (BD Diagnostic Systems, Sparks, MD, USA) and susceptibility testing was performed using BD Phoenix System (BD Diagnostic Systems, Sparks, MD, USA). ESBL confirmation was performed using cefepime and cefepime + clavulanic acid, ESBL Confirm (Chrom. AmpC) Kit (98012) (Rosco Diagnostica A/S, Taastrup, Denmark) when ceftriaxone and/or ceftazidime MICs were > 1 mg/L according to EUCAST Clinical breakpoints - bacteria (v 9.0) ('The European Committee on Antimicrobial Susceptibility Testing. Breakpoint Tables for Interpretation of MICs and Zone Diameters, Version 9.0, 2019.', n.d.). A selection of *E. coli s*trains with an ESBL negative phenotype and a cefoxitin MIC > 8 mg/L were selected as controls for further evaluation.

Selection of validation set 1 strains

We assessed all stored *E. coli* strains cultured between 2012 and 2015 at Amphia Hospital (Breda, The Netherlands), Elisabeth-TweeSteden Hospital (Tilburg, The Netherlands) and Bravis Hospital (Roosendaal and Bergen op Zoom, The Netherlands) which represents a Dutch hospital epidemiology to minimize selection bias. Strains identified as *E. coli* on the MALDI-TOF MS or VitekMS (bioMérieux, Marcy l'Etoile, France) were retrospectively selected from a collection of deep-frozen strains originated from blood cultures. ESBL confirmation was performed using the ESBL Confirm (Chrom. AmpC) Kit (98012). Selection was based upon ESBL negative phenotype and cefoxitin MIC > 8 mg/L BD obtained using BD Phoenix System or Vitek 2 (bioMérieux, Marcy l'Etoile, France). Strains were used as input for the validation of the algorithm.

Selection of validation set 2 strains

Three different institutes (department of Medical Microbiology, Erasmus MC, Rotterdam, The Netherlands; department of Medical Microbiology and Infection Control, Amsterdam UMC location VUmc, Amsterdam, The Netherlands; Central

Veterinary Institute part of Wageningen UR, Lelystad, The Netherlands), were asked to submit *pampC* confirmed *E. coli* strains to be evaluated in our algorithm.

DNA isolation, library preparation, and DNA sequencing of training set and validation set 2

Bacterial DNA was extracted by a CTAB-based method. Colonies were resuspended in 400 µL TE buffer (10 mM Tris pH8.0, 1 mM EDTA) and 50 µL of 10 mg/mL lysozyme was added. Samples were incubated for 60 min at 37°C, where after 75 µL of 0.7 mg/mL proteinase K in 10% SDS was added followed by an incubation for 10 min at 65°C. The samples were mixed with 100 µL of 5M NaCl and 100 µL CTAB/NaCl solution (1% N-cetyl-N,N,N,-trimethyl ammonium bromide in 0.7 M NaCl) and incubated for 10 min at 65°C. DNA was further isolated using chloroform/isoamylalcohol extraction. DNA was precipitated from the aqueous phase by adding an equal amount of 2-propanol and subsequent incubation for 20 min at -20°C. Samples were centrifuged for 10 min at 11.000*g*. DNA pellets were washed with 1 mL cold 75% ethanol and centrifuged for 5 min at 11.000*g*. DNA pellets were air-dried for 15 min at room temperature and dissolved in 100 µL TE buffer. DNA samples were quantified using the QuantiFluor dsDNA system (Promega, Madison, WI, USA). A fragmented genomic DNA library was prepared using a NexteraXT DNA sample preparation kit (Illumina, San Diego, CA, USA). Library purification was performed using Agencourt AMPure XP beads (Beckman Coulter, Bread, CA, USA). Subsequent sequencing was conducted in a paired-end 2 x 150 bp mode using an Illumina NextSeq500 sequencer (Illumina, San Diego, CA, USA).

DNA isolation, library preparation, and DNA sequencing of validation set 1

Colonies were suspended in 1000 µL TE buffer. Bacterial DNA was extracted using MagNA Pure LC Total Nucleic Acid Kit - High Performance on a MagNA Pure LC instrument (Roche Diagnostics International Ltd, Rotkreuz, Switzerland) according to the manufacturer's Protocol. A total of 200 µL bacterial solution was used for DNA extraction, together with Lysis/Binding buffer and proteinase K according to kit-protocol, with an elution volume of 100 µL. DNA concentration was normalized to 0.2 ng/µl by adding TE buffer to eluate. DNA samples were quantified using the Qubit Fluorometer dsDNA 3.0 system (Thermofisher Scientific, Waltham, MA, USA). A fragmented genomic DNA library was prepared using a NexteraXT DNA sample preparation kit. Library purification was performed using Agencourt AMPure XP beads. Subsequent sequencing was conducted in a paired-end 2 x 300 bp mode using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA).

Figure S1A Clockplot showing the distribution of *ampC* genotype in 84 *E. coli* strains of the training set. The legend is sorted in decreasing order of occurrence. The half circle indicates 50%, each genotype fills part of the circle to indicate the distribution of the genotypes.

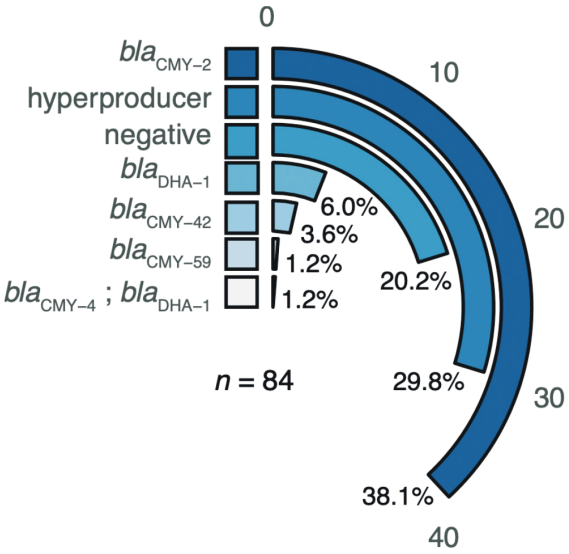

Figure S1B Clockplot showing the distribution of *ampC* genotype in 47 *E. coli* strains of the validation set 1. The full circle indicates 50%, each genotype fills part of the circle to indicate the distribution of the genotypes.

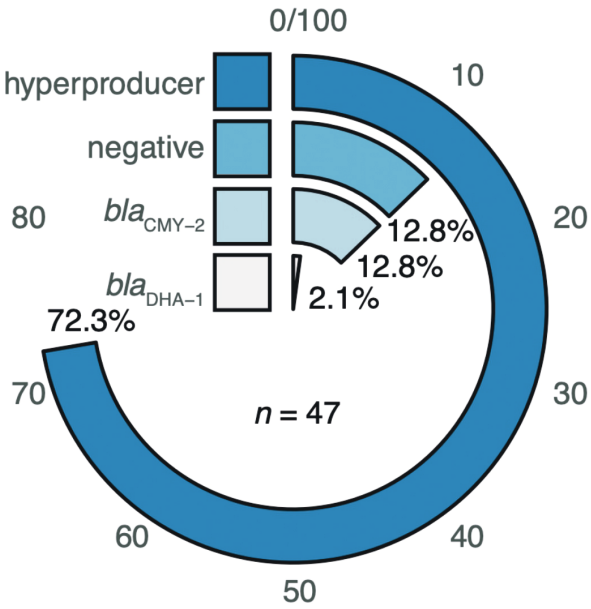

Figure S1C Clockplot showing the distribution of *ampC* genotype in 41 *E. coli* strains of the validation set 2. The full circle indicates 50%, each genotype fills part of the circle to indicate the distribution of the genotypes.

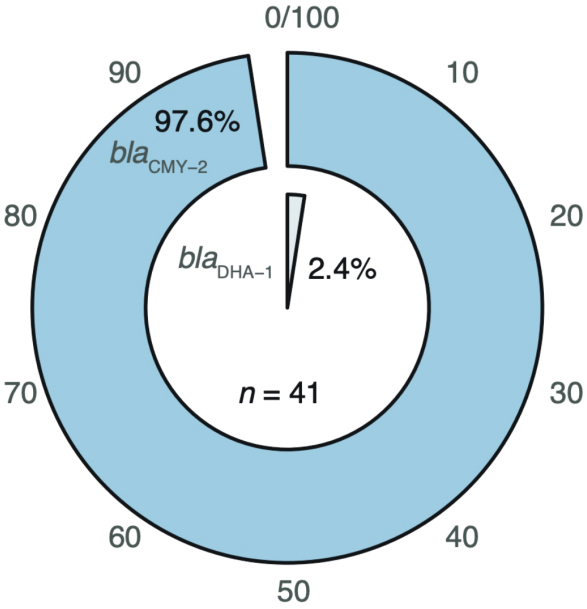

Figure S2 Boxplots of 172 *E. coli* strains E-test MICs grouped by genotype. MIC: Minimal Inhibitory Concentration, FOX: cefoxitin, CAZ: ceftazidime, CTX: cefotaxime.

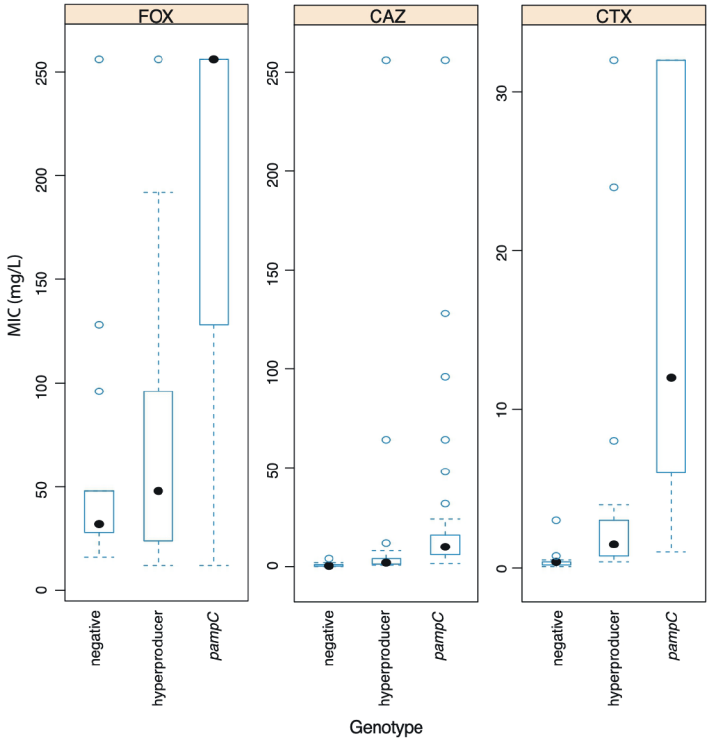

Figure S3 Boxplots of 172 *E. coli* strains AmpC confirm Kit inhibition zone differences (mm) grouped by genotype. Horizontal dashed line indicates test cutoff line as described by the manufacturer. This clearly indicates that the test is able to discriminate *pampC*-negative from hyperproducer/*pampC* but not between hyperproducer and *pampC* positive strains. CAZCX: ceftazidime with cloxacillin, CAZ: ceftazidime, CTXCM: cefotaxime with cloxacillin, CTX: cefotaxime

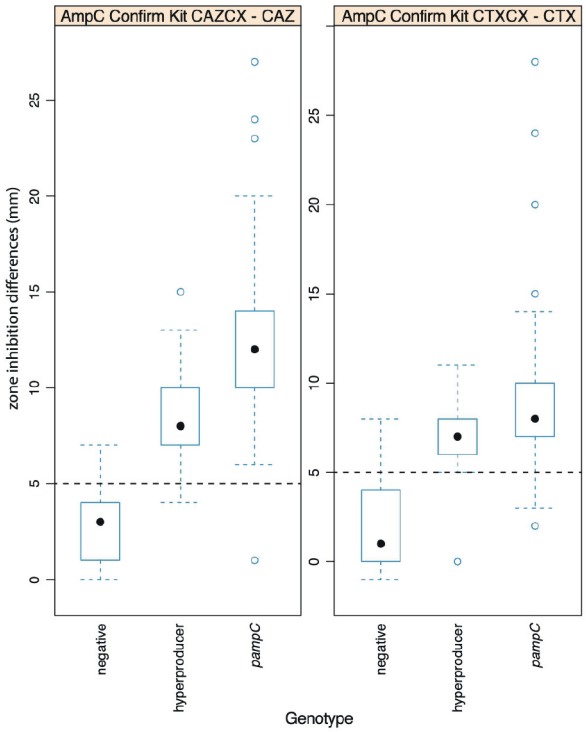

Figure S4 Boxplots of 172 *E. coli* strains MAST D68C inhibition zone differences (mm) grouped by genotype. A: cefpodoxime, B: cefpodoxime with ESBL inhibitor, C: cefpodoxime with AmpC inhibitor, D: cefpodoxime with ESBL and AmpC inhibitor

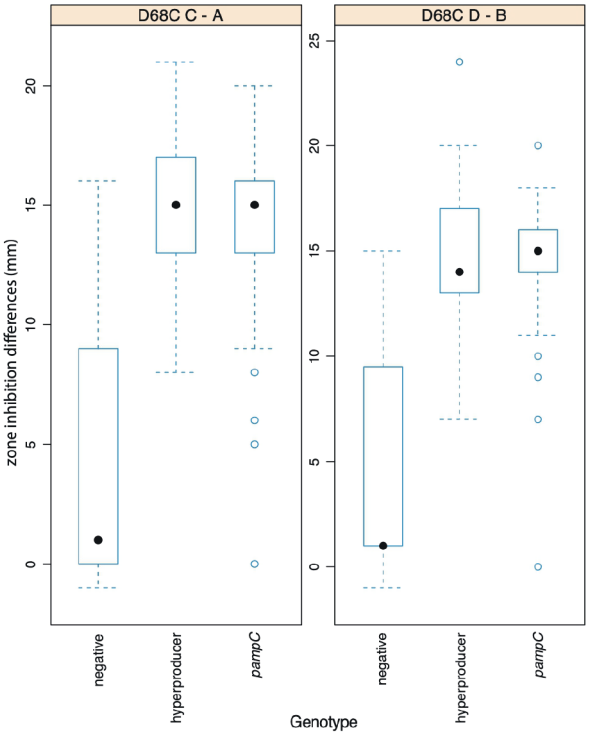

Figure S5 E-test MICs of 46 MAST D68C AmpC negative *E. coli strains* which are not included in any of the datasets but have been tested and excluded for validation set 1. Y-axis: counts indicate number of occurrences. X-axis: shows MICs in mg/L. Dashed lines indicate decision tree model MIC cutoffs. MIC: Minimal Inhibitory Concentration, FOX: cefoxitin, CAZ: ceftazidime, CTX: cefotaxime

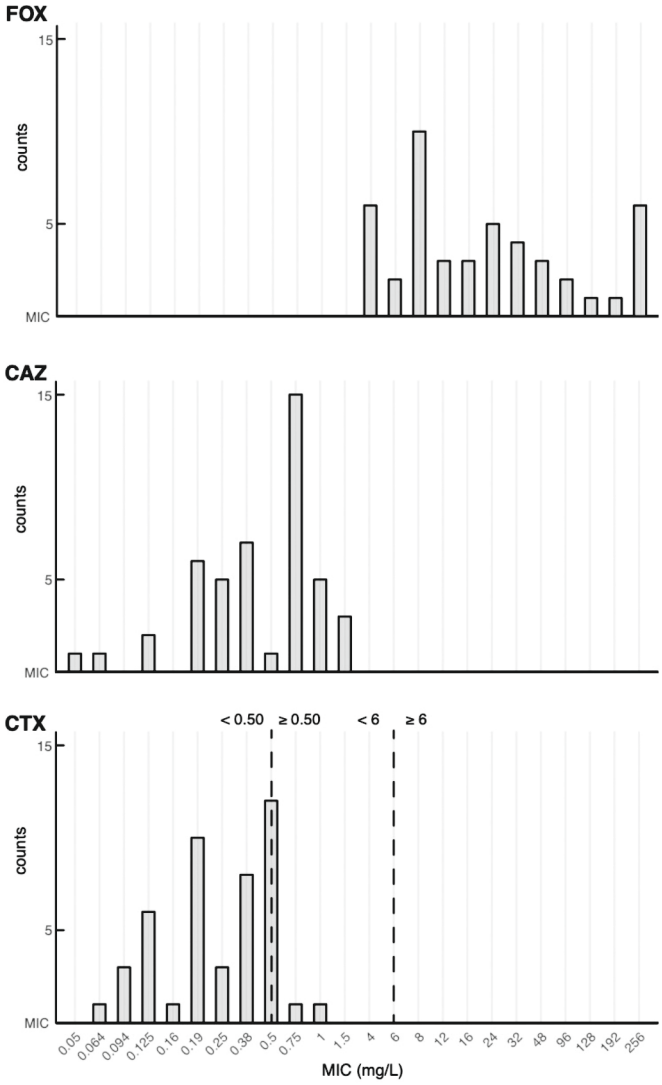

Table S1. Overview of Sequence Type, genotype and origin of all 172 *E. coli* strains

ST	Genotype	Host	*n* (%)	total per ST (% Total)
131				
	bla_{CMY-2}	human	8 (57.14)	
	hyperproducer	human	3 (21.43)	
	negative	human	3 (21.43)	14 (8.14)
38				
	bla_{CMY-2}	animal	6 (50)	
	bla_{CMY-2}	human	3 (25)	
	bla_{DHA-1}	human	1 (8.33)	
	hyperproducer	human	1 (8.33)	
	negative	human	1 (8.33)	12 (6.98)
73				
	hyperproducer	human	11 (91.67)	
	negative	human	1 (8.33)	12 (6.98)
429				
	bla_{CMY-2}	animal	5 (50)	
	bla_{CMY-2}	human	5 (50)	10 (5.81)
69				
	bla_{CMY-2}	human	7 (70)	
	bla_{DHA-1}	human	2 (20)	
	negative	human	1 (10)	10 (5.81)
88				
	bla_{CMY-2}	animal	1 (14.29)	
	hyperproducer	human	4 (57.14)	
	negative	human	2 (28.57)	7 (4.07)

Table S1. Continued.

ST	Genotype	Host	*n* (%)	total per ST (% Total)
12				
	$bla_{\text{CMY-2}}$	human	1 (16.67)	
	hyperproducer	human	5 (83.33)	6 (3.49)
95				
	hyperproducer	human	3 (75)	
	negative	human	1 (25)	4 (2.33)
405				
	negative	human	3 (100)	3 (1.74)
648				
	$bla_{\text{CMY-2}}$	human	1 (33.33)	
	$bla_{\text{CMY-4}}$; $bla_{\text{DHA-1}}$	human	1 (33.33)	
	$bla_{\text{CMY-42}}$	human	1 (33.33)	3 (1.74)
963				
	$bla_{\text{CMY-2}}$	human	3 (100)	3 (1.74)
101				
	$bla_{\text{CMY-2}}$	animal	1 (50)	
	negative	human	1 (50)	2 (1.16)
117				
	$bla_{\text{CMY-2}}$	animal	1 (50)	
	$bla_{\text{CMY-2}}$	human	1 (50)	2 (1.16)
1196				
	$bla_{\text{CMY-2}}$	animal	2 (100)	2 (1.16)
141				
	hyperproducer	human	2 (100)	2 (1.16)

Table S1. Continued.

ST	Genotype	Host	*n* (%)	total per ST (% Total)
1485				
	bla_{CMY-2}	animal	1 (50)	
	bla_{CMY-2}	human	1 (50)	2 (1.16)
345				
	hyperproducer	human	2 (100)	2 (1.16)
349				
	bla_{CMY-2}	human	1 (50)	
	bla_{DHA-1}	human	1 (50)	2 (1.16)
357				
	hyperproducer	human	2 (100)	2 (1.16)
394				
	bla_{DHA-1}	human	1 (50)	
	hyperproducer	human	1 (50)	2 (1.16)
93				
	bla_{CMY-2}	human	1 (50)	
	bla_{CMY-59}	human	1 (50)	2 (1.16)
973				
	bla_{CMY-2}	human	1 (50)	
	hyperproducer	human	1 (50)	2 (1.16)
Remaining				
				66 (38.37)
			Total	172 (100)

Table is ordered in descending order of number of strains per ST, ST: Sequence Type, Remaining: all strains from which no ST could be determined or ST occurred ones.

Table S2. Confusion matrix on performance of the 2-class decision tree model trained using the 84 *E. coli* strains of the training set on all sets

Training set (*n*=84)					**% (CI)**
		Reference		**Accuracy**	0.90 (0.82-0.96)
		non-*pampC*	***pampC***	**Sensitivity**	0.81 (0.66-0.91)
Prediction	**non-*pampC***	42	8	**Specificity**	1.00 (0.92-1.00)
	pampC	0	34		
	Total	42	42		
Validation set 1 (*n*=47)					**% (CI)**
		Reference		**Accuracy**	0.91 (0.80-0.98)
		non-*pampC*	***pampC***	**Sensitivity**	0.86 (0.42-1.00)
Prediction	**non-*pampC***	37	1	**Specificity**	0.93 (0.80-0.98)
	pampC	3	6		
	Total	40	7		
Validation set 2 (*n*=41)					**% (CI)**
		t		**Accuracy**	0.85 (0.71-0.94)
		non-*pampC*	***pampC***	**Sensitivity**	0.85 (0.71-0.94)
Prediction	**non-*pampC***	0	6	**Specificity**	NA NA
	pampC	0	35		
	Total	0	41		

CI, 95% confidence interval, NA: Not Applicable

Table S3. Confusion matrix on performance of the 3-class decision tree model trained using the 84 *E. coli* strains of the training set on all sets

Training set (*n*=84)				
		Reference		
		negative	**hyperproducer**	***pampC***
Prediction	**negative**	16	1	0
	hyperproducer	1	24	8
	pampC	0	0	34
	Total	17	25	42
Validation set 1 (*n*=47)				
		Reference		
		negative	**hyperproducer**	***pampC***
Prediction	**negative**	2	2	0
	hyperproducer	4	29	1
	pampC	0	3	6
	Total	6	34	7
Validation set 2 (*n*=41)				
		Reference		
		negative	**hyperproducer**	***pampC***
Prediction	**negative**	0	0	0
	hyperproducer	0	0	6
	pampC	0	0	35
	Total	0	0	41

Chapter 5

Detection of AmpC beta-lactamases in *Escherichia coli* using different screening agars

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Abstract

The aim of this study was to determine the performance of both cefotaxime and ceftazidime containing agars on the specificity and sensitivity for chromosomal AmpC-hyperproducing and plasmid AmpC harbouring *Escherichia coli* compared to ESBL-producing *E. coli* and *E. coli* without ESBL, pAmpC or cAmpC hyperproduction. Second, we evaluated the influence of adding cefoxitin to these agars for detection of both chromosomal AmpC-hyperproducing and plasmid AmpC harbouring *E. coli*.

Four different homemade screening agars with cefotaxime (1mg/L), ceftazidime (1mg/L), cefotaxime (1mg/L) with cefoxitin (8mg/L), and ceftazidime (1mg/L) with cefoxitin (8mg/L) were compared to each other for the identification of AmpC producing *E. coli*. A total of 40 isolates with plasmid encoded AmpC beta-lactamases, 40 isolates with alterations in the promoter/attenuator region of the AmpC gene leading to hyperproduction of the beta-lactamase, 40 isolates with ESBL genes and 39 isolates lacking both a AmpC and ESBL genotype were used to test the four agars.

The sensitivity and specificity were 100% (95% confidence interval (95% CI) 96.1% to 100%) and 48.1% (95% CI 38.6%-60.2%), respectively, for the cefotaxime agar; 100% (95% CI 96.1% to 100%) and 49.41% (95% CI 39.8%-61.4%), respectively, for the ceftazidime agar; 96.3% (95% CI 89.1% to 99.2%) and 77.2% (95% CI 66.7%-85.2%) respectively, for the cefotaxime with cefoxitin agar; 98.8% (95% CI) 92.6% to 99.6%) and 81.0% (95% CI 70.9%-88.3%) respectively, for the ceftazidime agar with cefoxitin. The main reason for false-positive results were ESBL-harbouring strains that grew on various agars; therefore, the specificity of each agar reported here was influenced mainly by the proportion of ESBL isolates tested. In conclusion addition of cefoxitin to cefotaxime and ceftazidime containing agars had little influence on sensitivity, but increased specificity for the detection of AmpC in *E. coli*.

Introduction

Over the past decades, the importance of resistant Enterobacteriales increased substantially due to the emergence of various resistance traits that inactivate most beta-lactam antibiotics. In *Escherichia coli*, resistance to 2nd and 3rd generation cephalosporins is most frequently due to the occurrence of plasmid-encoded ESBL, as well as both plasmid-encoded (*pampC*) and chromosomally-encoded (*campC*) AmpC genes. As the rapid and accurate detection of resistant bacteria is crucial for treatment and control, selective media for detection of extended-spectrum beta-lactamase-producing Enterobacteriales (ESBL-E) have been developed. A considerable number of studies were published on the performance of ESBL-E screening agars (Glupczynski *et al*. 2007; Réglier-Poupet *et al*. 2008; Randall *et al*. 2009; Al Naiemi *et al*. 2009; Overdevest *et al*. 2011). So far, few studies have focused on screening media for AmpC beta-lactamase-producing Enterobacteriales (AmpC-E).

The basis of most agars that are selective for ESBL is a adding a 3rd generation cephalosporin, e.g. cefotaxime or ceftazidime. Some ESBL-E screening agars use AmpC inhibitors, such as cloxacillin to suppress Enterobacteriales that intrinsically produce AmpC beta-lactamase on high level (e.g. Enterobacter spp) (Randall *et al*. 2009; Al Naiemi *et al*. 2009; Overdevest *et al*. 2011). Overgrowth of such species with intrinsic AmpC beta-lactamase hyperproduction makes it more difficult to screen for *E. coli* with acquired beta-lactamases, which may lead to a lower sensitivity of the ESBL-E screening agars (Randall *et al*. 2009). *E. coli* isolates produce chromosomally encoded AmpC (cAmpC) constitutively, but only on a low level. However, they may acquire alterations in the promoter/attenuator region leading to hyperproduction of cAmpC beta-lactamase (Tracz *et al*. 2005; 2007). Growth of both cAmpC and pAmpC producing Enterobacteriales is inhibited by cloxacillin. Cloxacillin containing agars may improve the yield of ESBL producing *E. coli* but they lead to a lower yield of both pAmpC and cAmpC beta-lactamase producing *E. coli*. One option to solve this problem could be to include an additional agar specifically developed for AmpC producing *E. coli* during screening.

According to the EUCAST guidelines combined resistance to 3rd generation cephalosporins and cefoxitin, a cephamycin antibiotic, may be used as phenotypic criteria for AmpC screening (Martinez and Simonsen 2017). Cefoxitin is highly active against ESBL producing bacteria, but has little activity against most AmpC producing bacteria (Jacoby 2009; Birnbaum *et al*. 1978). This makes cefoxitin useful in screening strategies for AmpC (Ingram *et al*. 2011; Polsfuss *et al*. 2011; Peter-Getzlaff *et al*. 2011).

A study by Reuland *et al* compared three different screening strategies of pAmpC Enterobacteriales based on susceptibility patterns(E. Ascelijn Reuland *et al*. 2014). These included: reduced susceptibility to cefotaxime and/or ceftazidime (>1 mg/L), reduced susceptibility to cefoxitin (> 8mg/L), or a combination of cefotaxime and/or ceftazidime (>1 mg/L). Using just cefoxitin during a screening resulted in high sensitivity (97%), but was not specific for AmpC (72%) as a porin deficiency can cause cefoxitin resistance as well. Combining reduced cefotaxime and/or ceftazidime susceptibility with reduced cefoxitin susceptibility increased the specificity to 90%, without significant loss of sensitivity (97%). Reuland *et al*. did not use a screening agar to differentiate pAmpC Enterobacteriales. We hypothesize that an agar which combines cefoxitin with either cefotaxime or ceftazidime may result in an AmpC specific screening agar, which could be of additional value to the use of an ESBL-agar with cloxacillin.

The first aim of this study was to determine the performance of both cefotaxime and ceftazidime containing agars on the specificity and sensitivity for AmpC-producing *E. coli* compared to ESBL-producing and AmpC/ESBL negative *E. coli*. Second, we evaluated the influence of adding cefoxitin to these agars for detection of AmpC producing *E. coli*.

Methods

Collection of isolates

A panel of 159 *E. coli* isolates was used for the evaluation of the AmpC screening agar. This panel consisted of 40 *E. coli* isolates harbouring pAmpC, 40 *E. coli* isolates with cAmpC mutations known to lead to hyperproduction (referred in this study as "cAmpC positive"), 40 ESBL-producing *E. coli* isolates and 39 *E. coli* without signs of ESBL, pAmpC or cAmpC hyperproduction based on genotype (referred in this study as "AmpC/ESBL negative"). The ESBL-producing *E. coli* and AmpC/ESBL negative *E. coli* isolates were confirmed to lack any known alterations in the promoter/attenuator region of the AmpC gene leading to hyperproduction of the beta-lactamase, as reported by Tracz *et al* (Tracz *et al*. 2007). Isolates were recovered at different study sites or from various clinical specimens, and were isolated from humans (n=141) and animals (n=18) (E. Den Drijver et al. 2018; Hordijk, Wagenaar, van de Giessen, et al. 2013; C. M. Dierikx et al. 2012; Marjolein F Q Kluytmans-Van Den Bergh et al. 2016; C. Dierikx et al. 2013; Ferdous M, Friedrich AW, Grundmann H, de Boer RF, Croughs PD, Islam MA, Kluytmans-van den Bergh MF, Kooistra-Smid AM 2016). More detailed information on

the recovery of the isolates is described in the additional file 1. Additional file 2 shows an overview of all strains.

Evaluation of AmpC agars

MacConkey agar no3 medium was obtained from Oxoid Limited (ThermoFisher, Basingstoke, Hampshire, United Kingdom). Both cefotaxime sodium salt and ceftazidime sodium salt were obtained from VWR International (Radnor, Pennsylvania, USA). Cefoxitin sodium salt was obtained from Sigma-Aldrich (Poole, Dorset, UK). MacConkey agar no3 was used as the basal medium to which different dilutions of antibiotics were added. Four different concentration combination were evaluated; 1. cefotaxime 1mg/L agar, 2. ceftazidime 1 mg/L agar, 3. cefotaxime 1mg/L with cefoxitin 8mg/L agar, and 4. ceftazidime 1 mg/L with cefoxitin 8mg/L agar.

All strains with AmpC or ESBL were cultured in a selective enrichment broth consisting of a TSB containing 0, 25 mg/L cefotaxime and 8mg/L vancomycin (TSB-VC, Mediaproducts, Groningen, The Netherlands). For strains without AmpC or ESBL a brain heart infusion broth was used (BHI, Mediaproducts, Groningen, The Netherlands), as using selective enrichment broth might inhibit growth. After overnight aerobic incubation for 18-24 hours (35°-37°), 10 µl of the broth was subcultured on the four different AmpC agars. Agars were incubated aerobically for 18-24 hours (35°-37°). Results were interpreted as growth or no growth.

Statistical analysis

Data were analyzed with Statistical Package for Social Science software (SPSS; IBM Corp., Armonk, New York, US; version 22). For sensitivity all strains positive for an acquired AmpC gene or alterations in promoter/attenuator AmpC related to hyperproduction were considered true positives. For specificity were all strains negative for an acquired AmpC gene and alterations in promoter/attenuator AmpC related to hyperproduction were considered true negatives. Confidence intervals (95% CI) were calculated according to the adjusted Wald method. The prevalence of ESBL in the panel of isolates was 25.16% (40 out of 159 isolates), due to the artificial nature of the collection set up. To analyse the influence of variations in ESBL prevalence on the specificity of both the cefotaxime 1mg/L + cefoxitin 8mg/L and the ceftazidime 1mg/L + cefoxitin 8mg/L agar, we remodelled our data for an ESBL prevalence range from 0 to 99%. Specificity of the cefotaxime 1mg/L + cefoxitin 8mg/L agar was calculated using following formula: 38 + (23/40 x number of ESBL isolates) / (38 + (23/40 x number of ESBL isolates) + (1 + 17/40 x number of ESBL isolates)).

Specificity of the ceftazidime 1mg/L + cefoxitin 8mg/L agar was calculated using following formula: 39 + (25/40 x number of ESBL isolates)/(39 + (25/40 x number of ESBL isolates) + (15/40 x number of ESBL isolates)). Prevalence of ESBL was calculated using the formula: number of ESBL isolates / (119 + number of ESBL isolates).

Results

The 159 *E. coli* isolates were evaluated for growth on the four different selective agar plates as shown in Table 1. Isolates hyperproducing cAmpC or producing pAmpC or ESBL *E. coli* grew on both cefotaxime and ceftazidime agar plates without cefoxitin. Of the AmpC/ESBL negative isolates, only one (2.6%) isolate grew on the cefotaxime agar. No growth of AmpC/ESBL negative isolates was seen on the ceftazidime agar. The cefotaxime + cefoxitin agar showed growth of 39 (97.5%) pAmpC isolates, 39 (97.5%) cAmpC isolates, 17 (42.5%) ESBL isolates and one (2.6%) AmpC/ESBL negative isolate. The ceftazidime + cefoxitin agar showed growth of 39 (97.5%) pAmpC isolates, 40 (100%) cAmpC isolates, 15 (37.5%) ESBL isolates and none of the AmpC/ESBL negative isolates. In both agars the pAmpC isolate that did not grow was the same and appeared to contain a bla_{ACC} gene. The ATCC control *E. coli* strain did not grow on any of the evaluated agars.

Table 1. Ability of 159 *E. coli* isolates to grow on four different AmpC screening agars

		Cefotaxime 1 mg/L		*Ceftazidime 1 mg/L*	
Characteristics	**No. of isolates**	**No Cefoxitin**	**8 mg/L Cefoxitin**	**No Cefoxitin**	**8 mg/L Cefoxitin**
pAmpC positive and ESBL negative	40	40 (100%)	39 (97.5%)	40 (100%)	39 (97.5%)
bla_{CMY-2}	31	31 (100%)	31 (100%)	31 (100%)	31 (100%)
bla_{CMY-42}	3	3 (100%)	3 (100%)	3 (100%)	3 (100%)
bla_{CMY-79}	1	1 (100%)	1 (100%)	1 (100%)	1 (100%)
bla_{DHA}	2	2 (100%)	2 (100%)	2 (100%)	2 (100%)
bla_{ACC}	2	2 (100%)	1 (50%)	2 (100%)	1 (50%)
bla_{MOX}	1	1 (100%)	1 (100%)	1 (100%)	1 (100%)

Table 1. Continued.

		Cefotaxime 1 mg/L		*Ceftazidime 1 mg/L*	
Characteristics	**No. of isolates**	**No Cefoxitin**	**8 mg/L Cefoxitin**	**No Cefoxitin**	**8 mg/L Cefoxitin**
cAmpC positive and ESBL negative	40	40 (100%)	39 (97.5%)	40 (100%)	40 (100%)
ESBL positive and AmpC negative	40	40 (100%)	17 (42.5%)	40 (100%)	15 (37.5%)
$bla_{CTX\text{-}M\text{-}1}$ group	28	28 (100%)	14 (50%)	28 (100%)	14 (50%)
$bla_{CTX\text{-}M\text{-}9}$ group	12	12 (100%)	3 (25%)	12 (100%)	1 (8.3%)
AmpC negative and ESBL negative	39	1 (2.6%)	1(2.6%)	0 (0%)	0 (0%)

Table 2 and 3 present the sensitivity and specificity values. Although the agars containing cefoxitin showed a slightly lower sensitivity then the agars without cefoxitin, all confidence intervals overlapped. Specificity was assessed for the 79 AmpC-negative isolates. The agars containing cefoxitin showed a better specificity than the agars without cefoxitin. The specificity of the cefotaxime 1mg/L + cefoxitin 8mg/L and ceftazidime 1mg/L + cefoxitin 8mg/L agar plates were similar, based upon overlap of confidence intervals. As described above, we found that ESBL isolates had the largest impact on specificity, since ~40% of them grew on the cefotaxime+cefoxitin or ceftazidime+cefoxitin agars.

Table 2 Sensitivity (with 95% confidence intervals) of different agars

		*Sensitivity of different agars**		
		pAmpC		cAmpC
Cefotaxime	40/40	1.00 (0.92-1.00)	40/40	1.00 (0.92-1.00)
Cefotaxime + Cefoxitin	39/40	0.98 (0.86-1.00)	39/40	0.98 (0.86-1.00)
Ceftazidime	40/40	1.00 (0.92-1.00)	40/40	1.00 (0.92-1.00)
Ceftazidime + Cefoxitin	39/40	0.98 (0.86-1.00)	40/40	1.00 (0.92-1.00)

**Sensitivity based on growth on agar (true positive) divided through all isolates of group (true positive + false negative)*

Table 3. Specificity (with 95% confidence intervals) of different agars

	*Specificity of agars**			
	ESBL-positive isolates		AmpC/ESBL-negative isolates	
Cefotaxime	0/40	0.00 (0.00-0.08)	38/39	0.97 (0.86-1.00)
Cefotaxime + Cefoxitin	23/40	0.58 (0.42-0.72)	38/39	0.97 (0.86-1.00)
Ceftazidime	0/40	0.00 (0.00-0.08)	39/39	1.00 (0.92-1.00
Ceftazidime + Cefoxitin	25/40	0.63 (0.47-0.76)	39/39	1.00 (0.92-1.00)

**Specificity based on no growth on agar (true negative) divided through all isolates of group (true negative + false positive).*

The prevalence of ESBL in the isolates tested was 25.16%, due to the artificial nature of the isolates chosen. Figures 1 and 2 show the relation between specificity of the cefotaxime 1mg/L + cefoxitin 8mg/L and of the ceftazidime 1mg/L + cefoxitin 8mg/L agar plotted versus ESBL prevalence. An increase in ESBL prevalence in the isolate collection would decrease specificity in a nonlinear function. According to our model a low ESBL prevalence e.g. 5% would lead to a specificity of the cefotaxime 1mg/L + cefoxitin 8mg/L agar of 92% and of the ceftazidime 1mg/L + cefoxitin 8mg/L agar of 95%. A high prevalence of e.g. 50% would decrease specificity of the cefotaxime 1mg/L + cefoxitin 8mg/L agar to 67% and of the ceftazidime 1mg/L + cefoxitin 8mg/L agar to 72%.

Figure 1. Model of expected specificity of cefotaxime 1mg/L + cefoxitin 8mg/L agar versus ESBL prevalence (%)

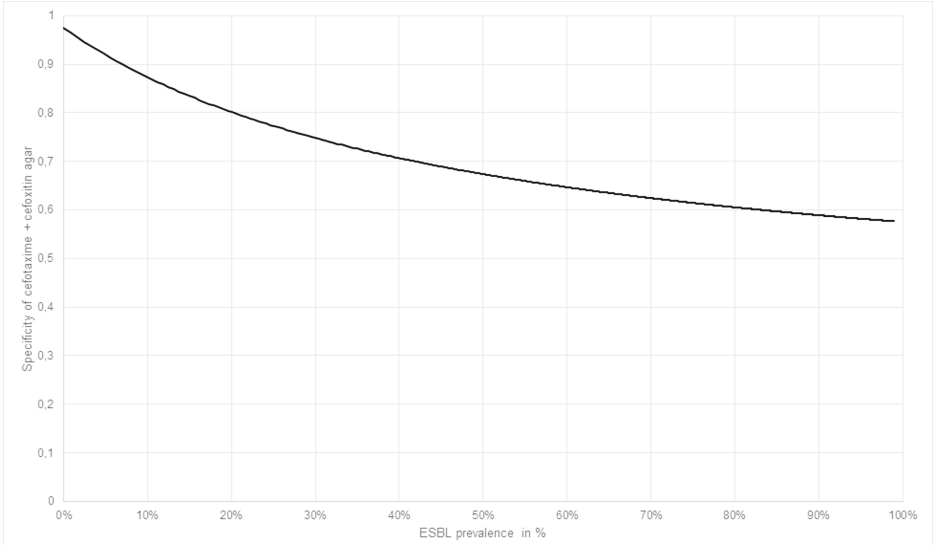

FIG 1 Model of expected specificity of cefotaxime 1mg/L + cefoxitin 8mg/L agar based upon ESBL prevalence (in %)

Figure 2. Model of expected specificity of ceftazidime 1mg/L + cefoxitin 8mg/L agar versus ESBL prevalence (%).

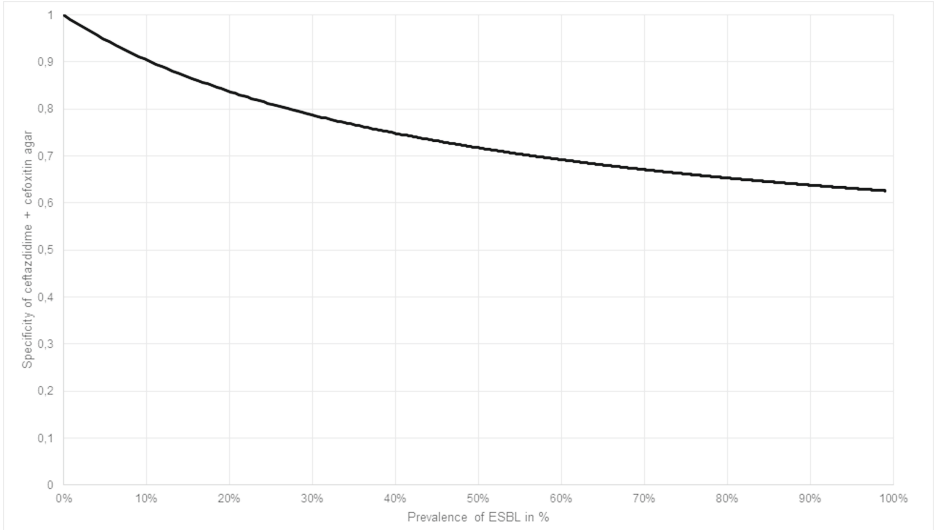

FIG 2 Model of expected specificity of ceftazidime 1mg/L + cefoxitin 8mg/L agar based upon ESBL prevalence (in %).

Discussion

Four different screening agars were evaluated on their ability to identify AmpC producing *E. coli*. Cefotaxime- and ceftazidime containing agar showed similar results in sensitivity and specificity for AmpC detection in the tested panel of *E. coli* isolates. The addition of cefoxitin increased specificity for AmpC detection, but did not influence sensitivity.

Although there are no studies on AmpC selective agars, these results are in line with studies showing that cefoxitin might be a useful screening additive for AmpC production (Ingram *et al*. 2011; Polsfuss *et al*. 2011; Peter-Getzlaff *et al*. 2011). Polsfuss *et al.* analysed the use of cefoxitin disc diffusion compared to cefotetan for different Enterobacteriales (Polsfuss *et al*. 2011). The study included 211 potential AmpC producers based on the basis of cefoxitin inhibition zone diameters of ≤18 mm, cefotetan inhibition zone diameters of ≤16 mm, and/or positive ESBL screening diameters according to CLSI guidelines. The potential AmpC producers were compared to 94 isolates that tested negative in the three mentioned screening criteria. All isolates were subjected to an AmpC multiplex PCR and promoter/attenuator regions of *E. coli* isolates were sequenced. The detection of a pAmpC gene and/or known promoter/attenuator mutations leading to AmpC hyperproduction were considered as gold standard. The majority of acquired AmpC beta-lactamases were of the CMY-group and DHA-group, none of the strains possessed beta-lactamases of the ACC, FOX, MOX or ACT/MIR groups. Cefoxitin showed a sensitivity of 97,4% and a specificity of 78,7%. The use of cefotetan, another type of cephamycin, was tested as well, but although this method showed a better specificity (99,3%), sensitivity was much lower (52,6%).

As already described, Reuland *et al.* compared different screening methods based upon reduced 3rd generation cephalosporin susceptibility for plasmid-encoded AmpC in Enterobacteriales (E. Ascelijn Reuland *et al*. 2014). The study collection consisted out of 356 Enterobacteriales resistant to a third generation cephalosporins (cefotaxime and/or ceftazidime) and/or cefoxitin, of which 68.8% was determined as *E. coli*. A total of 34 pAmpC containing isolates (28 *E. coli*) were detected containing genes from the CMY-group n=29 ; DHA-group n=4 and ACC-group n=1. No analysis on chromosomal AmpC hyperproducers was performed. The strategy using reduced susceptibility to cefotaxime and/or ceftazidime together with reduced susceptibility to cefoxitin showed a sensitivity of 97% and a specificity of 90%. The only isolate not detected contained a bla_{ACC}-gene.

In our study one isolate containing an ACC-gene did not grow on the cefoxitin containing agars. Although most acquired AmpC beta-lactamases are inhibited by

cephamycins, the ACC-group remains an exception (Jacoby 2009). This beta-lactamase seems to be less common as CMY-group AmpC beta-lactamases. Nevertheless, ACC-type enzymes have been detected in several countries in Europe (Bauernfeind *et al*. 1999; Girlich *et al*. 2000; Miró *et al*. 2005). Outbreaks have been reported as well (Nadjar *et al*. 2000). The use of cefoxitin containing agars may have limitations in areas where the ACC-group is prevalent.

Clearly, there are more limitations in the present study. First, this study is an analytical exploration of sensitivity and specificity based upon retrospectively selected strains. Although the panel of strains was selected on genotype, all strains were obtained from clinical or study collections in which different culture screening criteria were used. We tried to minimize selection bias based upon our screening criteria, but the distribution of our collection may not be completely comparable to a clinical setting. The use of mainly CMY-group pAmpC isolates may have led to overestimation of sensitivity in some degree. However, the CMY-group seems to be the predominant *pampC* gene in clinical settings, and the cefoxitin containing agars may be therefore applicable in these setting for e.g. screening of AmpC rectal carriage.

We remodelled specificity for the cefotaxime 1mg/L + cefoxitin 8mg/L and ceftazidime 1mg/L + cefoxitin 8mg/L agar, as the number of ESBL isolates seems to influence agar specificity. We expect that in a low endemic setting of ESBL specificity of both cefoxitin containing agars will increase. In the Netherlands prevalence of ESBL rectal carriage has been found to range from 5% in a teaching hospital in the South of the Netherlands (25) to 8,6% in general practices in Amsterdam (Willemsen *et al*. 2015; E. A. Reuland *et al*. 2016). Numbers on rectal carriage in North-America are scarce, but a recent prevalence study on ESBL infections in a hospital in the United States found ranges between 4.7% to 13.4% (Hoffman-Roberts *et al*. 2016). It is likely that screenings agars in area's similar to the Dutch setting would have high specificity. In an ESBL high endemic area specificity of the agar will be lower.

Our study was performed using strains cultured in semi-selective pre-enrichment broth. This pre-enrichment step was included to mimic our laboratory algorithm for screening of multiresistant Enterobacterales in rectal samples. Former studies on the screening of third generation cephalosporin resistance have shown that a pre-enrichment strategy results in higher yield compared to direct plating (M. F.Q. Kluytmans-Van Den Bergh *et al*. 2015; Nathalie Jazmati, Hein, and Hamprecht 2016; N. Jazmati, Jazmati, and Hamprecht 2017). We assume that that the use of a pre-enrichment strategy in clinical setting will result in a similar detection range, though inoculum of the original sample may differ. A limit of detection is difficult to obtain, as it is not well-known which load in

clinical samples is comparable to an in vitro setting. Our strategy using the combination of pre-enrichment and selective screening agars was applied in a prevalence study on AmpC in the Amphia hospital (E. Den Drijver *et al*. 2018). Yield of AmpC *E. coli* was comparable to other Dutch prevalence studies (E. Ascelijn Reuland *et al*. 2015; Van Hoek *et al*. 2015). However, a more elaborate evaluation of clinical samples is needed to confirm our results on sensitivity and specificity of our screening strategy.

In this study the use of screening agars was only assessed on *E. coli*, as this is one of the most prevalent Enterobacteriales with acquired beta-lactamase genes. We expect the agar to be useful for other AmpC producing Enterobacteriales, however sensitivity and specificity for other species may differ. For example, *K. pneumoniae* without pAmpCs can be cefoxitin-resistant due to loss of porin expression (Martínez-Martínez 2008). Furthermore, not all existing beta-lactamase groups were included. No *E. coli* isolates containing AmpC FOX and ACT/MIR groups nor the ESBL SHV and TEM or other ESBL groups nor carbapenemases were tested. Co-expression of AmpC and ESBL in *E. coli* isolates lacked as well. Although, beta-lactamases from the CTX-M, CMY and DHA groups seem to be most prevalent in the European setting (David M. Livermore *et al*. 2007; Jacoby 2009), future analysis is needed to see if results are applicable to other Enterobacteriales, beta-lactamase gene groups and beta-lactamase gene combinations as well.

The AmpC screening agars are not able to differentiate pAmpC vs cAmpC in *E. coli*. Both mechanisms cause elevated 3rd generation cephalosporin MICs, and MIC distributions of cAmpC and pAmpC tend to overlap (Edquist *et al*. 2013; Aarestrup *et al*. 2010).This makes it difficult to create a specific pAmpC or cAmpC screening agar. EUCAST guidelines advise a PCR to distinguish both mechanisms. A screening agar on AmpC may limit the amount of strains for molecular confirmation.

Conclusions

Cefotaxime or ceftazidime had a similar sensitivity and specificity for AmpC in screenings agar in our isolate panel. Addition of cefoxitin to create a more AmpC selective agar had little influence on sensitivity, but increased specificity. Our results apply mainly for bla_{CMY}-type producing *E. coli* and further studies are needed to evaluate an agar containing cefotaxime or ceftazidime with cefoxitin in a clinical setting. We expect the screening agars to be feasible to screen for AmpC rectal carriage, when using pre-enrichment similar to current ESBL screening agar strategies. So, agars containing

cefotaxime or ceftazidime with cefoxitin do not seem to be inferior in sensitivity compared to agars without cefoxitin, and may be promising when used additional to e.g. a ESBL specific screenings agar for screening of 3rd generation cephalosporin resistance in *E. coli*.

Declarations

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Competing interests

The authors declare no competing interests.

Authors' contributions

E.D. and J.A.J.W.K. conceived of and designed the study ; J.S. and C.V. were responsible for laboratory processing of the isolates ; J.J.V. conducted molecular analysis of local strains ; K.V., J.W.R., A.M.D.K.S., and M.F.Q.K. provided isolates for the strain collection and revised and approved the manuscript. E.D. performed the analyses and wrote the first draft of the manuscript with input from J.A.J.W.K. All authors contributed to preparation of the final manuscript.

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Supplementary data

Supplement 1. Detailed supplementary "Materials and Methods"

AmpC E. coli isolates from SOM study

In total 27 *E. coli* isolates were selected from the SOM-study based upon the presence of pAmpC encoding genes in the whole genome sequence (WGS) data (Marjolein F Q Kluytmans-Van Den Bergh *et al.* 2016; Kluytmans-van den Bergh *et al.* 2019). In this study perianal swabs taken from hospital patients, were pre-enriched using selective tryptic soy broth (TSB) and subsequently cultured on EbSA screening agar (Cepheid Benelux, Apeldoorn, the Netherlands). Sequencing was performed as described by Kluytmans-van den Bergh *et al.* (Marjolein F Q Kluytmans-Van Den Bergh *et al.* 2016). The assembled genomes were uploaded to the online bioinformatics tools ResFinder v2.1 and MLST v1.8 (Center for Genomic Epidemiology, Technical University of Denmark, Lingby, Denmark (version 2.1) (Zankari *et al.* 2012; Alessandra Carattoli *et al.* 2014) . ESBL- and pAmpC-encoding genes were reported when at least 60% of the length of the best matching gene in the ResFinder database was covered with a sequence identity of at least 90%. Conventional MLST sequence types were based on the Achtman MLST scheme (Wirth *et al.* 2006). In addition, the assembled sequences of each isolate were aligned against the promoter/attenuator region of the cAmpC gene of the *E. coli* K-12 strain MG1655 (GenBank database accession number U00096 (https://www.ncbi.nlm.nih.gov/nuccore/U00096)) using CLC Genomic Workbench version 8.5 (CLC Bio, Qiagen, Hilden, Germany). The level of hyperproduction of AmpC was based on a former study by Tracz *et al.* (Tracz et al. 2007).

AmpC E. coli isolates from Amphia prevalence screening

In total 22 cAmpC hyperproducing and 4 pAmpC containing *E. coli* isolates were selected from prevalence screening which had been performed in the Amphia hospital described by Den Drijver *et al.* (E. Den Drijver et al. 2018). Rectal swabs taken from hospital patients were pre-enriched using selective TSB and subsequently cultured on MacConkey agar plate containing cefotaxime (1 mg/L) or MacConkey double agar plate containing cefotaxime (1 mg/L) with cefoxitin (8 mg/L) one side and ceftazidime (1 mg/L) with cefoxitin (8mg/L) other side (Mediaproducts, Groningen, The Netherlands). Presence of pAmpC was analyzed with micro-array MDR CT103 (Check-Points, Wageningen, the Netherlands), alterations in the promoter and attenuator region were analysed with Sanger sequencing as previously described (Stéphane Corvec et al. 2003).

pAmpC-encoding clinical E. coli isolates from Amphia hospital

In total 9 pAmpC producing *E. coli* isolates were selected retrospectively from our laboratory database based upon the presence of pAmpC encoding genes. In the laboratory protocol for AmpC screening on clinical samples, *E. coli* isolates with cefotaxime and/or ceftazidime MIC > 1mg/l combined with cefoxitin MIC ≥8mg/l are screened for AmpC production using D68C AmpC & ESBL Detection Set (Mastdiscs, Mastgroup Ltd, Bootle, United Kingdom). The presence of *pampC* genes and/or alterations in the promoter/attenuator region were evaluated similar as in the Amphia prevalence screening described by Den Drijver *et al.* (E. Den Drijver et al. 2018).

AmpC E. coli isolates from Wageningen University prevalence study

In total 18 cAmpC hyperproducing *E. coli* isolates were selected retrospectively from a former prevalence study among livestock (e.g. cattle, pigs and broilers) by the Wageningen University. Screening swabs were cultured on MacConkey agar (product no. 212123, Becton Dickinson)+1 mg/L cefotaxime (Sigma-Aldrich, Germany)] and inoculated in a selective pre-enrichment broth (Luria–Bertani broth containing 1 mg/L cefotaxime). Phenotypic and genotypic confirmation with micro-array and sanger sequencing were performed similar as described by Dierikx *et al.* and Hordijk *et al.* (C. M. Dierikx et al. 2012; C. Dierikx et al. 2013; Hordijk, Wagenaar, Kant, et al. 2013).

ESBL-encoding clinical E. coli isolates from Amphia hospital

In total 40 ESBL producing *E. coli* isolates were selected retrospectively from our laboratory database of blood culture *E. coli* isolates containing of CTX-M encoding genes. In the in-laboratory protocol for ESBL screening on clinical samples, *E. coli* isolates with cefotaxime and/or ceftazidime MIC > 1mg/l are screened for ESBL production using combination disk diffusion method for cefotaxime, ceftazidime, and cefepime with and without clavulanic acid (Rosco, Taastrup, Denmark) and interpreted according to manufacturer's instructions. WGS was performed in UMCG using MiSeq (Illumina, San Diego, United States) and assembled with CLC Genomics Workbench 9.0, 9.0.1 or 9.5.2 (Qiagen, Hilden, Germany) as was previously described in more detail by Kluytmans-van den Bergh *et al.* (Marjolein F Q Kluytmans-Van Den Bergh et al. 2016). Assembly, identification of resistance genes and MLST type and analysis of promoter/attenuator region were performed as described for the SOM study isolates.

Non-AmpC/non-ESBL producing E. coli from STEC-ID-net study

In total 39 *E. coli* isolates obtained from stools of patients in a former multicentre prospective study, STEC-ID-net, performed from April 2013 to March 2014 in the Dutch regions of Groningen and Rotterdam were selected based on the absence of both AmpC, ESBL genes and known promoter/attenuator mutations related to AmpC hyperproduction (Ferdous M, Friedrich AW, Grundmann H, de Boer RF, Croughs PD, Islam MA, Kluytmans-van den Bergh MF, Kooistra-Smid AM 2016). Identification of resistance genes and MLST type and analysis of promoter/attenuator regions were performed as described above (pAmpC SOM study isolates) using the already available WGS data.

Additional file 2 for this chapter is available online via:
https://www.biorxiv.org/content/biorxiv/early/2019/09/30/787085/DC1/embed/media-1.pdf?download=true

Chapter 6

Limited genetic diversity of *bla*CMY-2-containing IncI1-pST12 plasmids from Enterobacteriaceae of human and broiler chicken origin in the Netherlands

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Abstract

Distinguishing epidemiologically related and unrelated plasmids is essential to confirm plasmid transmission. We compared IncI1–pST12 plasmids from both human and livestock origin and explored the degree of sequence similarity between plasmids from Enterobacteriaceae with different epidemiological links. Short-read sequence data of Enterobacteriaceae cultured from humans and broilers were screened for the presence of both a bla_{CMY-2} gene and an IncI1–pST12 replicon. Isolates were long-read sequenced on a MinION sequencer (OxfordNanopore Technologies). After plasmid reconstruction using hybrid assembly, pairwise single nucleotide polymorphisms (SNPs) were determined. The plasmids were annotated, and a pan-genome was constructed to compare genes variably present between the different plasmids. Nine *Escherichia coli* sequences of broiler origin, four *Escherichia coli* sequences, and one *Salmonella enterica* sequence of human origin were selected for the current analysis. A circular contig with the IncI1–pST12 replicon and bla_{CMY-2} gene was extracted from the assembly graph of all fourteen isolates. Analysis of the IncI1–pST12 plasmids revealed a low number of SNP differences (range of 0–9 SNPs). The range of SNP differences overlapped in isolates with different epidemiological links. One-hundred and twelve from a total of 113 genes of the pan-genome were present in all plasmid constructs. Next generation sequencing analysis of bla_{CMY-2}-containing IncI1–pST12 plasmids isolated from Enterobacteriaceae with different epidemiological links show a high degree of sequence similarity in terms of SNP differences and the number of shared genes. Therefore, statements on the horizontal transfer of these plasmids based on genetic identity should be made with caution.

Keywords: AmpC beta-lactamase; plasmid; IncI1; bla_{CMY-2}

Introduction

Antimicrobial resistance in Gram-negative bacteria is a worldwide growing public health problem (Davies and Davies 2010; Premanandh, Samara, and Mazen 2016). The gut is an important reservoir for resistant Gram-negative bacteria, both in humans and livestock (Carlet 2012; A. Carattoli 2008). Antimicrobial resistance in livestock has been suggested as a potential source for resistance in humans, with a growing number of studies published on this potential transmission route for antimicrobial resistance mechanisms in Gram-negative bacteria (C. Dierikx *et al*. 2013; Ewers *et al*. 2012; Berg *et al*. 2017). AmpC beta-lactamase-production is an example of these mechanisms as a potential source for 3rd generation cephalosporin resistance in Gram-negative bacteria (Jacoby 2009).

Plasmids are an important vector for antimicrobial resistance dissemination with genes for various resistance mechanisms (e.g., AmpC beta-lactamase genes) being located on these mobile genetic elements. Incompatibility group I1 (IncI1) plasmids of the plasmid sequence type (pST) 12 have been associated with the spread of bla_{CMY-2}, which is the most common AmpC beta-lactamase gene (Accogli *et al*. 2013; Alessandra Carattoli *et al*. 2018; Hansen *et al*. 2016). Recent studies show that the sequence of IncI1 plasmids is highly conserved (Pietsch *et al*. 2018; Roer *et al*. 2019; Castellanos *et al*. 2019; Valcek *et al*. 2019; Shirakawa *et al*. 2020). Most studies to date are based on short-read sequence mechanisms (Pietsch *et al*. 2018; Castellanos *et al*. 2019; Shirakawa *et al*. 2020). However, it remains challenging to study plasmid transmission using short-read sequencing data alone. Repeated sequences, often shared between plasmid and chromosomal DNA, hinder the assembly of the bacterial genome from short-read data, often resulting in contigs of which the origin, either plasmid or chromosomal, cannot be resolved (Stohr *et al*. 2020). This limits the interpretation of plasmid transmission by not providing accurate prediction of the total plasmid sequence. Recently, a combination of short- and long-read sequence data provided an accurate analysis, such as shown in a recent study on IncI1 plasmids of pST3 and pST7 (Valcek *et al*. 2019). Everything considered, the number of studies using combined short- and long-read sequencing data of IncI1-pST12 plasmids from human and livestock origin is still limited. The transmission of antimicrobial resistant bacteria within and between domains is predominantly based on the comparison of bacterial chromosome. However, when only typing the bacterial chromosome, the transmission of resistance gene-containing plasmids can go undetected. Although plasmid replicon typing combined with pMLST data can be useful to monitor the spread of plasmids through populations, more accurate

distinguishing of related from non-related plasmids based on molecular characteristics (e.g., number of single nucleotide polymorphisms (SNP) differences) is essential for using sequence data to detect plasmid transmission. We hypothesize that a combination of short- and long-read sequence data of bla_{CMY-2} containing IncI1–pST12 plasmids reveal highly conserved plasmid sequencing, which complicates distinguishing plasmid transmission between epidemiologically related and unrelated isolates. The objective of the current study is to determine the relatedness between IncI1–pST12 plasmids of epidemiologically related and unrelated Enterobacteriaceae isolates from humans and livestock, and we explore the possibility of accurately distinguishing related from unrelated samples based on plasmid sequencing data alone.

Materials and Methods

Collection of Isolates

AmpC *E. coli* Isolates from i-4-1-Health Dutch-Belgian Cross-Border Project

As part of the i-4-1-Health project, human and broiler samples were collected as described by Kluytmans-van den Bergh *et al* (Kluytmans-van Den Bergh *et al*. 2019). After vortexing, the nylon-flocked swabs in 2 mL Cary–Blair medium (FecalSwab®, Copan Italy, Brescia, Italy) were plated on a blood agar plate (growth control, performed since 2011), and the liquid Cary–Blair medium was mixed in tryptic soy broth (TSB) and incubated for 18–24 h (35–37 °C). Broths were subcultured on an AmpC selective MacConkey agar containing cefotaxime and cefoxitin (1 and 8 mg/mL, respectively) on half the plate and ceftazidime and cefoxitin (1 and 8 mg/mL, respectively) on the other half of the plate (Mediaproducts, Groningen, The Netherlands) (Drijver *et al*. 2019). For all oxidase-negative isolates that grew on either side of the selective agar plates, species identification was performed by automated mass spectrometry systems (VitekMS, bioMérieux, Marcy l'Etoile, France). Susceptibility testing was performed using Vitek 2 (bioMérieux, Marcy l'Etoile, France). The presence of AmpC in all oxidase-negative isolates was phenotypically confirmed using the D68C AmpC & ESBL Detection Set (Mastdiscs, Mastgroup Ltd., Bootle, UK) and interpreted according to the manufacturer's instructions. All phenotypically confirmed isolates were sequenced using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). DNA isolation and sequencing were performed as described by Coolen *et al* (Coolen *et al*. 2019). De novo assembly and error correction were performed using SPAdes version 3.9.1 (Bankevich *et al*. 2012).

AmpC E. coli Isolates from Amphia Prevalence Screening

pampC gene containing *E. coli* isolates were selected from a prevalence screening, which had been performed in the Amphia hospital described by Den Drijver *et al.* (E. Den Drijver *et al*. 2018). Rectal swabs taken from hospital patients were pre-enriched using selective TSB containing cefotaxime (0.25 mg/L) and vancomycin (8 mg/L) and subsequently cultured on a MacConkey agar plate containing cefotaxime (1 mg/L) or a MacConkey double agar plate containing cefotaxime and cefoxitin (1 and 8 mg/mL, respectively) on half the plate and ceftazidime and cefoxitin (1 and 8 mg/mL, respectively) on the other half of the plate (Mediaproducts, Groningen, The Netherlands) (Drijver *et al*. 2019). For all oxidase-negative isolates that grew on either side of the selective agar plates, species identification was performed by automated mass spectrometry systems (VitekMS, bioMérieux, Marcy l'Etoile, France). Susceptibility testing was performed using Vitek 2 (bioMérieux, Marcy l'Etoile, France). The presence of AmpC in all oxidase-negative isolates was phenotypically confirmed using the D68C AmpC & ESBL Detection Set (Mastdiscs, Mastgroup Ltd., Bootle, UK) and interpreted according to the manufacturer's instructions. All phenotypically confirmed isolates were sequenced in the University of Groningen Medical Center (UMCG) using MiSeq (Illumina, San Diego, CA, USA) and assembled with CLC Genomics Workbench 9.0, 9.0.1 or 9.5.2 (Qiagen, Hilden, Germany) as was previously described in more detail by Kluytmans-van den Bergh *et al.* (Marjolein F Q Kluytmans-Van Den Bergh *et al*. 2016).

pAmpC-encoding Clinical Isolates from Elisabeth-Tweesteden Hospital

Suspected *pampC* gene containing *E. coli* isolates from blood cultures were selected retrospectively from our laboratory database based upon the presence of a phenotype (cefoxitin minimal inhibitory concentration (MIC) > 8 mg/L and/or cefotaxime MIC ≥ 1mg/L and/or ceftazidime MIC ≥ 1mg/L. One *Salmonella enterica* serotype Kentucky isolate from a faecal sample was selected from our laboratory database based upon the presence of an AmpC suspected phenotype (cefoxitin MIC > 8 mg/L and/or cefotaxime MIC ≥ 1mg/L and/or ceftazidime MIC ≥ 1mg/L). The isolates were recultured from deep frozen samples on blood agar and identified using the MALDI-TOF MS (BD Diagnostic Systems, Sparks, MD, USA). Susceptibility testing was performed using a Phoenix Automated Microbiology System (BD Diagnostic Systems, Sparks, MD, USA). The isolates were sequenced using an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). DNA isolation and sequencing were performed as described by Coolen *et al.* (Coolen *et al*. 2019). De novo assembly and error correction were performed using SPAdes version 3.9.1 (Bankevich *et al*. 2012).

Whole-Genome Bioinformatics Analysis of Short-Read Sequencing Data

The presence of acquired resistance genes was identified by uploading assembled genomes to the ResFinder web service of the Center for Genomic Epidemiology (version 3.1) (Zankari *et al*. 2012). The presence of plasmid replicons and the typing of a specific IncI plasmid was performed using pMLST (version 2.0) (Alessandra Carattoli *et al*. 2014). The genomes were selected based on a 100% match to blaCMY-2 and IncI-pST12. Typing of a specific multi locus sequence type (MLST) was performed using the MLST web service of the Center for Genomic Epidemiology (version 2.0), and *fim* typing was performed using FimTyper (version 1.0), Center for Genomic Epidemiology (Camacho *et al*. 2009; Larsen *et al*. 2012).

Long-Read Sequencing and Hybrid Assembly

No more than two isolates of the same flock or patient belonging to the same MLST were selected for further long-read sequencing.

All isolates were long-read sequenced on a MinION sequencer using the FLO-MIN106D flow cell and the Rapid Barcoding Sequencing Kit SQK RBK004 according to the standard protocol provided by the manufacturer (Oxford Nanopore Technologies, Oxford, UK). A hybrid assembly of long-read and short-read sequence data was performed using Unicycler v.0.8.4 (Wick *et al*. 2017). Whole-genome MLST (wgMLST) (core and accessory genome) was performed for all isolates using Ridom SeqSphere+, version 4.1.9 (Ridom, Münster, Germany). Species-specific wgMLST typing schemes were used as described previously (Marjolein F Q Kluytmans-Van Den Bergh *et al*. 2016). The pairwise genetic difference between isolates of the same species was calculated by dividing the total number of allele differences by the total number of shared alleles from the typing scheme present in both sequences, using a pairwise ignoring missing values approach. Genetic relatedness was determined using the thresholds for wgMLST-based genetic distance of 0.0095, as described previously (Marjolein F Q Kluytmans-Van Den Bergh *et al*. 2016).

Plasmid Analysis

The genomes created using the hybrid assembly were uploaded to the online bioinformatics tools ResFinder v.2.1, VirulenceFinder v.1.2 and PlasmidFinder v.1.2. (Center for Genomic Epidemiology, Technical University of Denmark, Lingby, Denmark) (Zankari *et al*. 2012; Alessandra Carattoli *et al*. 2014; Joensen *et al*. 2014). Circular components created by the hybrid assembly that were smaller than 1000 kb and that contained an IncI1-pST12 plasmid replicon and a $bla_{\text{CMY-2}}$ gene were extracted from

the assembly graph using BANDAGE v0.8.1. (Wick *et al*. 2015). All extracted plasmid components were annotated using Prokka v1.13.3 (Seemann 2014). Using snippy v4.4.59 (https://github.com/tseemann/snippy), the number of single nucleotide polymorphisms (SNPs) was determined between the extracted plasmid components using a *bla*CMY-2 gene containing IncI1–pST12 plasmid extracted from the GenBank (accession number: MH472638.1) as reference (Roer *et al*. 2019). A pan-genome was constructed, and a gene presence or absence was determined for all extracted plasmid components using roary v3.12 (Page *et al*. 2015). All extracted plasmids consisting of a single circular contig were aligned using GView 1.7 and progressiveMAUVE v2.4.0 to detect possible rearrangements (Petkau *et al*. 2010; Darling, Mau, and Perna 2010). If a hypervariable region is identified, the sequence of this region and its flanking regions are extracted using biopython v1.37. Moreover, segments (A, B, C, D) and flanking genes (*PilV* and *rci*) of a previously described hypervariable shufflon region of the IncI1 replicon containing plasmids (GenBank accession nr: AB027308.1) were BLAST searched in the extracted hypervariable regions (Brouwer *et al*. 2015; 2019).

Classification of Pairwise Comparisons

Pairwise comparisons of assembled plasmids were classified according to the known epidemiological link between the isolates: (i) same sample; (ii) same ward/flock but different sample; (iii) same location (hospital or farm) but different ward/flock and sample; (iv) same domain (human or broiler) but different location, ward/flock and sample; and (v) no known epidemiological link, i.e., different domain, location, ward/floc, and sample.

Ethical Statement

The I-4-1-Healt study was judged to be beyond the scope of the Dutch Medical Research Involving Human Subjects Act and the Belgian Law on Experiments on Humans, dated 7 May 2004. Written or verbal informed consent for data collection and taking a faecal, perianal, or gastrointestinal stoma swab for microbiological culture is obtained from all participants or their legal representatives. For the veterinary domain, approval by an animal welfare body is not required. All human data are anonymized, i.e., data cannot be directly or indirectly related to their source. Data on institutions and farms are pseudonymized, i.e., identifying information is replaced by a code, and a key file that links this code to the identifying information is kept separate from the research data.

Results

Isolate Characteristics

A total of 2508 human cases from four different hospitals and 119 broilers from 14 different farms were screened for the presence of plasmid encoded AmpC genes, e.g bla_{CMY-2} (Table S1). In 107 isolates, an AmpC phenotype was confirmed based on the D68C AmpC & ESBL Detection Set. Sixteen of 107 isolates contained both an IncI1 pST12 and a bla_{CMY-2} gene (Table S1). Based upon the above-mentioned selection criteria, fourteen isolates were included for long-read sequencing analysis, i.e., thirteen *E. coli* and one *Salmonella enterica*, serotype Kentucky (Table 1). Nine of the *E. coli* isolates were from one broiler farm; the other isolates were from human origin. The *E. coli* isolates included five different MLSTs and *fim* types. Based on wgMLST analysis, four different clusters could be identified (Figure 1, Table 1, and Table S2). Additional information regarding antimicrobial resistance phenotype and genotype of the included isolates is provided in Table S3.

Figure 1 Neighbor-joining tree representing the whole-genome multi locus sequence type (wgMLST) analysis of the different *E. coli* isolates included in the study. Isolates belonging to the same clonal clusters are represented in the identical colours.

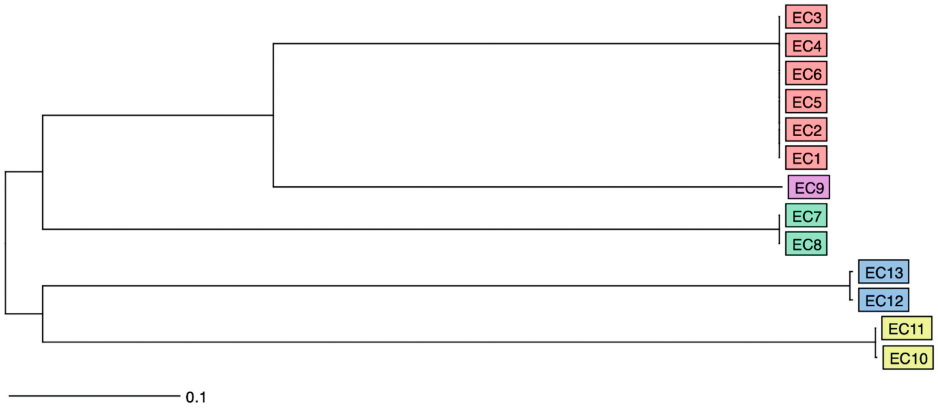

Table 1. Descriptive characteristics of fourteen IncI1–pST12 and bla_{CMY-2} containing isolates.

Isolate no.	Species	MultilocusST[a]	wgMLST cluster	Fim	Origin	Sample location	Flock or ward	Sample source	Month and Year of isolation	Accession no.
EC1	*E. coli*	ST665	1	fimH30	Broiler	Farm 1,	Flock 1	Faecal swab 1	Nov 2017	ERS4591617
EC2	*E. coli*	ST665	1	fimH30	Broiler	Farm 1	Flock 1	Faecal swab 2	Nov 2017	ERS4591618
EC3	*E. coli*	ST665	1	fimH30	Broiler	Farm 1	Flock 2	Faecal swab 3	Nov 2017	ERS4591619
EC4	*E. coli*	ST665	1	fimH30	Broiler	Farm 1	Flock 2	Faecal swab 4	Nov 2017	ERS4591620
EC5	*E. coli*	ST665	1	fimH30	Broiler	Farm 1	Flock 3	Faecal swab 5	Nov 2017	ERS4591621
EC6	*E. coli*	ST665	1	fimH30	Broiler	Farm 1	Flock 3	Faecal swab 6	Nov 2017	ERS4591622
EC7	*E. coli*	ST86	2	fimH289	Broiler	Farm 1	Flock 3	Faecal swab 5	Nov 2017	ERS4591623
EC8	*E. coli*	ST86	2	fimH289	Broiler	Farm 1	Flock 3	Faecal swab 7	Nov 2017	ERS4591624
EC9	*E. coli*	ST6856		fimH71	Broiler	Farm 1	Flock 3	Faecal swab 6	Nov 2017	ERS4591625
EC10	*E. coli*	ST131	3	fimH22	Human	Hospital 1	Ward 1	Blood 1	Oct 2013	ERS4591626
EC11	*E. coli*	ST131	3	fimH22	Human	Hospital 2	Ward 1	Blood 2	Jul 2014	ERS4591627
EC12	*E. coli*	ST973	4	fimH95	Human	Hospital 3	Ward 1	Rectal swab 1	Dec 2017	ERS4591628
EC13	*E. coli*	ST973	4	fimH95	Human	Hospital 3	Ward 2	Rectal swab 2	Dec 2017	ERS4591629
SE1	*Salmonella enteritidis*	-		-	Human	Primary care unit	n.a.	Faeces	Aug 2018	ERS4591630

a. MLST according to Enterobase (http://enterobase.warwick.ac.uk/)

Plasmid Analysis

In the hybrid assembly of fourteen sequences, both the IncI1–pST12 replicon gene and bla_{CMY-2} gene were located on a single circular contig ranging in size from 98,410 to 98,999 bp. No additional antimicrobial resistance or virulence genes were detected on any of the extracted plasmids. The number of SNP's detected between the fourteen plasmids ranged from zero to nine SNPs (Table 2). When comparing the plasmids extracted from the selected isolates to a publicly available IncI1–pST12 bla_{CMY-2} gene-containing plasmid extracted from the GenBank (accession number: MH472638.1), the number of SNPs detected ranged from 0 to 7 (Table 2). A small SNP difference was seen between epidemiologically related strains with a maximum difference of two SNPs. The range of SNP differences overlapped between epidemiologically related and unrelated plasmids (Table 3). The median number of SNP differences of plasmids in a different domain or different location, but the same domain was higher than in the other three pairwise comparison groups.

The total number of genes detected in the fourteen plasmids was 113, of which 112 were detected in all plasmids. One gene was present only in one plasmid (pEC11) and encoded for a hypothetical protein. An alignment of coding regions of the fourteen plasmids revealed no rearrangements between the described plasmids (Figure 2). However, progressive MAUVE alignment of non-coding regions revealed a small highly variable region of 519 to 1096 bp in all plasmids (Figure S1). This hypervariable region and approximately 2125 bp of the flanking sequence were extracted from all plasmids. The genes *PilV* and *rci* were detected in the flanking regions of the hypervariable region of all plasmids (Table 4). Moreover, in all plasmids, either one (B) or two (A, B) shufflon segments were detected in the extracted hypervariable region of the various plasmids (Table 4). No rearrangements were detected in any of the other regions.

Table 2. Number of SNP's detected between the 14 extracted plasmids and GenBank reference plasmid MH472638.1.

	pEC1	pEC2	pEC3	pEC4	pEC5	pEC6	pEC7	pEC8	pEC9	pEC10	pEC11	pEC12	pEC13	pSE1	MH472638.1
pEC1	0	2	2	2	2	2	3	3	3	3	3	9	8	6	2
pEC2	2	0	0	0	0	0	1	1	1	1	1	7	6	4	0
pEC3	2	0	0	0	0	0	1	1	1	1	1	7	6	4	0
pEC4	2	0	0	0	0	0	1	1	1	1	1	7	6	4	0
pEC5	2	0	0	0	0	0	1	1	1	1	1	7	6	4	0
pEC6	2	0	0	0	0	0	1	1	1	1	1	7	6	4	0
pEC7	3	1	1	1	1	1	0	0	0	2	2	8	7	5	1
pEC8	3	1	1	1	1	1	0	0	0	2	2	8	7	5	1
pEC9	3	1	1	1	1	1	0	0	0	2	2	8	7	5	1
pEC10	3	1	1	1	1	1	2	2	2	0	0	8	7	5	1
pEC11	3	1	1	1	1	1	2	2	2	0	0	8	7	5	1
pEC12	9	7	7	7	7	7	8	8	8	8	8	0	1	5	7
pEC13	8	6	6	6	6	6	7	7	7	7	7	1	0	4	6
pSE1	6	4	4	4	4	4	5	5	5	5	5	5	4	0	4
MH472638.1	2	0	0	0	0	0	1	1	1	1	1	7	6	4	0

Table 3. Median and range of SNP differences in pairwise comparisons per EPI link

		SNP differences	
	n of pairwise comparisons	Median	Range
Same sample[(i)]	2	1	1
Same flock, different sample[(ii)]	10	0.5	0-2
Same location, different ward/flock[(iii)]	25	1	0-3
Same domain, different location[(iv)]	9	5	0-8
Different domain[(v)]	45	4	1-9

Figure 2. GView alignment of the various plasmid sequences. Each arrow represents a coding sequence and not necessarily transcriptional direction; gene names are depicted as generated by prokka.

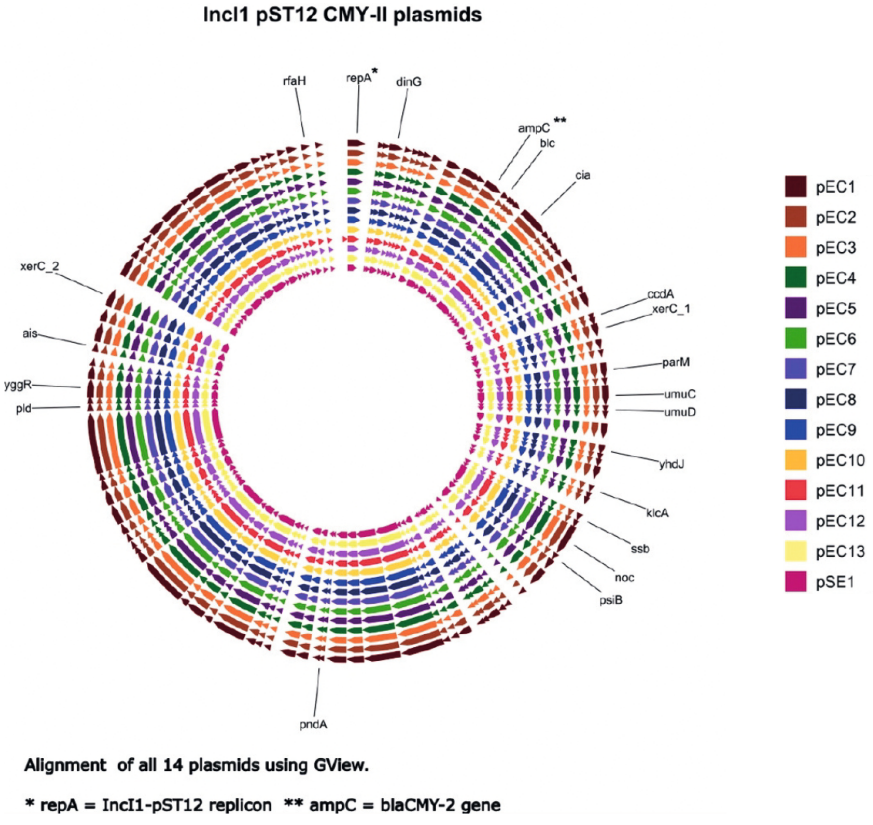

Table 4. Shufflon segments in variable regions of the different plasmids included (direction: ′5–′3).

Plasmid	Shufflon Segments			
pEC1	*PilV*	A	B	*rci*
pEC2	*PilV*	A	B	*rci*
pEC3	*PilV*	A	B	*rci*
pEC4	*PilV*	B	*rci*	
pEC5	*PilV*	B	*rci*	
pEC6	*PilV*	A	B	*rci*
pEC7	*PilV*	B	*rci*	
pEC8	*PilV*	A	B	*rci*
pEC9	*PilV*	A	B	*rci*
pEC10	*PilV*	A	B	*rci*
pEC11	*PilV*	B	A	*rci*
pEC12	*PilV*	B	*rci*	
pEC13	*PilV*	B	*rci*	
pSE1	*PilV*	B	*rci*	

Discussion

The current study included *E. coli* isolates of various sequence types and a *S. enterica* isolate, which were from both human and broiler origin. Plasmid analysis based on short- and long-read sequence data of bla_{CMY-2} containing IncI1-pST12 plasmids from the included isolates revealed a low number of SNP differences and a high number of shared genes between the various plasmids extracted. Despite the tendency of median SNP increase from epidemiologically related to unrelated plasmids, the range in number of SNPs detected overlapped between every classified epidemiological link in the current study. A small SNP difference was seen between epidemiologically related strains with

a maximum difference of two SNPs. Furthermore, only one gene was variably present between the different plasmids, and no rearrangements were observed apart from a small, highly variable region. This area is the formerly described highly variable shufflon region at the C-terminal end of the PilV protein (Brouwer *et al*. 2015; 2019).

A high degree of similarity between IncI1–pST12 plasmids was previously reported (Pietsch *et al*. 2018; Roer *et al*. 2019; Castellanos *et al*. 2019; Shirakawa *et al*. 2020). However, all of the studies either contained only plasmids extracted from one *E. coli* sequence type (ST131) or the included plasmids were primarily of poultry origin (Roer *et al*. 2019; Castellanos *et al*. 2019). All of the studies used either gene presence/absence-based or SNP-based analysis, but not both, possibly missing subtle differences between various plasmids. Shirakawa *et al* used a combination of short-read sequence data of different bla_{CMY-2}-containing plasmids from Japanese poultry and human origin, together with plasmid sequence data retrieved from the National Center for Biotechnology Information nucleotide database (https://www.ncbi.nlm.nih.gov/) to perform an extensive plasmid comparative analysis(Shirakawa *et al*. 2020). Their clustering analysis showed a high similarity among the IncI1–pST12 plasmids as well; however, this study did not provide further detail on the SNP differences of possible rearrangements within the plasmid sequences. Moreover, these studies predominately used in silico reference-based plasmid reconstructions of short-read sequence data rather than performing a hybrid assembly of both short- and long-read sequence data. A recent study by Valcek *et al.* on IncI1–pST3 and IncI1–pST7 plasmids showed that using combined long-read and short-read sequencing data improves the accuracy of a full plasmid analysis, e.g., of rearrangements (Valcek *et al*. 2019). The current study is the first study describing plasmid differences using both gene presence/absence-based and SNP-based analysis. Moreover, rearrangements between the different plasmids could be detected such as those shown in the hypervariable region, which were missed in previous studies based on only short-read sequences.

Several studies have described outbreaks with bla_{CMY-2}-harbouring Enterobacteriaceae (Matsumura *et al*. 2015; Wendorf *et al*. 2015; I. F. Huang *et al*. 2005; Kameyama *et al*. 2015). Since the bla_{CMY-2} is predominantly located on plasmids, horizontal transfer of the plasmid in an outbreak can go undetected if only typing of the bacterial chromosome is performed. Distinguishing epidemiologically related and unrelated plasmids is essential to confirm plasmid transmission in an outbreak. Therefore, statements on the horizontal transfer of these plasmids based on genetic identity should be made with caution. However, given the conservation of the IncI1–pST12 plasmids, they could instead be used as a tool to monitor the speed and breadth of spread of these plasmids through populations, either different in place of origin or bacterial host.

The current study is the first to explore bla_{CMY-2}-containing IncI1–pST12 plasmids from related and unrelated isolates, using combined short- and long-read sequencing data. Moreover, this study includes isolates from different species, sequence types, and domains, both from human and broiler origin. Two different comparison techniques, either gene presence/absence and SNP differences, were used. Furthermore, combining long-read and short-read sequence data provided full plasmid analysis, including the presence of rearrangements.

By combining the isolate collections from three different studies, we screened a relatively large amount of human and broiler cases. However, due to low prevalence of bla_{CMY-2} in the Netherlands, our sample size remained relatively small. This results in the main limitation of the current study, as the small sample size precludes the use of statistical test and caution must be applied, as the findings should be confirmed in a study with a larger sample size. Preferably, such a study should include isolates of different species, sequence types, and origin of isolation containing IncI1–pST12 plasmids. Furthermore, the current study only included plasmids of broilers isolated in one farm; therefore, other plasmids of veterinary origin should be added in future studies to confirm our findings.

In conclusion, IncI1–pST12 plasmids of epidemiologically related and unrelated Enterobacteriaceae of both human and broiler origin in the current explorative study show a high degree of sequence similarity in terms of SNP differences and the number of shared genes.

Supplementary Materials

Supplementary materials can be found at https://www.mdpi.com/2076-2607/8/11/1755/s1, Tables S1–S3, and Figure S1.

Author Contributions

Conceptualization, E.P.M.d.D., J.J.J.M.S., J.J.V. and J.A.J.W.K.; methodology, E.P.M.d.D. and J.J.J.M.S.; software, J.J.J.M.S.; formal analysis, E.P.M.d.D. and J.J.J.M.S.; investigation, E.P.M.d.D., J.J.J.M.S., C.V. and F.C.V.; resources, J.J.V., A.S., M.F.Q.K.-v.d.B. and J.A.J.W.K.; data curation, E.P.M.d.D. and J.J.J.M.S.; writing—original draft preparation, E.P.M.d.D. and J.J.J.M.S.; writing—review and editing, J.J.V., C.V., F.C.V.,

A.S., M.F.Q.K.-v.d.B. and J.A.J.W.K.; visualization, J.J.J.M.S.; supervision, J.J.V. and J.A.J.W.K.; project administration, M.F.Q.K.-v.d.B. and J.A.J.W.K.; funding acquisition, J.A.J.W.K. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Supplementary data

Supplementary table S1. Number screened cases per study on AmpC producing isolates, number of sequenced isolates based on AmpC phenotype per study collection, isolates containing both a IncI1-pST12 replicon and a bla_{CMY-2} gene, and included isolates int the current study.

Collection	Total number of screened cases		Number of sequenced isolates based on AmpC phenotype		N of isolates containing IncI1-pST12 replicon and bla_{CMY-2} gene		N of isolates included in study	
	Human	Broiler	Human	Broiler	Human	Broiler	Human	Broiler
I-4-1 Health study	380	119	19	22	0	11	0	9
Amphia prevalence screening 2017	378	0	14	0	2	0	2	0
E. coli blood cultures 2013-2015	1749	0	51	0	2	0	2	0
Salmonella enteritidis faecal culture	1	0	1	0	1	0	1	0

Supplementary table S2. Distance matrix containing number of allele differences between the included *E. coli* isolates based on wgMLST

	EC1	EC2	EC3	EC4	EC5	EC6	EC7	EC8	EC9	EC10	EC11	EC12	EC13
EC1	0	0	0	0	0	0	2051	2051	1369	2318	2324	2288	2286
EC2	0	0	0	0	0	0	2053	2053	1370	2320	2326	2290	2288
EC3	0	0	0	0	0	0	2050	2050	1367	2317	2323	2287	2285
EC4	0	0	0	0	0	0	2051	2051	1368	2318	2324	2288	2286
EC5	0	0	0	0	0	0	2053	2053	1370	2320	2326	2290	2288
EC6	0	0	0	0	0	0	2053	2053	1370	2320	2326	2290	2288
EC7	2051	2053	2050	2051	2053	2053	0	1	2021	2338	2344	2304	2302
EC8	2051	2053	2050	2051	2053	2053	1	0	2021	2338	2344	2304	2302
EC9	1369	1370	1367	1368	1370	1370	2021	2021	0	2273	2279	2244	2242
EC10	2318	2320	2317	2318	2320	2320	2338	2338	2273	0	1	2363	2361
EC11	2324	2326	2323	2324	2326	2326	2344	2344	2279	1	0	2369	2367
EC12	2288	2290	2287	2288	2290	2290	2304	2304	2244	2363	2369	0	7
EC13	2286	2288	2285	2286	2288	2288	2302	2302	2242	2361	2367	7	0

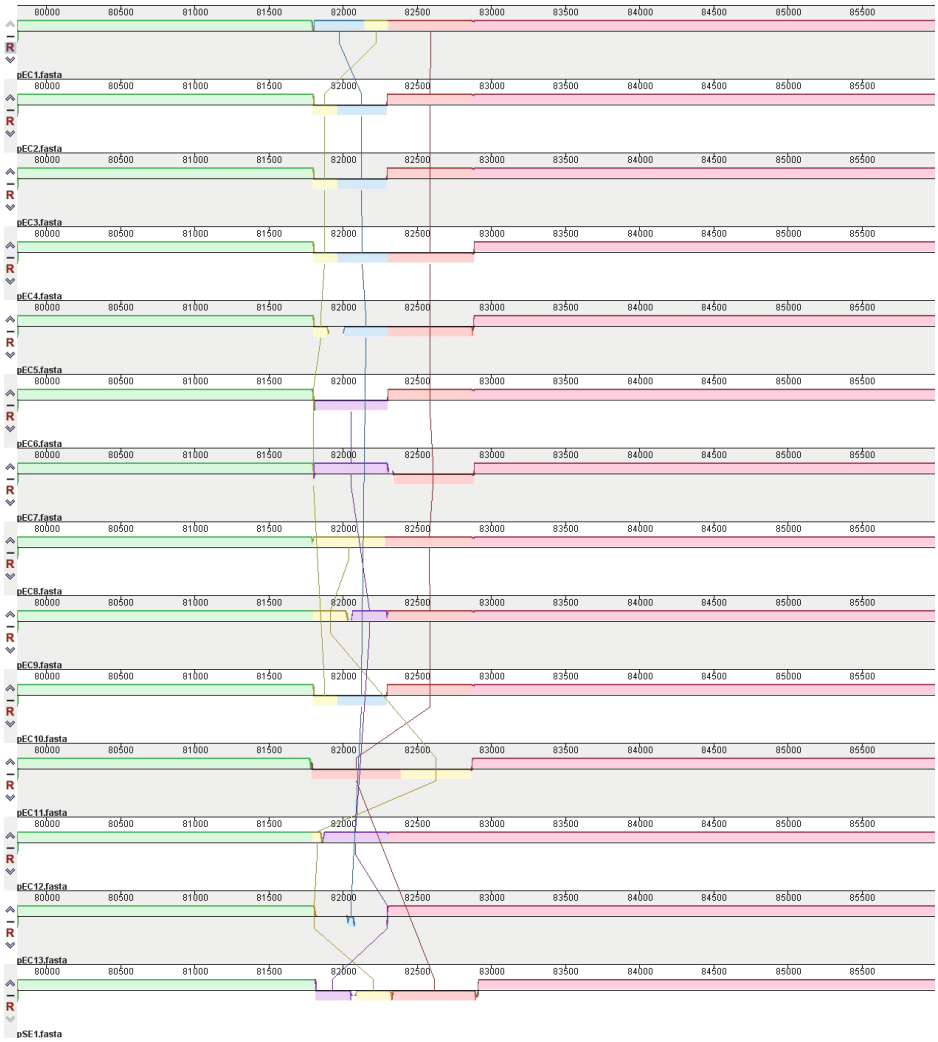
Supplementary table 3. Overview of resistance genes and phenotype of the fourteen IncI1 pST12 and *bla*CMY-2 containing isolates

Isolate no.	Species	MultilocusST[a]	Resistance genes	Piperacillin-Tazobactam MIC	Cefotaxime/ Ceftriaxone MIC	Ceftazidime MIC	Cefoxitin MIC	Accession no.[b]
EC1	*E. coli*	ST665	bla_{CMY-2}, $bla_{TEM-1b,}$ *aada1, strA, strB, sul1, sul2, dfra1*	8 mg/L	4 mg/L	32 mg/L	≥64 mg/L	ERS4591617
EC2	*E. coli*	ST665	bla_{CMY-2}, $bla_{TEM-1b,}$ *aada1, strA, strB, sul1, sul2, dfra1*	8 mg/L	8 mg/L	32 mg/L	≥64 mg/L	ERS4591618
EC3	*E. coli*	ST665	bla_{CMY-2}, $bla_{TEM-1b,}$ *aada1, strA, strB, sul1, sul2, dfra1*	8 mg/L	4 mg/L	32 mg/L	≥64 mg/L	ERS4591619
EC4	*E. coli*	ST665	bla_{CMY-2}*aada1, strA, strB, sul1, sul2, dfra1*	8 mg/L	8 mg/L	32 mg/L	≥64 mg/L	ERS4591620
EC5	*E. coli*	ST665	bla_{CMY-2}, $bla_{TEM-1b,}$ *aada1, strA, strB, sul1, sul2, dfra1*	8 mg/L	4 mg/L	32 mg/L	≥64 mg/L	ERS4591621
EC6	*E. coli*	ST665	bla_{CMY-2}, $bla_{TEM-1b,}$ *aada1, strA, strB, sul1, sul2, dfra1*	8 mg/L	4 mg/L	32 mg/L	≥64 mg/L	ERS4591622
EC7	*E. coli*	ST86	bla_{CMY-2}, $bla_{TEM-1b,}$, *strA, strB, sul2, dfra1*	≤4 mg/L	4 mg/L	8 mg/L	32 mg/L	ERS4591623
EC8	*E. coli*	ST86	bla_{CMY-2}, $bla_{TEM-1b,}$ *strA, strB, sul2, dfra1*	≤4 mg/L	8 mg/L	8 mg/L	32 mg/L	ERS4591624

Supplementary table 3. Continued.

Isolate no.	Species	MultilocusST[a]	Resistance genes	Piperacillin-Tazobactam MIC	Cefotaxime/ Ceftriaxone MIC	Ceftazidime MIC	Cefoxitin MIC	Accession no.[b]
EC9	*E. coli*	ST6856	bla_{CMY-2}, $bla_{TEM-1b,}$	8 mg/L	8 mg/L	32 mg/L	≥64 mg/L	ERS4591625
EC10	*E. coli*	ST131	bla_{CMY-2}, $bla_{TEM-1b,}$ *QnrS1, aac(6')-Ib-cr, aadA16-like*[c]*, ARR-3, sul1, dfrA27, tet(A)-like*[c]	≥128 mg/L	≥64 mg/L	≥64 mg/L	≥64 mg/L	ERS4591626
EC11	*E. coli*	ST131	bla_{CMY-2}, $bla_{TEM-1b,}$ *QnrS1*	8 mg/L	8 mg/L	16 mg/L	≥64 mg/L	ERS4591627
EC12	*E. coli*	ST973	bla_{CMY-2}	8 mg/L	>64 mg/L	32 mg/L	≥64 mg/L	ERS4591628
EC13	*E. coli*	ST973	bla_{CMY-2}	8 mg/L	8 mg/L	32 mg/L	≥64 mg/L	ERS4591629
SE1	*Salmonella enteritidis*	n.a	bla_{CMY-2}, $bla_{TEM-1b,}$ a*ac(6')-Iaa-like, rmtB, catA2-like*[c]*, tet(A)-like*[c]	8 mg/L	>4 mg/L	>16 mg/L	>16 mg/L	ERS4591630

Figure S1. Hypervariable region.



Chapter 7

Genome-wide analysis in *Escherichia coli* unravels a high level of genetic homoplasy associated with cefotaxime resistance

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Abstract

Cefotaxime (CTX) is a third-generation cephalosporin (3GC) commonly used to treat infections caused by *Escherichia coli*. Two genetic mechanisms have been associated with 3GC resistance in *E. coli*. The first is the conjugative transfer of a plasmid harbouring antibiotic-resistance genes. The second is the introduction of mutations in the promoter region of the *ampC* beta-lactamase gene that cause chromosome-encoded beta-lactamase hyperproduction. A wide variety of promoter mutations related to AmpC hyperproduction have been described. However, their link to CTX resistance has not been reported. We recultured 172 cefoxitin-resistant *E. coli* isolates with known CTX minimum inhibitory concentrations and performed genome-wide analysis of homoplastic mutations associated with CTX resistance by comparing Illumina whole-genome sequencing data of all isolates to a PacBio sequenced reference chromosome. We mapped the mutations on the reference chromosome and determined their occurrence in the phylogeny, revealing extreme homoplasy at the −42 position of the *ampC* promoter. The 24 occurrences of a T at the −42 position rather than the wild-type C, resulted from 18 independent C>T mutations in five phylogroups. The −42 C>T mutation was only observed in *E. coli* lacking a plasmid-encoded *ampC* gene. The association of the −42 C>T mutation with CTX resistance was confirmed to be significant (false discovery rate <0.05). To conclude, genome-wide analysis of homoplasy in combination with CTX resistance identifies the −42 C>T mutation of the *ampC* promotor as significantly associated with CTX resistance and underlines the role of recurrent mutations in the spread of antibiotic resistance.

Impact Statement

In the past decades, the worldwide spread of extended-spectrum beta-lactamases (ESBLs) has led to a substantial increase in the prevalence of resistant common pathogens, thereby restricting available treatment options. Although acquired resistance genes, e.g. ESBLs, get most attention, chromosome-encoded resistance mechanisms may play an important role as well. In *Escherichia coli,* chromosome-encoded beta-lactam resistance can be caused by alterations in the promoter region of the *ampC* gene. To improve our understanding of how frequently these alterations occur, a comprehensive interpretation of the evolution of these mutations is essential. In the current study, we apply genome-wide homoplasy analysis to better perceive adaptation of the *E.*

coli genome to antibiotics. Thereby, this study grants insights into how chromosome-encoded antibiotic resistance evolves and, by combining genome-wide association studies with homoplasy analyses, provides potential strategies for future association studies into the causes of antibiotics resistance.

Keywords: *ampC*, bioinformatics, *Escherichia coli*, genomics, whole-genome sequencing

Data summary

All data is available from the National Center for Biotechnology Information (NCBI) under BioProject number PRJNA592140. Raw Illumina sequencing data and metadata for all 171 *Escherichia coli* isolates used in this study is available from the NCBI Sequence Read Archive database under accession numbers SAMN15052485 to SAMN15052655. The full reference chromosome of ampC_0069 is available via GenBank accession number CP046396.1 and NCBI Reference Sequence NZ_CP046396.1. The scripts used to calculate homoplasy-based association analysis are available from GitHub (https://github.com/JordyCoolen/hombaampC) under MIT license.

Introduction

Escherichia coli is an important pathogen in both community and healthcare-associated infections (Weinstein, Gaynes, and Edwards 2005; J. D. D. Pitout 2012). In the past decades, a substantial increase in resistance to third-generation cephalosporin (3GC) antibiotics in *E. coli* has been observed worldwide, mainly caused by the production of extended-spectrum beta-lactamases (ESBLs) and AmpC beta-lactamases, restricting available treatment options for common infections (Jacoby 2009). AmpC beta-lactamases differ from ESBL as they hydrolyse not only broad-spectrum penicillins and cephalosporins, but also cephamycins. Moreover, AmpC beta-lactamases are not inhibited by ESBL-inhibitors like clavulanic acid, limiting antibiotic treatment options even further (Jacoby 2009). A widely used screening method for AmpC production is the use of susceptibility to cefoxitin (FOX), a member of the cephamycins (Martinez and Simonsen 2017).

Although *ampC* beta-lactamase genes can be plasmid-encoded (plasmid-mediated *ampC*, *pampC*), they are also encoded on the chromosomes of numerous *Enterobacterales* . *E. coli* naturally carries a chromosome-mediated *ampC* (*campC*) gene but, unlike most other *Enterobacterales* , this gene is non-inducible due to the absence of the *ampR* regulator gene (Jacoby 2009). Chromosomal AmpC production in *E. coli* is exclusively regulated by promoter and attenuator mechanisms. This results in constitutive low-level c*ampC* expression that still allows the use of 3GC antibiotics, such as cefotaxime (CTX), to treat *E. coli* infections (Jacoby 2009). However, various mutations in the promoter/attenuator region of *E. coli* may cause constitutive hyperexpression of *campC*, thereby increasing the minimum inhibitory concentrations (MICs) for broad-spectrum penicillins and cephalosporins and limiting appropriate treatment options (Tracz *et al*. 2005; 2007).

A wide variety of promoter and attenuator mutations have been related to AmpC hyperproduction (Tracz *et al*. 2007). AmpC hyperproduction is primarily caused by alterations of the *ampC* promoter region, leading to a promoter sequence that more closely resembles the *E. coli* consensus σ^{70} promoter with a TTGACA −35 box separated by 17 bp from a TATAAT −10 box. These alterations can be divided into different variants associated with, for example, an alternate displaced promoter box, a promoter box mutation or an alternate spacer length due to insertions (Tracz *et al*. 2007). Furthermore, mutations of the attenuator sequence can lead to changes in the hairpin structure that strengthen the effect of promoter alterations on AmpC hyperproduction. In the study by Tracz *et al.* on FOX-resistant *E. coli* isolated from Canadian hospitals, 52 variants of

the promoter and attenuator region were described (Tracz *et al*. 2007). Tracz *et al*. used a two-step quantitative reverse-transcriptase PCR (qRT-PCR) to determine the effect of promoter/attenuator variants on *ampC* expression. Various mutations were related to different delta–delta cycle threshold values in the qRT-PCR and corresponding variations in FOX resistance. An interesting observation that emerged from this study was that the −32T>A and the −42C>T mutation were the major alterations that strengthened the *ampC* promoter. Both result in a consensus −35 box. Although it is known that AmpC hyperproduction leads to FOX resistance, as studied by Tracz *et al*., the effects of various mutations on resistance to a 3GC antibiotic such as CTX have not been explored. This is relevant because CTX is commonly used in the treatment of patients with severe *E. coli* infections, often in combination with selective digestive tract decontamination in intensive care units (de Smet *et al*. 2009; Aardema *et al*. 2020).

While previous research mainly focused on the chromosomal AmpC resistance mechanism and the impact of AmpC hyperproduction, there is a lack in knowledge and understanding of the evolutionary origin of these promoter/attenuator variants. Notably, it is unexplored how the two most prominent promoter mutations, −32T>A and −42C>T, are distributed over the *E. coli* phylogeny and therewith how often they occur. More precisely, literature shows selective pressure can lead to convergent evolution that results in the reoccurrence of a mutation in multiple isolates independently and in separate lineages (Wake, Wake, and Specht 2011). This phenomenon is named homoplasy (Crispell, Balaz, and Gordon 2019). A consistency index can be calculated to quantify homoplasy by dividing the minimum number of changes on the phylogeny by the number of different nucleotides observed at that site minus one, effectively quantifying how often the same mutation occurred in a phylogenetic tree (Kluge and Farris 1969). One can use the consistency index to recognize genomic locations subjected to homoplasy, and relate the SNP positions that are inconsistent with the phylogeny to antibiotic resistance, as has been done, for example, in multiple studies on *Mycobacteria* spp. (Farhat *et al*. 2013; Mortimer, Weber, and Pepperell 2018; Ruesen *et al*. 2018; Miotto *et al*. 2014).

In the present study, we hypothesize that some of the mutations in the *ampC* promoter/attenuator region are homoplastic and are associated with CTX resistance. To test our hypothesis, we performed genome-wide homoplasy analysis and combined it with a genome-wide analysis of polymorphisms associated with CTX resistance by constructing an *E. coli* reference chromosome and combining it with whole-genome sequencing (WGS) data of 172 both FOX resistant and ESBL-negative *E. coli* isolates from human and animal origin previously collected by our research group (Coolen *et al*. 2019).

Methods

Isolate selection, DNA isolation, library preparation and DNA sequencing

One hundred and seventy-two both FOX resistant (MIC >8 mg l^{-1}) and ESBL-negative *E. coli* isolates previously used by our study group were selected in the present study (Table S1, available with the online version of this article) (Coolen *et al*. 2019). To summarize the methods, DNA isolation was performed as previously described (Coolen *et al*. 2019), library preparations were performed using an Illumina Nextera XT library preparation kit, and DNA sequencing was performed using an Illumina NextSeq 500 to generate 2×150 bp paired-end reads or 2×300 bp reads on an Illumina MiSeq. *De novo* assembly was also performed identically to the method as described previously [16] using SPAdes version 3.11.1 (Bankevich *et al*. 2012).

Phylogroup and multilocus sequence typing (MLST)

Phylogroup stratification was performed using ClermonTyping version 1.4.0 (Beghain *et al*. 2018). MLST sequence types (STs) were derived from the contigs using Mlst version 2.5 PubMLST (31 October 2017) (Jolley and Maiden 2010; Seemann, T, n.d.).

Obtaining the ampC promoter/attenuator region

To detect the promoter/attenuator region, a custom blast database was created using the 271 bp fragment as described by Peter-Getzlaff *et al.* using *E. coli* K-12 strain ER3413 (accession no. CP009789.1) (Altschul *et al*. 1990; Peter-Getzlaff *et al.* 2011). ABRicate version 0.8.9 was used to locate matching regions per sample and these were extracted and converted into multi-fasta format using a custom Python script (Seemann, T, n.d.). Strains were labelled AmpC putative hyperproducer when promoter mutations were found, as previously identified by Caroff *et al*., Siu *et al.* and Tracz *et al.* (Caroff N, Espaze E, Gautreau D, Richet H 2000; Tracz *et al*. 2007; Siu *et al*. 2003).

pampC detection

Detection of *pampC* genes was performed by using ABRicate version 0.5 and ResFinder database (16/02/2018) as described by Coolen *et al.* (Coolen *et al*. 2019).

PacBio single-molecule real-time (SMRT) sequencing of an E. coli isolate

To enable an accurate SNP analysis, a reference chromosome of an *E. coli* isolate from our collection (ampC_0069) was constructed using PacBio SMRT sequencing. For sequencing, genomic DNA (gDNA) was extracted using a bacterial gDNA isolation kit (Norgen Biotek). A single *E. coli* isolate was subjected to DNA shearing using Covaris

g-TUBEs for 30 s at 11 000 r.p.m. Each DNA sample was separated into two aliquots. Size selection was performed using a 0.75% agarose cassette and marker S1 on the BluePippin system (Sage Science) to obtain either 4–8 kb or 4–12 kb DNA fragments. This size selection was chosen to maintain all DNA fragments, including these originating from plasmids (data not used in this study). Library preparation was performed using the PacBio SMRTbell template prep kit 1.0 (Pacific Biosciences). For cost-effectiveness, samples were barcoded and pooled with other samples that are not relevant for this study. Sequencing was conducted using the PacBio Sequel I (Pacific Biosciences) on a Sequel SMRT Cell 1M v2 (Pacific Biosciences) with a movie time of 10 h (and 186 min pre-extension time). Subreads per sample were obtained by extracting the bam files using SMRT Link version 5.1.0.26412 (Pacific Biosciences).

Chromosomal reconstruction using de novo hybrid assembly

To obtain a full-length chromosome, Unicycler version 0.4.7 (settings: --mode bold) was used, combining Illumina NextSeq 500 2×150 bp paired-end reads with PacBio Sequel SMRT subreads (Wick *et al*. 2017). Because Unicycler requires fasta reads as input, the subreads in bam format were converted to fasta by using bam2fasta version 1.1.1 from pbbioconda (https://github.com/PacificBiosciences/pbbioconda) prior to *de novo* hybrid assembly. The full circular chromosome was uploaded to the National Center for Biotechnology Information (NCBI) database and annotated using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 4.10 (Tatusova *et al*. 2016; Haft *et al*. 2018).

SNP analysis using E. coli reference ampC_0069

Alignment of Illumina reads and SNP calling was performed for all isolates to the reference chromosome of *E. coli* isolate ampC_0069 using Snippy version 4.3.6 (https://github.com/tseemann/snippy). A full-length alignment (fullSNP) and a coreSNP alignment containing SNP positions shared among all isolates were generated by using snippy-core version 4.3.6 (https://github.com/tseemann/snippy).

Inferring of phylogeny

A phylogenetic tree was inferred by using the coreSNP alignment as input for FastTree(MP) version 2.1.3 SSE3 (settings: -nt -gtr) (M. N. Price, Dehal, and Arkin 2010).

Detection of homoplasy

The consistency index for all nucleotide positions on the chromosome was calculated using HomoplasyFinder version 0.0.0.9000 (Crispell, Balaz, and Gordon 2019). The

coreSNP phylogeny was used as true phylogeny and the consistency index was calculated using the multiple sequence alignments fullSNP alignment as input.

Relating mutations to CTX resistance

To assess whether certain mutations were linked to CTX resistance, all non-plasmid-harbouring *ampC E. coli* isolates were used. CTX resistance was defined using European Committee on Antimicrobial Susceptibility Testing (EUCAST) guideline standards of CTX MIC>2 mg l^{-1} (The European Committee on Antimicrobial Susceptibility Testing. 2020). CTX MIC results were obtained from our previous study (Coolen *et al*. 2019). For each nucleotide position on the reference chromosome, the numbers of resistant and sensitive isolates were counted and tested for adenine versus all other nucleotides, thymine versus all other nucleotides, cytosine versus all other nucleotides, and guanine versus all other nucleotides, creating a contingency table and performing a Fisher's exact test in R 3.6.1 (Mehta and Patel 1983). To correct for multiple testing, *P* values were adjusted using false discovery rate (FDR) (Benjamini and Hochberg 1995).

Selection of genomic positions of interest

Genomic positions with potential roles in CTX resistance were identified based on FDR ≤0.05 for CTX and a consistency index of ≤0.05882353. Annotation of mutation positions was obtained by using the genome annotation of the reference chromosome (GenBank accession no. CP046396) and applying snpEff (version 4.3 t) (Cingolani *et al*. 2012). The EnteroBase core-genome MLST and whole-genome MLST schemes were used to distinguish core and accessory genes (Z. Zhou *et al*. 2020).

Recombination analysis

Gubbins version 2.4.1. (settings: -f 30) was used to detect recombination regions with coreSNP alignment and tree as input (Croucher *et al*. 2015).

Pyseer analysis

To compare our homoplasy-associated analysis method to an alternative method, we used Pyseer (version 1.3.6) (Lees *et al*. 2018). In short, variant calling files (VCFs) were obtained from snippy, and bcftools (version 1.11) was used to merge and filter the VCFs from all samples to a single VCF (Li *et al*. 2009). A phylogenetic distance file was calculated by using the phylogeny_distance.py included in Pyseer on the corrected for recombination phylogeny of Gubbins. Finally, the distance, trait and VCF file was used to run Pyseer (default fixed effects) with settings --min-af 0.01, --max-af 0.99.

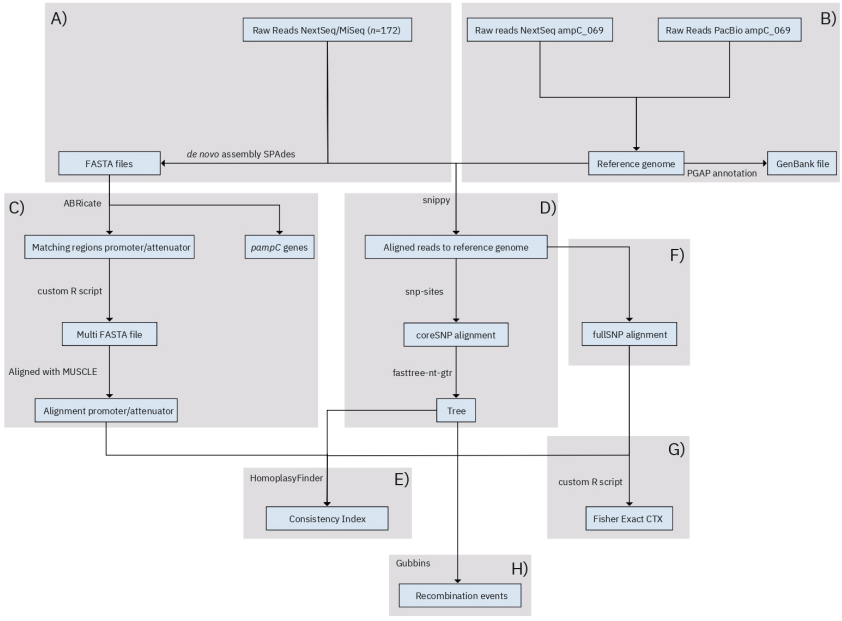
Visualization of data

The interactive tree of life web-based tool (iTOL) version 5.3 was used to visualize the phylogenetic tree (Letunic and Bork 2019). Information about CTX resistance, presence of the *pampC* gene, *campC* hyperproduction as defined, MLST and phylogroup, as well as alignments of promoter and attenuator region, were incorporated into the visualization. The sequence logo of the promoter and the attenuator alignment were generated using the web-based application WebLogo, version 3.7 (http://weblogo.threeplusone.com) (Crooks *et al*. 2004). A chromosome ideogram of the *E. coli* isolate ampC_0069 reference chromosome was visualized using the circos software package, version 0.69-8 (Krzywinski *et al*. 2009). Consistency index scores and significant mutations associated with CTX resistance were plotted in the ideogram. Gubbins results were displayed by using Phandango (Hadfield *et al*. 2018).

Overview of the method

A workflow graph of the method is visualized in Fig. 1, using the web-based application yEd Live version 4.4.2 (https://www.yworks.com/yed-live/).

Figure 1. Schematic of the workflow used to perform the homoplasy-based association analysis. Starting from the top, (a) the *de novo* assembly of the NextSeq/MiSeq reads and (b) the hybrid assembly of the reference chromosome ampC_069. On the left side, (c) the alignment of promoter/attenuator region. In the middle, (d) the coreSNP analysis for the phylogeny used in (e) the homoplasy analysis combined with (f) the fullSNP data, on the right, which was also used for (g) the statistics (Fisher›s exact test and FDR) to relate CTX resistance to SNP positions. (h) Inferring recombination events using Gubbins.



Results

E. coli collection

To study genetic homoplasy events in suspected AmpC-producing *E. coli,* FOX-resistant ESBL phenotype negative *E. coli* isolates (*n*=172) were selected, as previously described by Coolen *et al.* (see Table S1) (Coolen *et al*. 2019). The entire collection was subjected to WGS, followed by *de novo* assembly of the sequence reads to obtain contigs.

MLST and phylogroup variants

To access the genetic diversity of our *E. coli* collection, we identified both MLST and phylogroup variants of each of the 172 *E. coli* isolates. A total of 75 different STs were identified, of which ST131 (8.1%, *n*=14), ST38 (7.0%, *n*=12) and ST73 (7.0%, *n*=12) were the most prevalent. The STs of 13 isolates are unknown. Phylogroup stratification revealed that the isolates belonged to eight different phylogroups (Table 1). Phylogroup B1, B2 and D were the most prevalent. One isolate belonged to *E . coli* clade IV (strain no. ampC_0128).

ampC promoter and attenuator variants

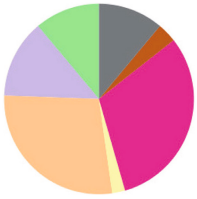
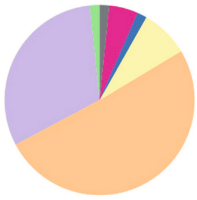
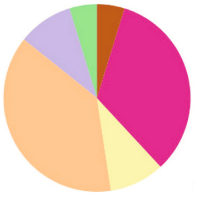
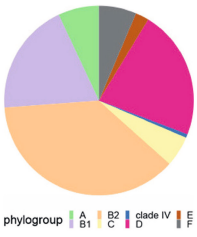
We examined the whole *E. coli* genome. However, we firstly focused on mutations in the *ampC* promoter and attenuator region. Previously described mutations in the *ampC* promoter region that according to described literature lead to 'hyperproduction' of AmpC were detected in 61 (35.5%) of the isolates (see Tables S1 and S2) (Caroff N, Espaze E, Gautreau D, Richet H 2000; Tracz *et al*. 2007; Siu *et al*. 2003). These isolates were, therefore, labelled as putative hyperproducers. Analysis of the promotor area (−42 to −8) resulted in 20 different variants and the wild-type (see Table 1). In the attenuator region (+17 to +37), 18 different variants were identified (see Table 1). One isolate (ampC_0128) showed an unusual promoter variant, a four nucleotide deletion (−45_−42delATCC). Moreover, an insertion (21_22insG) of unknown function was detected in the attenuator of this isolate (ampC_0128) as well (see Tables S1 and S2).

pampC variants

As we aimed to associate chromosomal mutations with CTX resistance, differentiation of *pampC*-harbouring isolates from non-*pampC*-harbouring isolates was required. Genomic analysis showed that 90 (52.3%) of the isolates harboured a *pampC* gene of which $bla_{\text{CMY-2}}$ was the most prevalent (*n*=78). One isolate harboured two different *pampC* genes ($bla_{\text{CMY-4}}$ and $bla_{\text{DHA-1}}$) (ampC_0119). One isolate contained

a $bla_{CTX\text{-}M\text{-}27}$ gene combined with a $bla_{CMY\text{-}2}$ gene (ampC_0114), but was ESBL disc test negative (see Table S1). In 21 (12.2%) of the isolates, neither *pampC* nor described mutations related to AmpC hyperproduction were detected and are noted as putative low-level AmpC producers.

Table 1. Table of the distribution of the different AmpC promoter and attenuator variants, as well as the numbers of different MLST STs and phylogroups per grouped genotype (*pampC*, putative hyperproducers and putative low-level AmpC producers)

Genotype	Isolate	Promoter variant	Attenuator variant	MLST	Phylogroup
pampC	*n*=90	3 variants	6 variants	44 STs and 4 unknown	
Putative hyperproducers	*n*=61	13 variants	14 variants	30 STs and 5 unknown	
Putative low-level AmpC producers	*n*=21	8 variants	5 variants	14 STs and 4 unknown	
Total	*n*=172	21 variants	18 variants	75 STs and 13 unknown	

Reference chromosome

To be able to reconstruct an accurate phylogeny, we selected *E. coli* isolate ampC_0069, one of the strains of the study, to use as self-constructed reference chromosome for SNP calling. The reference chromosome was constructed through a hybrid assembly of *n*=44231 09 2×150 bp Illumina NextSeq 500 paired-end reads together with *n*=218475 PacBio Sequel SMRT subreads (median 5640 bp). This resulted in a high-quality full circular chromosome of *E. coli* isolate ampC_0069, with a size of 5056572 bp. This isolate belongs to ST648 and contains a plasmid-encoded bla_{CMY-42}. The full circular chromosome was uploaded to GenBank under accession number CP046396 and was used for further analysis. Genome annotation with the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) identified 4720 coding sequences.

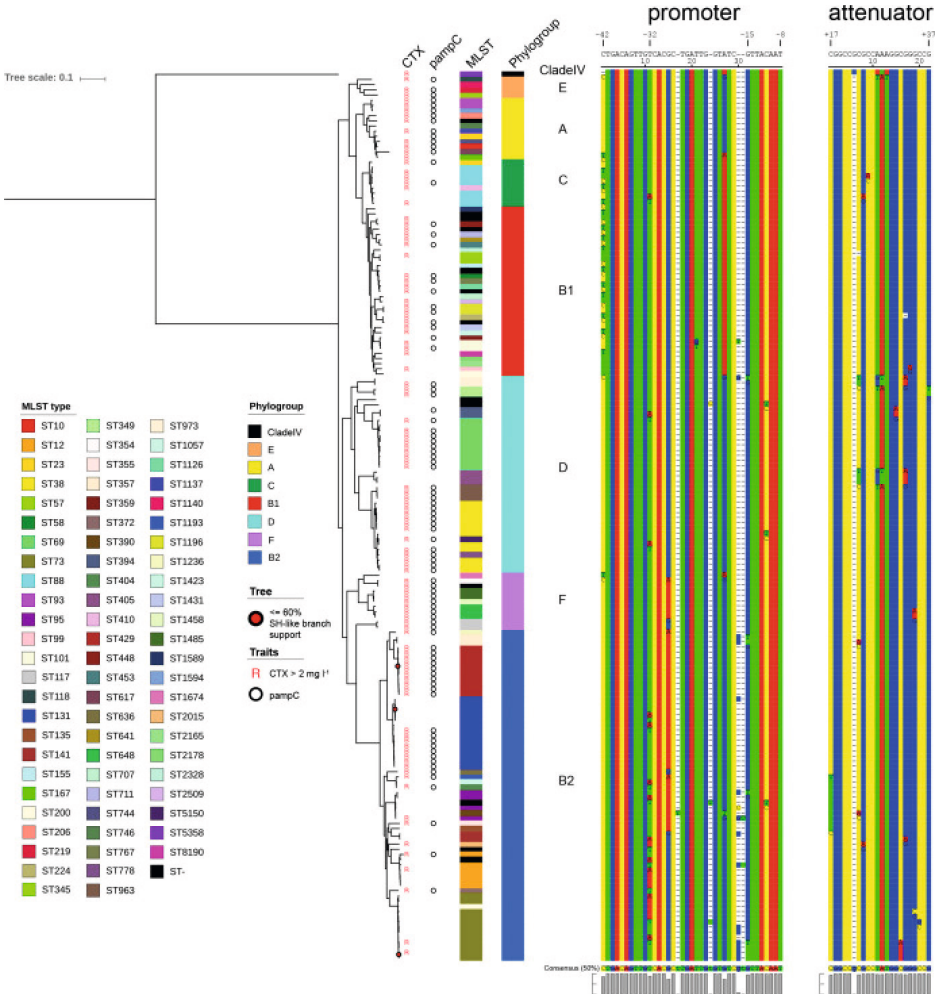
SNP calling

The mapping of the reads of all isolates to the reference chromosome *E. coli* ampC_0069 (accession no. CP046396) resulted in a coreSNP alignment containing 314200 variable core SNP positions. For further details per isolate see Table S3. To validate our SNP calling method, we compared the ampC_0069 Illumina NextSeq 500 paired-end reads to the reference chromosome of ampC_0069, resulting in 0 SNPs detected, supporting that the SNP calling data and method produce no false positives.

Phylogenetic tree based on coreSNP

The coreSNP alignment was used for further analysis. Fig. 2 illustrates the approximately maximum-likelihood phylogenetic tree of all 172 isolates based on the coreSNP alignment. The tree has a robust topology as indicated by the SH-like (Shimodaira–Hasegawa like) branch support, only three positions have a value ≤60% (M. N. Price, Dehal, and Arkin 2010). When focusing on the *ampC* promoter mutations, they were most prevalent in phylogroups B1, B2 and C, although they were present in all phylogroups except phylogroup E that lacked mutations in either the promoter or attenuator region. Interestingly, two positions previously highlighted by Tracz *et al*., −42 and −32, are only mutated in the absence of a *pampC* gene, even in isolates with a similar MLST ST (ST12, ST88 and ST131). The −42C>T mutation, which results in an alternate displaced promoter box and, therefore, leads to increased resistance (Tracz *et al*. 2007), is present in 24 isolates in five distinct phylogroups and in 17 separate phylogenetic branches, indicating that this mutation is homoplastic. Additionally, the −32T>A mutation in the promoter, previously also associated with resistance [6], is present in 20 isolates in three distinct phylogroups and in 14 separate phylogenetic branches. To quantify the level of homoplasy, we calculated the consistency index.

Figure 2. Approximately maximum-likelihood phylogenetic tree of all 172 *E. coli* isolates based on the coreSNP alignment with the resistance for CTX, *pampC* gene presence, MLST STs, phylogroups, and the alignments of the promoter and the attenuator region. Positions with a SH-like (Shimodaira–Hasegawa like) branch support ≤60% are indicated as red dots. Scale bar indicates branch length calculated by FastTree.



Genomic homoplastic mutations

We calculated the consistency index for all SNP positions on the *E. coli* reference chromosome. A low consistency index value for a position indicates a high degree of inconsistency with the chromosomal phylogeny and can be calculated by HomoplasyFinder as described in earlier studies (Crispell, Balaz, and Gordon 2019; Crispell *et al*. 2019; Van Dorp *et al*. 2020). As can be observed in Fig. 3, results clearly

indicate position −42 (4470140) and −32 (4470150) are the lowest scoring positions on the consistency index concerning the promoter and attenuator region, respectively, 0.05882353 and 0.07142857 (see also Fig. S1). To access how extreme these values are, we calculated the consistency index for all SNP positions in the chromosome (see Fig. S2). All consistency indexes <1.0 are plotted in the outer ring (ring A) of Fig. 4. Results show that only 9640 out of 5056572 positions (0.19%) had a consistency index ≤0.07142857 (see Fig. 4, ring a, cut-off is indicated by a black circle). This clearly indicates that positions with low consistency indexes are rare, but not unique. Although these 9640 positions have a low consistency index, we do not yet know their relation to CTX resistance.

Figure 3. WebLogo sequence logo with both bits and probability score for the promoter and the attenuator region. Consistency index and the minimum number of changes on the tree per position are represented in the bar charts below the sequence logos. Orange bars are positions containing SNPs that are tested for homoplasy. Blue bars are positions containing insertions that are tested for homoplasy.

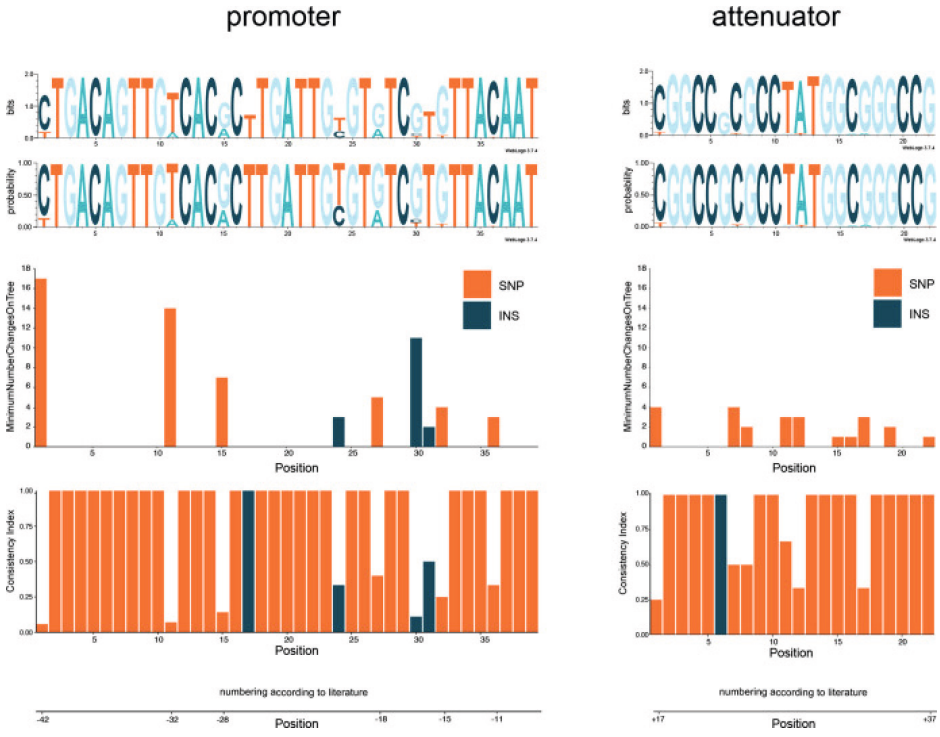
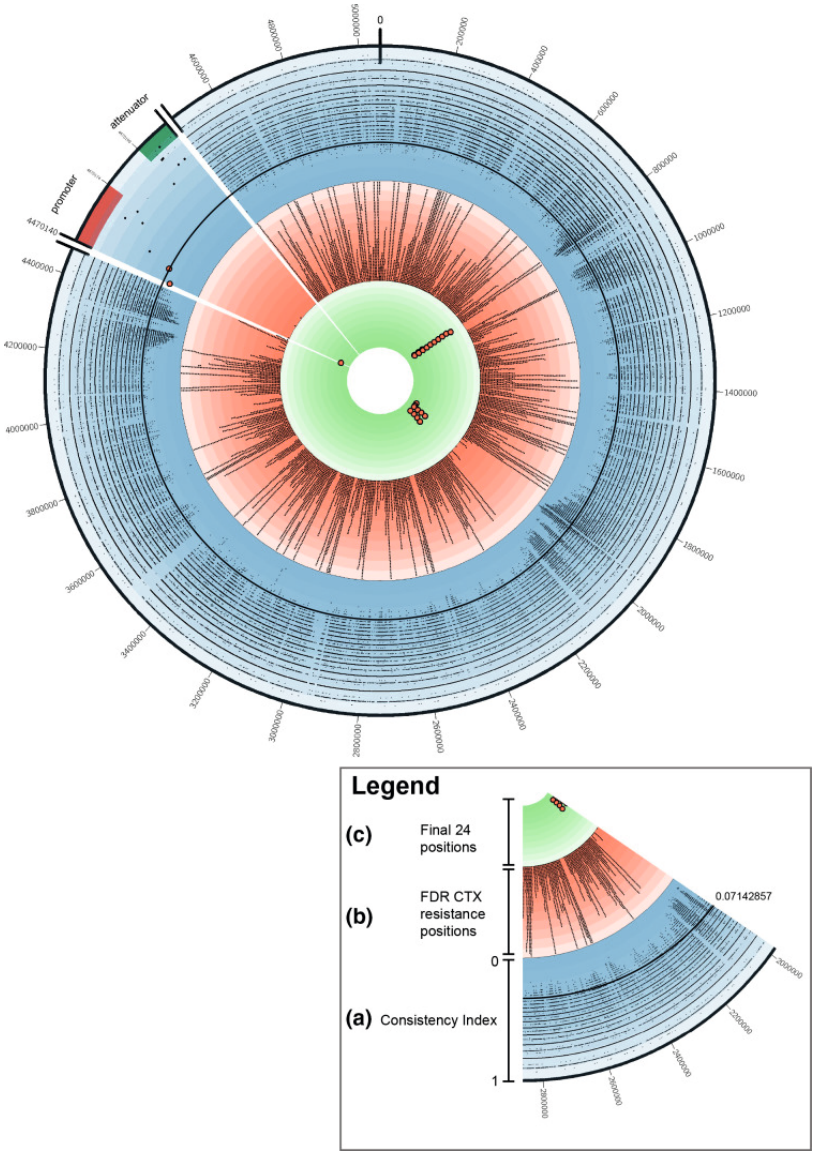


Figure 4. circos plot for the full chromosome of ampC_0069 (accession no. CP046396) with, per position, the various metrics used. (a) The blue coloured ring represents the consistency index results per genomic position. The two red dots indicate the −42 and −32 position on the promoter. The black circle line indicates the 0.07142857 consistency index value. (b) The ring with a red background shows all positions that were significantly associated with CTX resistance in all non-pampC-harbouring *E. coli* isolates. Larger bars pointing outwards indicate multiple significant associated positions in a small genomic region. (c) The ring with the green background shows all 24 positions that have a low consistency index of ≤0.05882353 and are significantly associated with CTX resistance in all non-pampC-harbouring *E. coli* isolates.



CTX-resistance measurements

CTX MIC measurements from Coolen *et al.* in relation to the genotype of the *E. coli* isolates are shown in Table S1 (Coolen *et al.* 2019). Eighty-four of ninety (93.3%) *pampC*-harbouring *E. coli* were CTX resistant (MIC >2 mg l^{-1}) based on EUCAST clinical breakpoints. Twenty-two of sixty-one (36.1%) isolates categorized as putative hyperproducers based on Tracz *et al.* were CTX resistant, primarily isolates with the −42 (*n*=15, 62.5% of isolates with −42 mutation) or −32 mutation (*n*=2, 10.0% of isolates with −32 mutation). The *pampC* genes never occurred simultaneously with the −42 or −32 mutations in any of these isolates. As depicted in Fig. 2, the non-*pampC* strains with a phenotype of CTX >2 mg l^{-1} were present in all phylogroups, although CTX-resistant isolates with the −42 or −32 mutation were predominantly present in phylogroups B1, B2 and C.

Genotype to phenotype

To be able to link *E. coli* chromosomal mutations to CTX resistance, we excluded all *E. coli* isolates with a plasmid containing an *ampC* beta-lactamase gene. The association of SNPs with the CTX-resistance phenotype (MIC >2 mg l^{-1}) was tested in the remaining 82 isolates using Fisher's exact test. After FDR correction to 0.05, 45 998 significant positions were found (see Fig. 4, ring b). Mutation C>T on position −42 of the *ampC* promoter was found to be significantly associated with CTX resistance (FDR=0.034). However, position −32 A>T was not significantly associated with CTX resistance (FDR=1).

Homoplasy-based association analysis

Combining the outcome of the homoplasy analysis with the significant CTX-resistance-associated positions results in genomic positions associated with CTX resistance that have evolved multiple times independently. After selecting the lowest scoring consistency index positions, ≤0.05882353, 24 relevant genomic positions were identified that had both a low consistency index and a significant association with CTX resistance. Most notably, 1 of these 24 positions is position −42. Only 2 mutations of those 24 that were located in genes were non-synonymous: a (conservative) missense mutation in the type II secretion system protein L (*gspL*) gene leading to a Ser330Thr alteration, and a mutation in the hydroxyethylthiazole kinase (*thiM*) gene resulting in a Thr122Ala alteration according to the annotation of *E. coli* strain ampC_0069 (accession no. CP046396). In addition to the non-synonymous mutation found on the *gspL* gene, eight synonymous mutations are

also located in genes annotated as being part of the type II secretion system. A complete overview is presented in Tables 2 and S4.

Recombination analysis

To verify whether the level of homoplasy could be a result of recombination, we used the Genealogies Unbiased By recomBinations In Nucleotide Sequences (Gubbins) algorithm to predict recombination events in our isolate collection (Croucher *et al*. 2015). This analysis showed frequent recombination events in our 172 *E. coli* isolates (see Fig. S3). Results illustrate that recombination blocks cover the region of the *gspL* and the *thiM* gene, and their high homoplasy levels could, thus, be due to recurrent recombination rather than independent mutations. Nonetheless, position −42 in the *ampC* promoter is not located in a region affected by recombination, as shown in Fig. S3. Moreover, when inferring the phylogenetic tree corrected for recombination events as obtained from the Gubbins analysis, the −42 C>T mutation actually occurred in 18 independent branches rather than the 17 branches in the uncorrected tree. This supports our previous result that this mutation is homoplastic, and not the result of a recurrent recombination event.

Pyseer analysis

To add additional support to our findings, we used Pyseer as an alternative method to compare the 24 positions identified with the homoplasy-based association analysis (see Table 2). Pyseer identified 65 501 unique significant mutations associated with CTX resistance with filter *P* value ≤0.05 and 1097 unique positions with filter and likelihood ratio test (lrt) *P* value ≤0.05. Of the 24 positions identified with the homoplasy-based association analyses, we identified 8 positions also reported by the Pyseer method (see Table 2). Furthermore, the Pyseer method identified 6 complex mutation variants and a total of 14 positions that have multiple mutation variants overlapping the same genomic positions as found by the homoplasy-based method. The most dominant position associated with CTX resistance is the −42 C>T promoter mutation as indicated by both methods. No further positions on the promoter or the attenuator were found significantly associated with CTX resistance.

Table 2. The *n*=24 positions with a significant association with cefotaxime resistance (FDR ≤0.05) and a Consistency Index ≤ 0.05882353.

Homoplasy-based association analysis										pyseer				
Genomic position	**mutation**	**Counts ACGT**	**Min changes**	**CI**	**crosstab**	**AF**	**pvalue Fisher**	**FDR**	**Gene name**	**Genomic position**	**mutation**	**AF**	**filterpvalue**	**lrtpvalue**
810581	A>G	41:0:122:0	18	0.056	0:22:26:34	6.83E-01	8.11E-05	0.039	*glcE*	810581	A>G	0.683	1.87E-04	2.09E-02
810791	G>T	0:0:54:109	17	0.059	0:22:28:32	6.59E-01	1.68E-05	0.035	*glcE*	810791*	**G**GGT>**T**GGC	0.646	4.08E-04	1.94E-02
815680	C>T	0:69:0:93	18	0.056	2:20:34:26	5.61E-01	1.06E-04	0.042	*glcA*	815680	C>T	0.561	1.20E-04	2.07E-02
824522	T>C	0:82:0:73	18	0.056	2:20:36:24	5.37E-01	3.58E-05	0.036	*gspC*	824522*	T>C	0.378	8.08E-06	1.00E+00
828695	A>G	79:0:74:0	18	0.056	3:19:41:19	4.63E-01	1.14E-05	0.034	*gspF*	828695	A>G	0.463	1.08E-05	3.26E-02
830684	T>C	0:80:0:73	18	0.056	2:20:37:23	5.24E-01	1.61E-05	0.034	*gspI*	830684*	T>C	0.488	3.74E-05	1.06E-01
830708	A>G	68:0:85:0	19	0.053	2:20:36:24	5.37E-01	3.58E-05	0.036	*gspI*	830708*	A>G	0.11	6.41E-01	5.02E-01
830732	T>C	0:82:0:71	17	0.059	2:20:36:24	5.37E-01	3.58E-05	0.036	*gspI*	830732	T>C	0.537	4.20E-05	4.19E-02
831564	G>A	71:0:83:0	17	0.059	5:17:45:15	3.90E-01	2.76E-05	0.036	*gspK*	831564	G>A	0.39	1.71E-05	2.37E-01
832152	T>C	0:86:0:72	18	0.056	2:20:28:22	5.83E-01	1.08E-05	0.034	*gspK*	832152*	**C**GTCG>**T**GTCA	0.195	6.94E-03	2.29E-01
832287	A>T	81:0:0:79	17	0.059	2:20:37:23	5.24E-01	1.61E-05	0.034	*gspK*	832287*	T>A	0.232	6.73E-02	5.73E-01
833451	A>T	64:0:0:98	20	0.05	1:21:34:26	5.73E-01	1.03E-05	0.034	*gspL*	833451*	T>A	0.378	1.69E-04	9.04E-02
843887	G>A	64:0:40:0	19	0.053	21:1:29:31	3.90E-01	7.00E-05	0.036	unnamed	843884*	GTT**G**CGC>TCG**A**CGT	0.354	4.08E-04	1.08E-01
1863004	A>G	126:0:46:0	18	0.056	9:13:53:7	2.44E-01	3.37E-05	0.036	*thiM*	1862989*	CTGTCTCATAAGCC**A**ACCGCC > TTATCTTTTAAACC**G**GCAGCG	0.232	4.55E-05	3.30E-02
1911551	T>C	0:116:0:44	17	0.059	16:6:14:46	6.34E-01	7.02E-05	0.036	unnamed	1911551*	C>T*	0.28	1.22E-03	5.28E-01

Table 2. Continued.

Homoplasy-based association analysis										pyseer				
Genomic position	**mutation**	**Counts ACGT**	**Min changes**	**CI**	**crosstab**	**AF**	**pvalue Fisher**	**FDR**	**Gene name**	**Genomic position**	**mutation**	**AF**	**filterpvalue**	**lrtpvalue**
1946016	A>G	58:0:91:0	23	0.043	17:5:17:43	5.85E-01	1.03E-04	0.041	*ugd*	1946016*	A>G*	0.402	5.02E-02	4.92E-01
1946067	G>T	0:0:65:84	22	0.045	18:4:20:40	5.37E-01	1.25E-04	0.05	non-coding region	1946067	G>T	0.537	9.58E-05	2.26E-01
1946072	T>A	84:0:0:65	22	0.045	18:4:20:40	5.37E-01	1.25E-04	0.05	non-coding region	1946072	T>A	0.537	9.58E-05	2.26E-01
1952745	A>G	131:0:40:0	28	0.036	9:13:52:8	2.56E-01	7.67E-05	0.037	*hisD*	1952745	G>A	0.732	6.54E-05	7.96E-03
2051214	T>A	137:0:0:34	19	0.053	11:11:5:55	8.05E-01	1.00E-04	0.041	unnamed	2051211*	CAC**T**>TAC**A**	0.659	3.92E-03	4.87E-01
2051220	T>A	137:0:0:34	19	0.053	11:11:5:55	8.05E-01	1.00E-04	0.041	unnamed	2051220*	T>A	0.793	4.79E-06	5.72E-02
2057518	A>G	111:0:60:0	19	0.053	7:15:49:11	3.17E-01	3.89E-05	0.036	*dcm*	2057506*	ATGTTTCCCTGC**G**CAGCGAGT > CTGCTATCCGGC**A**CAACGTATT	0.0122	9.66E-02	1.00E+00
2068593	G>A	45:0:123:0	17	0.059	9:13:52:8	2.56E-01	7.67E-05	0.037	*fliM*	2068593	G>A	0.256	2.60E-05	1.28E-01
4470140	C>T	0:147:0:24	17	0.059	7:15:51:9	2.93E-01	8.35E-06	0.034	*ampC promoter*	4470140	C>T	0.293	2.74E-06	5.42E-03

pyseer identified multiple mutation variants, affecting the outcome, only the position with the highest allele frequency (AF) is reported which overlaps the result of the homoplasy-based association analysis. Sequences in the pyseer mutation of the pyseer outcome that are* **bold *are the corresponding position with the tested genomic position.*

crosstab: R/other:R/mutation:S/other:S/mutation.

AF: allele frequency, CI: Consistency Index, FDR: false discovery rate, mu: mutation, lrt: Likelihood ratio test.

Discussion

We present a genome-wide analysis in which homoplastic mutations are associated with antibiotic resistance in *E. coli* . By comparing WGS data of 172 *E. coli* isolates to a reference chromosome, we were able to reconstruct the evolution of the genomes and therewith map recurrent events, allowing us to detect homoplasy associated with CTX resistance.

Our foremost finding is the significant association of the −42C>T mutation, in the *ampC* promoter, with CTX resistance that evolved independently at least 17 times in five distinct phylogroups. The −42C>T mutation has been confirmed in former studies to result in AmpC hyperproduction in *E. coli* (Caroff N, Espaze E, Gautreau D, Richet H 2000; Tracz *et al*. 2007; Nelson and Elisha 1999). Nelson *et al.* demonstrated an 8 to 18 times increase in activity of AmpC when cloning the promoter upstream a *lac* operon (Nelson and Elisha 1999). Conversely, Caroff *et al.* found a decrease in expression of AmpC when cloning the promoter with a −42T>C mutation in a pKK232-8 reporter plasmid with a chloramphenicol acetyltransferase gene (Caroff N, Espaze E, Gautreau D, Richet H 2000). Tracz *et al.* confirmed that the −42C>T mutation has the strongest effect on the *ampC* promoter, resulting in a high expression of the *ampC* gene as detected by qRT-PCR (Tracz *et al*. 2007). Despite the fact that the −42C>T mutation has such a strong effect on AmpC production, the effect of the mutation on CTX MICs had not been confirmed. Moreover, the contribution of convergent evolution on this position relative to the role of the expansion of a clone with a beneficial mutation at this position has not been determined. That being the case, this study provides evidence that this −42 C>T mutation is not a result of a recombination event and most likely evolved many times independently.

In the current study, we see a strong correlation between the −42C>T mutation and CTX resistance, even though there were exceptions, as not all isolates with this mutation were considered resistant according to EUCAST guidelines. As described by Coolen *et al*., the MIC for CTX in putative AmpC hyperproducers was generally higher than in the putative low-level AmpC producers, though the range in CTX MICs overlapped (Coolen *et al*. 2019). Yet, the lowest MIC measured in the isolates with the −42C>T mutation was 0.75 mg l^{-1}, which is at the higher end of the EUCAST epidemiological cut-off values (ECOFF) distribution. The variation in phenotypical testing could be an explanation, although an interplay of AmpC hyperproduction and other strain-specific factors as previously described by Tracz *et al.* may also be considered (Tracz *et al*. 2007).

In order to avoid biasing towards a single method, in our case the homoplasy-based association method, we performed the analysis using Pyseer on the same data set.

The outcome of the Pyseer analysis provided a similar number of CTX-resistance-associated mutations and also confirmed the strong association of the −42 C>T mutation. Nonetheless, when zooming in on the identified positions by the homoplasy-based method, not all 24 mutations were significantly associated with CTX by Pyseer. An explanation for this discrepancy is that the combination of snippy and Pyseer identifies various mutation variants on the same overlapping genomic region by stratifying it as complex, indels and/or SNP. This greatly affects the power of the test on certain positions. An example of the differences in mutation variants is illustrated in Table 2.

Remarkably, we observed that the −42C>T mutation never occurs in the presence of a *pampC* gene (0 out of 24 cases). This was even noticed in isolates with the same MLST, i.e. ST88 −42C>T (*n*=3) and *pampC* (*n*=1), suggesting preferred exclusivity for one of the resistance mechanisms. One study mentioned the co-occurrence of the −42C>T mutation and a *pampC* gene in only 1 out of 36 strains (Mulvey *et al*. 2005). One could speculate that the exclusivity is a matter of what arrives first, the plasmid or the mutation, after which there is no selective advantage for the second mechanism, or that there is actually a fitness cost to having both the mutation and the plasmid relative to having only the mutation or the plasmid. This hypothesis might be a start for future studies to determine the relative fitness and resistance provided by the −42C>T mutation relative to isolates harbouring a *pampC* gene.

The study performed by Tracz *et al.* showed that position −32T>A on the promotor of *ampC* associates with AmpC hyperproduction that results in elevated MIC levels for FOX (Tracz *et al*. 2007). Surprisingly, in the current study, no significant association of −32T>A with CTX resistance was noticed despite its low consistency index. Only 2 out of 20 isolates with the −32T>A were CTX resistant, 4 out of 20 showed an intermediate elevated CTX MIC, and 14 were susceptible to CTX. Although we do not know under which conditions this mutation did arise, it can be speculated that the high level of homoplasy at the −32 position is associated with a different trait, e.g. resistance against another antibiotic.

Prior studies discovered the importance of mutations in the promoter elements. Although an existing promoter is often copied upstream to the gene, a *de novo* promoter can also evolve out of an existing sequence region. Random sequences can even evolve expression comparable to the wild-type promoter elements after only a single mutation (Yona, Alm, and Gore 2018). This means that the *de novo* creation of a promoter region within the *E. coli* may be much more often the result of mutation rather than a rearrangement. Furthermore, these promotor elements evolve to only a few forms, indicating convergent evolution (Liu and Libchaber 2006), as also observed in the

present study. All encountered variants seem to result in a sequence that resembles the *E. coli* consensus σ^{70} promoter more than the wild-type sequence they are derived from (Tracz *et al*. 2007).

Next to the −42C>T promoter mutation, we detected 23 other positions in our analysis that are associated with CTX resistance and have extremely high levels of homoplasy. Most of these are synonymous mutations, with only two missense mutations (*thiM* and *gspL*) found. It is remarkable that one missense mutation (p.Ser330Thr) is located in *gspL* that encodes a protein of the type II secretion system. The type II secretion system is used by many Gram-negative bacteria to translocate folded proteins from the periplasm, through the outer membrane, into the extracellular milieu (Korotkov, Sandkvist, and Hol 2012). The system is composed of 12–15 different general secretory pathway (Gsp) proteins and is related to virulence of various pathogenic *E. coli* , e.g. EHEC (enterohaemorrhagic *E. coli*) and UPEC (uropathogenic *E. coli*) (Ho *et al*. 2008; Baldi *et al*. 2012; Kulkarni *et al*. 2009). It could be that in our selection of mainly clinical samples a certain predilection has occurred towards isolates with particular virulence traits and not based on mechanistic benefits. The *gspL* gene has been described as being part of the accessory genome of *E. coli* (Dunne *et al*. 2017). Our study supports this finding as some strains did not harbour this gene. Additionally, we found evidence that recombination events in the type II secretion system could be the underlying cause of the extreme homoplasy levels. Still, it is remarkable that missense mutation p.Ser330Thr in the *gspL* gene correlates with the CTX-resistance trait even though it is most likely caused by a recombination event. To the best of our knowledge, no relationship between the type II secretion system and CTX resistance has been observed before. One could hypothesize that the mutation is a secondary adaptation needed to cope with the elevated AmpC production, as the peptidoglycan (PG) layer is affected by AmpC hyperproduction and the type II secretion system contains proteins that are partly localized in the periplasm (Vanderlinde *et al*. 2017; Juan *et al*. 2018).

The use of genomic data to detect homoplasy events is an accepted scientific technique (Read and Massey 2014; Chen and Shapiro 2015; B. J. Shapiro et al. 2009). In *Mycobacterium tuberculosis* , it is a well-known method for identifying advantageous mutations, as they are likely to be associated with phenotypes such as drug resistance, heightened transmissibility or host adaptation (Farhat *et al*. 2013; Mortimer, Weber, and Pepperell 2018; Ruesen *et al*. 2018; Miotto *et al*. 2014). In other species, e.g. *Staphylococcus aureus* and *Burkholderia pseudomallei* , the method has been effectively applied to identify mutations associated with antibiotic resistance or virulence-associated genes (Alam *et al*. 2014; Sahl *et al*. 2015). Homoplasy-based

association analysis limits phylogenetic bias by correcting for genetic relatedness of strains with the same phenotype, thereby increasing statistical power to find true associations (Ruesen *et al*. 2018). Taking this into account, the use of homoplasy-based association analysis seems viable to relate polymorphic sites to phenotypic traits in bacteria. Still, studies on other genera than mycobacteria are scarce. To our knowledge, no homoplasy studies have used this method on *E. coli*.

The increase of 3GC resistance imposes a clinical threat by restricting treatment options and it is essential to understand the underlying resistance mechanisms. To be able to explore these mechanisms, we selected primarily clinical *E. coli* strains. The current study is directed on exploring AmpC-mediated CTX resistance. Therefore, we included isolates that are already suspected for increased AmpC production based on elevated FOX resistance. Since a random sample of *E. coli* would limit finding homoplasy-based associated promoter mutations with CTX resistance. A downside of these selection criteria might be that we over-estimated certain genetic variants associated with the trait, as we do not know the frequency of these variants in the general population. Despite the fact that the spontaneous mutation rate in *E. coli* is relatively low (H. Lee *et al*. 2012), it is still likely that this particular mutation occurs often in the general population, given the vast amounts of *E. coli* in the environment (Tenaillon *et al*. 2010), providing ample opportunities for adaptation to antibiotics and arguing for antibiotics of which genomic adaptation requires multiple mutations in order to develop resistance.

The findings of this study have a number of implications for future practice. This study not only grants insights into how chromosome-encoded antibiotic resistance evolves, but also provides potential strategies for future homoplasy-based association studies. Furthermore, the use of genome-wide homoplasy-based analysis could be applied to optimize outbreak analysis. Prior studies have optimized outbreak analysis by removing recombinant regions (Escobar-Páramo *et al*. 2004; L. B. Price *et al*. 2013). Homoplasy events disturbs the true phylogeny; hence, removing genomic positions that are heavily affected by homoplasy could improve tree topology, thereby refining outbreak analysis, although this strategy is still under debate (Hedge and Wilson 2014).

Conclusions

To conclude, our method demonstrates extreme levels of homoplasy in *E. coli* that are significantly associated with CTX resistance. Greater access to WGS data provides new opportunities to perform large-scale genome-wide analysis. Homoplasy-based methods can have a potential role in future studies as they constitute an effective strategy to relate phenotypic traits to variable genomic positions.

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Author contributions

H. F. L. W., J. A. J. W. K. and M. A. H. conceived and supervised the study. J. P. M. C., E. P. M. D., E. K., J. A. S., J. J. V., W. J. G. M. and K. N. performed the data acquisition. J. P. M. C. and E. P. M. D. performed the data analysis. J. P. M. C. performed bioinformatic analysis. J. P. M. C., E. P. M. D. and M. A. H. performed the data interpretation and wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare that there are no conflicts of interest.

Footnotes

Abbreviations: *campC*, chromosome-mediated *ampC*; CTX, cefotaxime; ESBL, extended-spectrum beta-lactamase; EUCAST, European Committee on Antimicrobial Susceptibility Testing; FDR, false discovery rate; FOX, cefoxitin; 3GC, third-generation cephalosporin; MIC, minimum inhibitory concentration; MLST, multilocus sequence typing; NCBI, National Center for Biotechnology Information; *pampC*, plasmid-mediated *ampC*; qRT-PCR, quantitative reverse-transcriptase PCR; SMRT, single-molecule real-time; ST, sequence type; VCF, variant calling file; WGS, whole-genome sequencing.

All supporting data, code and protocols have been provided within the article or through supplementary data files. Four supplementary tables and three supplementary figures are available with the online version of this article.

Supplementary data

Table S1 for this chapter is available online via:
https://www.microbiologyresearch.org/content/journal/mgen/10.1099/mgen.0.000556#supplementary_data:~:text=Supplementary%20material%202-,EXCEL,-Most%20read%20this

Figure S1. Violin plots of the $\log_{10}$ Consistency Indexes of the promoter and attenuator.

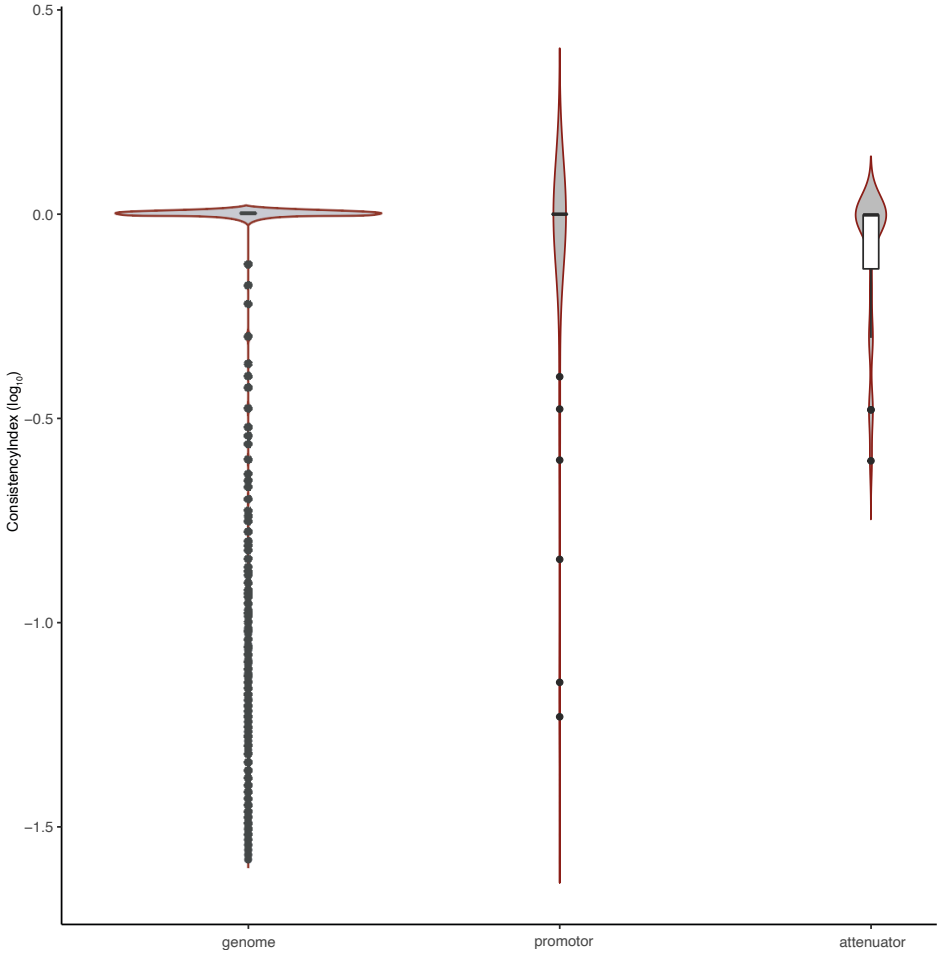


Figure S2. Distribution of the log10 Consistency Indexes of all genomic position based on the *E. coli* ampC_0069 reference genome, compared to the log10 Consistency Indexes of the promoter and attenuator region.

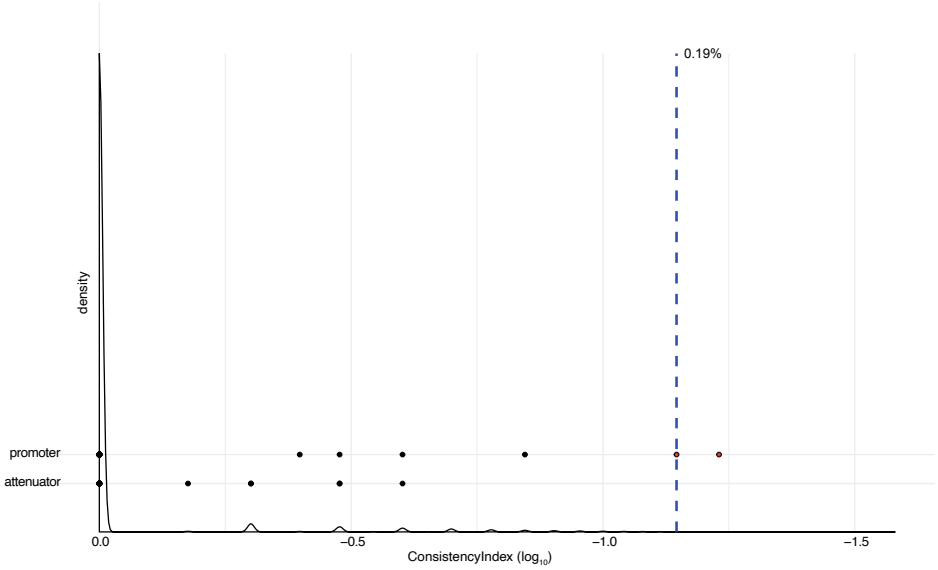
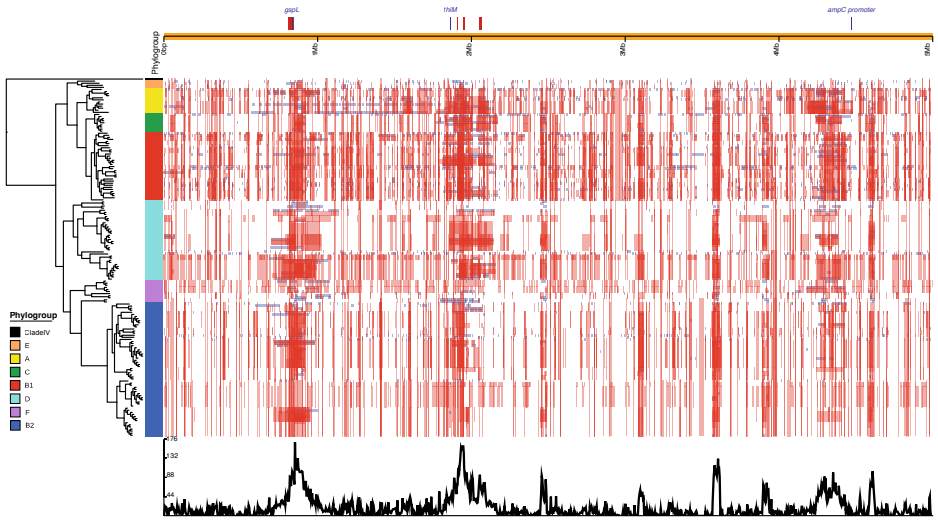


Figure S3. Recombination events inferred from all 172 *E. coli* isolates by Gubbins displayed along the approximately maximum-likelihood phylogenetic tree based on the coreSNP alignment. Phylogroups are depicted as in FIG 2. Gubbins blocks are coloured red if they are ancestral, and blue if they only affect one isolate. The line graph represents the recombination prevalence along the sequence. The 24 positions with a significant association with cefotaxime resistance (FDR ≤0.05) and a consistency index ≤0.05882353 are indicated on the top of the figure. The two missense mutations and *ampC* promoter region are displayed in blue



Chapter 8

In silico estimates of plasmid copy number are associated with increased resistance to cefotaxime, ceftazidime, and piperacillin/tazobactam in CMY-2-producing *Escherichia coli*

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Manuscript in preparation

Abstract

The *bla*$_{CMY-2}$ gene is the most prevalent plasmid-encoded *ampC* gene found in *Escherichia coli*. The production of *bla*$_{CMY-2}$ beta-lactamases is associated with resistance to third-generation cephalosporins and most beta-lactam/beta-lactamase inhibitor combinations. However, the minimal inhibitory concentrations for cephalosporins tend to vary between different CMY-2-producing isolates. Differences in plasmid copy number may be causing this variation in beta-lactam susceptibility. The objective of the present study was to identify determinants of increased resistance to cefotaxime, ceftazidime and piperacillin-tazobactam for *E. coli* isolates that encode *bla*$_{CMY-2}$ without co-expression of extended-spectrum beta-lactamase (ESBL), carbapenemase genes or chromosomal AmpC hyperproduction. The present study compared sequencing depth between chromosomal and plasmid-encoded scaffolds from whole genome sequencing data of twenty *bla*$_{CMY-2}$ encoded *E. coli* isolates. The ratio was used as an estimated plasmid copy number. A significantly higher estimated plasmid copy number was found in CMY-2 producing *E. coli* isolates with high MIC values for cefotaxime, ceftazidime, and piperacillin-tazobactam as compared to *E. coli* isolates with low MIC values. These results provide evidence that whole genome sequencing data not only confirms the presence of resistance genes but also supports evidence that plasmid copy numbers are related to the MICs of the bacterium.

Introduction

The increase in antimicrobial resistance has become a major public health threat (Paterson 2006; J. D. Pitout and Laupland 2008). Enterobacterales cause a considerable number of nosocomial and community-acquired bacterial infections, and resistance to the commonly used beta-lactam antibiotics may complicate optimal antibiotic treatment (Harris 2015; Rodríguez-Baño *et al*. 2018). The plasmid-encoded resistance genes play a central role in this phenomenon, as the horizontal spread of mobile genetic elements may cause transmission of resistance genes (Philippon, Arlet, and Jacoby 2002). The emergence of extended-spectrum beta-lactamase producing Enterobacterales (ESBL-E) has been the subject of numerous studies during the last decades. Increasingly other plasmid-encoded beta-lactamases, such as plasmid-mediated AmpC (pAmpC) beta-lactamases, are being reported (Park *et al*. 2013; Mata *et al*. 2010; Miró *et al*. 2013).

The most prevalent *pampC* genes found in *E. coli* in Western Europe are $bla_{\text{CMY-2}}$ -like genes (P Bogaerts *et al*. 2010; Stéphane Corvec *et al*. 2007). Production of this beta-lactamase is associated with resistance to third-generation cephalosporins and most beta-lactam/beta-lactamase inhibitor combinations, although Minimal Inhibitory Concentrations (MICs) tend to differ between isolates (Kurpiel and Hanson 2012; Edquist *et al*. 2013; Skalweit *et al*. 2013). It is known that increased plasmid copy numbers influence antibiotic susceptibility in several bacterial species. San Millan *et al*. showed an increase in the copy number of a ROB-1 beta-lactamase gene in *Haemophilus influenzae* under antibiotic pressure, which resulted in higher ampicillin resistance levels (Millan *et al*. 2015). Subsequently, Santos-Lopez *et al*. revealed an inverse mechanism in which lower plasmid copy numbers resulted in lower MICs in H. influenzae (Santos-Lopez *et al*. 2017). Elevated copy plasmid numbers have been associated with increased resistance in carbapenemase-producing Enterobacterales (Shen *et al*. 2020; Becker *et al*. 2016). This increase in MIC can occur with other resistance mechanisms, e.g., a decrease in membrane porins or changes in the promoter sequence of the beta-lactamase. Nonetheless, Shen *et al*. confirmed copy number increase as an independent cause of increased resistance as well (Shen *et al*. 2020). Kurpiel *et al*. have shown that *E. coli* strains with an increased copy number of an IncI1 plasmid harbouring a $bla_{\text{CMY-2}}$ gene were associated with increased resistance to piperacillin-tazobactam (Kurpiel and Hanson 2012). It is unknown if copy numbers of $bla_{\text{CMY-2}}$ encoded plasmids may influence MICs of third-generation cephalosporins such as cefotaxime or ceftazidime.

Becker *et al*. referred to variations in copy number of small plasmids being related to differences in sequencing depth in whole genome sequencing (WGS) data (Becker *et al*.

2016). The underlying assumption is that differences in coverage between chromosomal and plasmid-related DNA sequence data may be used as a derivative of the plasmid copy number. A similar method was used by Pena-Gonzalez *et al*. to estimate the copy number of pXO1/pXO2-like plasmids in Bacillus anthracis isolates, revealing consistent results compared to quantitative PCR analysis (Pena-Gonzalez *et al*. 2018). The present study uses the sequencing depth of the bla_{CMY-2} containing scaffolds to estimate the copy number by comparing this data with the sequencing depth of the chromosome-encoded household genes of the *E. coli* isolates.

The present explorative study aims to evaluate whether the susceptibility for cefotaxime, ceftazidime and piperacillin-tazobactam in CMY-2-producing *E. coli* is associated with the copy number of the bla_{CMY-2-}containing plasmid.

Materials and methods

Selection of isolates

A national collection of 2,005 phenotypic ESBL-E isolates was prospectively gathered in the SoM study, a multicenter cluster-randomized study isolation strategies for ESBL-E positive patients in 14 Dutch hospitals between 2011 and 2014 (Marjolein F Q Kluytmans-Van Den Bergh *et al*. 2016; Kluytmans-van den Bergh *et al*. 2019). Isolates were obtained from routine clinical cultures and perianal screening cultures that were taken during ward-based prevalence surveys as part of the project. The methods used to detect ESBL-E have been described previously (Marjolein F Q Kluytmans-Van Den Bergh *et al*. 2016). In 160 (8%) of the 2,005 phenotypic ESBL-E isolates, no ESBL-encoding gene could be detected. Of those, twenty (13%) were *E. coli* isolates that contained bla_{CMY-2} and were included in the present study.

Antimicrobial susceptibility testing

The susceptibility for cefotaxime (CTX), ceftazidime (CAZ) and piperacillin-tazobactam (TZP) was measured with the antibiotic gradient on a strip method (CTX and CAZ: E-test (bioMérieux, Marcy l'Etoile, France); TZP: MIC test strip (Liofilchem, Roseto degli Abruzzi, Italy)).

Short-read sequence data and de novo assembly

Raw short-read sequence data were previously generated on a MiSeq or a HiSeq 2500 sequencer (Illumina, San Diego, CA, USA) as described previously (Marjolein F Q

Kluytmans-Van Den Bergh *et al*. 2016). De novo assembly was performed using CLC Genomics Workbench 8.5.1 (Qiagen), with optimal word sizes based on the maximum N50 (the largest scaffold length, N, such that 50% of the assembled genome size is contained in scaffolds with a length of at least N) value. Assembled genomes were excluded from further analyses if they did not meet the previously described quality criteria for coverage (mean matched read depth), the number of scaffolds, N50, maximum scaffold length, and percentage of expected genome size (Marjolein F Q Kluytmans-Van Den Bergh *et al*. 2016).

Analysis of assembled genomes

Assembled genomes were uploaded to the online bioinformatics tools ResFinder v2.1 and MLST v1.8 (Center for Genomic Epidemiology, Technical University of Denmark, Lyngby, Denmark) (Zankari *et al*. 2012; Larsen *et al*. 2012). Acquired resistance genes were reported when at least 60% of the length of the best matching gene in the ResFinder database was covered with a sequence identity of at least 90%. Conventional MLST sequence types were based on the Achtman MLST scheme 23. For each assembled genome, the promoter/attenuator region of the chromosomal AmpC (cAmpC) gene was aligned with that of *E. coli* K-12 MG1655 (GenBank accession number U00096) using CLC Genomics Workbench 8.5.1. wgMLST was performed using Ridom SeqSphere+ (Ridom, Münster, Germany) as described previously (Marjolein F Q Kluytmans-Van Den Bergh *et al*. 2016).

CLC Genomics Workbench 8.5.1. (Qiagen) was used to obtain the coverage for 1) the whole genome assembly; 2) the scaffolds containing the $bla_{\text{CMY-2}}$ gene, and 3) the scaffolds containing either one of the seven *E. coli* MLST genes (*adk, fumC, gyrB, icd, mdh, purA and recA*) (Wirth *et al*. 2006). The ratio between the coverage of the $bla_{\text{CMY-2}}$-encoding scaffold and the coverage of the whole genome assembly was used as an in-silico estimate for the plasmid copy number (ePCN). The ratio between the coverage of the MLST-gene-containing scaffolds and the coverage of the whole genome was used to validate the use of the coverage of the whole genome assembly as a reference in the calculation of the ePCN.

Statistical analysis

The Mann-Whitney U test was used to test for differences in median ePCN value between ‘low-MIC isolates’ and ‘high-MIC isolates’. Data was analysed with Statistical Package for Social Science software (SPSS; IBM Corp., Armonk, New York, US; version 22).

Accession numbers

All raw reads were previously submitted to the European Nucleotide Archive (ENA) of the European Bioinformatics Institute (EBI) under the study accession number PRJEB64326.

Results

All genome assemblies met the previously described quality criteria for coverage, number of scaffolds, N50, maximum scaffold length, and percentage of expected genome size (Supplementary table S1) (Marjolein F Q Kluytmans-Van Den Bergh *et al*. 2016).

Table 1 shows the results of antimicrobial susceptibility testing of the twenty CMY-2 positive *E. coli* isolates. The MIC values for CTX, CAZ, and TZP showed a bimodal distribution (Figures 1, 2, and 3). Based on this bimodality, isolates were divided into a low-MIC and high-MIC group using the highest MIC in the detectable range as a cut-off value (≥256 mg/L). The distribution of ePCN between the low-MIC and high-MIC groups differed consistently (Figure 4). Isolate 16 showed low resistance for CTX but a high ePCN and formed the only exception (Figure 4.). Median ePCN was 1.98 (Interquartile Range (IQR) 1.60-4.76) for the CTX low-MIC group vs. 7.22 (IQR 4.24-9.61) for the CTX high-MIC group ($p=0.012$); 1.89 (IQR 1.57-4.12) for the CAZ low-MIC groups vs. 7.22 (IQR 4.78-9.83) for the CAZ high-MIC group ($p<0.0005$); 1.86 (IQR 1.53-2.80) in the TZP low-MIC-group vs. 6.06 (IQR 4.23-9.61) in the TZP high-MIC group ($p<0.0005$). A significantly higher CMY-2 ePCN was detected in the isolates with high MIC values for CTX, CAZ, and TZP compared to the isolates with low MIC values.

Table 1. Antimicrobial susceptibility and genetic characterisation of 20 ESBL-negative CMY-2-positive *E.coli* isolates.

		E-test (mg/L)				
Isolate no.	**MLST**	**CTX**	**CAZ**	**TZP**	**ePCN CMY-2**	**number of acquired resistance encoding gene other than CMY-2**
1	ST357	12	16	4	1.847	0
2	ST131	16	24	8	1.362	4
3	ST131	24	32	8	4.003	1
4	ST648	48	32	8	5.344	8
5	ST683	16	24	32	1.900	4
6	ST131	12	16	4	1.615	0
7	ST457	≥256	≥256	≥256	9.941	0
8	ST5454	≥256	64	≥256	4.161	12
9	ST1196	≥256	64	≥256	4.266	11
10	ST38	≥256	≥256	≥256	9.500	0
11	ST23	48	≥256	≥256	5.392	3
12	ST59	32	≥256	≥256	2.066	0
13	ST1722	≥256	≥256	≥256	7.721	0
14	ST4118	8	24	8	2.399	4
15	ST1487	16	32	24	1.559	10
16	ST131	64	≥256	≥256	10.446	0
17	ST131	32	64	16	1.448	0
18	ST131	24	≥256	≥256	4.571	0
19	ST1485	16	24	8	1.870	6
20	ST345	≥256	≥256	≥256	6.725	5

Figure 1. Distribution of 20 ESBL-negative CMY-2-positive *E.coli i*solates according to cefotaxime susceptibility

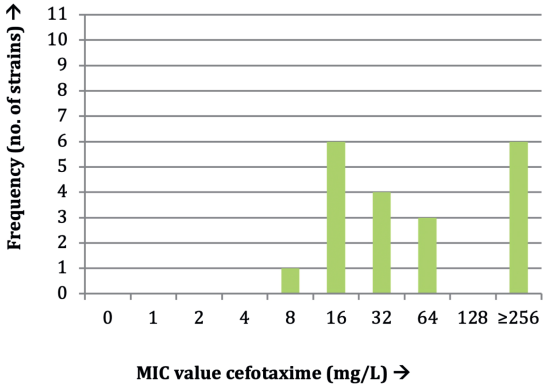


Figure 2. Distribution of 20 ESBL-negative CMY-2- positive ESBL-negative *E.coli i*solates according to ceftazidime susceptibility

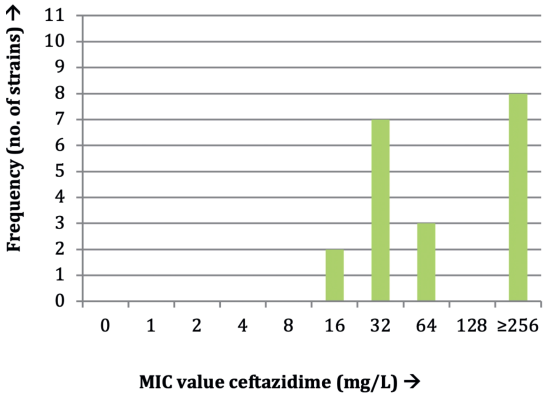


Figure 3. Distribution of 20 ESBL-negative CMY-2-positive *E.coli i*solates according to piperacillin-tazobactam susceptibility.

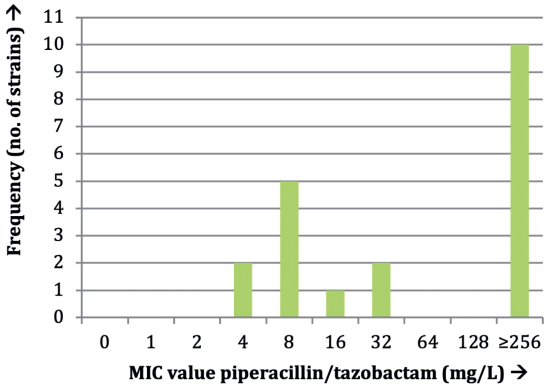
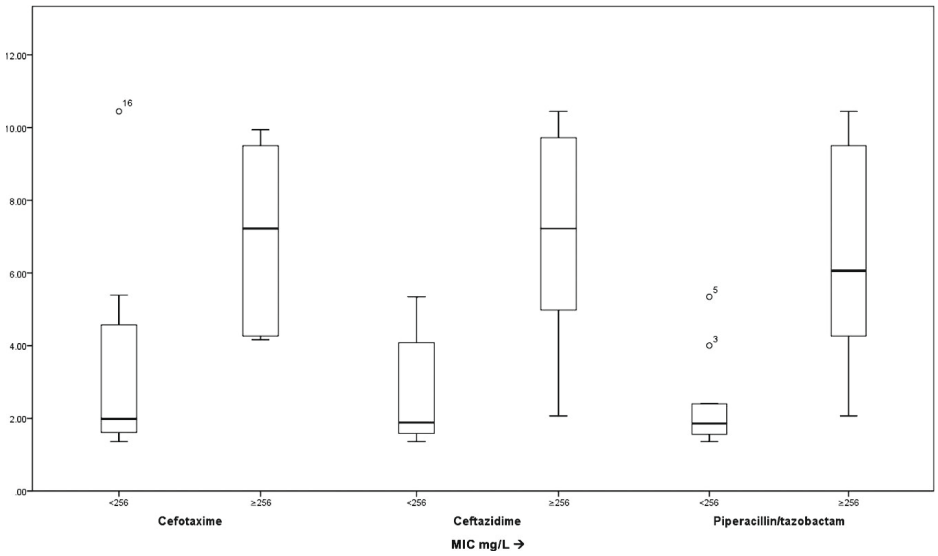


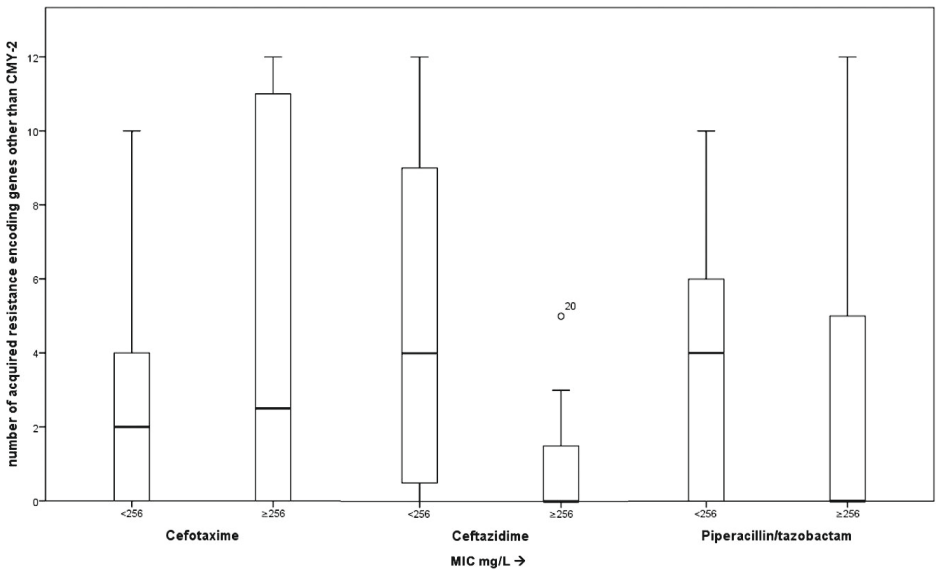
Figure 4. Boxplots of ePCN for CMY-2-producing *E. coli* isolates with an MIC (minimal inhibitory concentration) for cefotaxime, ceftazidime and piperacillin/tazobactam. Box plot explanation: bottom of the box, lower quartile; top of the box, upper quartile; line in the middle, median; end of the whiskers, lowest data point still within 1.5 times the interquartile range (IQR) below lower quartile and highest data point still within 1.5 times the IQR above upper quartile; ○ symbol outside the whiskers: outliers with isolate number.



Isolates in the high-MIC group for CAZ and TZP showed a lower number of acquired resistance genes other than *bla*CMY-2 compared to the isolates in the low-MIC group. (Figure 5.). The median number of acquired resistance genes was 2 (IQR 0-4.50) for the CTX low-MIC group vs. 2.5 (IQR 0-11.25) for the CTX high-MIC-group; 4 (IQR 0.25-9.50) for the CAZ low-MIC groups vs. 0 (IQR 0-2.25) for the CAZ high-MIC group; 4 (IQR 0-6.50) in the TZP low-MIC-group vs. 0 (IQR 0-6.50) in the TZP high-MIC group. This result was significant for CAZ (p=0.03) but not for TZP (0.48) (Figure 5).

The median ratio between the coverage of scaffolds containing either one of the seven *E. coli* MLST genes and the coverage of the whole-genome assembly was 0.88 (IQR 0.81-0.96).

Figure 5. Boxplots of number of acquired resistance encoding genes other than CMY-2 for CMY-2-producing *E. coli* isolates with an MIC (minimal inhibitory concentration) for cefotaxime, ceftazidime and piperacillin/tazobactam. Box plot explanation: bottom of the box, lower quartile; top of the box, upper quartile; line in the middle, median; end of the whiskers, lowest data point still within 1.5 times the interquartile range (IQR) below lower quartile and highest data point still within 1.5 times the IQR above upper quartile; ○ symbol outside the whiskers: outliers with isolate number.



Mutations in the promoter/attenuator region of the chromosomal *ampC* gene related to hyperproduction of bla_{AmpC}, as described by Tracz *et al*., were not detected (Tracz *et al*. 2007).

Table 1 shows the MLST-typing results, including six isolates with MLST sequence type (ST) 131. The wgMLST analysis revealed that two ST131 isolates (16 and 17), obtained from the same patient, were genetically closely related. Because of their differences in susceptibility and ePCN, both isolates were included in further analysis. The other isolates were obtained from separate patients and were genetically distinct based on wgMLST analysis.

Discussion

The present study aimed to evaluate whether an increased estimated copy number of *bla*CMY-2 carrying plasmids is associated with reduced susceptibility for CTX, CAZ, and TZP in ESBL-negative *E. coli* isolates. Based on the assumption that differences in sequencing depth between chromosomal and plasmid-encoded DNA sequence data correlate with plasmid copy number, an estimated plasmid copy number (ePCN) was calculated. A significantly higher ePCN of the $bla_{\text{CMY-2}}$ gene was found in isolates with high MIC values for CTX, CAZ, and TZP compared to isolates with low MIC values.

The results in the present study are in accordance with the study of Kurpiel *et al*. (Kurpiel and Hanson 2012), although using another method, describing an association between an increased plasmid copy number of the $bla_{\text{CMY-2}}$ gene and higher MICs for piperacillin-tazobactam using real-time reverse transcriptase PCR and Southern hybridization. Molecular analysis showed alterations in the sequence of the loop region of the Inc antisense RNA between low- and high-resistant strains, which might be associated with plasmid copy number regulation in IncI1 plasmids.

The present study detected fewer acquired resistance genes other than $bla_{\text{CMY-2}}$ in the high-MIC group for CAZ and TZP compared to the isolates in the low-MIC group. It is known that the presence of plasmids can have a negative effect on the fitness of bacteria (San Millan and MacLean 2017; Hall *et al*. 2017). The occurrence of multiple plasmids simultaneously seems to have a higher fitness cost than the presence of a single plasmid (Gama, Zilhão, and Dionisio 2018). The presence of specific antibiotic genes on the plasmid may also influence the bacterium's fitness as well (Rajer and Sandegren 2022). The results of the current study indicate that in the presence of one high copy number resistance gene, the presence of multiple resistance genes may pose a disadvantage for the bacterium. The interactions between multiple plasmids and the interactions with the micro-organism containing them are complex, and further studies are needed to unravel the complexity of these mechanisms.

In the present study, WGS data are used to estimate the plasmid copy number and relate this to the phenotypical susceptibility of *E. coli* isolates. Multiple methods have been developed to estimate plasmid copy numbers, from luciferase-based densitometry to quantitative PCR techniques and, more recently, droplet digital PCR (Providenti *et al*. 2006; Coronado *et al*. 1994; C. Lee *et al*. 2006; Plotka, Wozniak, and Kaczorowski 2017). These methods have proven to be accurate in determining plasmid copy numbers. However, they can be labour intensive and time-consuming. New improvements in WGS, automation, and bioinformatics pipelines make it easier to upscale the number

of samples and make the process more efficient (Deurenberg *et al*. 2017; Quainoo *et al*. 2017). Moreover, the use of WGS data makes it not only possible to analyse acquired resistance genes but also to screen for alterations that may lead to hyperproduction of chromosomal-encoded genes, e.g., the chromosomal *ampC* in *E. coli*. Furthermore, the clonal relatedness of the strains can be analyzed using wgMLST (Marjolein F Q Kluytmans-Van Den Bergh *et al*. 2016; Quainoo *et al*. 2017). This makes WGS a versatile method for the analysis of resistance mechanisms.

A limitation in the present study could be the use of the whole genome coverage as a reference for the ePCN calculation. As coverage of plasmid-encoded DNA is included in this average coverage, this might result in an overestimation of actual chromosomal coverage, and a higher plasmid copy number may increase this effect. The coverage of household MLST genes was slightly lower than the overall genome coverage in all isolates, although the ratio did not differ substantially. To limit this effect, the median coverage could be used, for example, in PLACNET, PlasmidSpades, and PlasmidSeeker in which the hypothesis of copy number differences in chromosomal-encoded and plasmid-encoded DNA is used to assemble plasmids (Lanza *et al*. 2014; Antipov *et al*. 2016; Roosaare *et al*. 2018).

In the calculation of the estimated plasmid copy number, it was assumed that there were no repeats of chromosomal-encoded genes present, so-called copy number variations (CNVs). These CNVs could lead to an overestimation of the actual chromosomal coverage. Although we cannot exclude that CNVs were present, we do not expect that this would have influenced our conclusion.

The present study shows that an increased estimated plasmid copy number of $bla_{\text{CMY-2}}$ carrying plasmids is associated with reduced susceptibility for CTX, CAZ, and TZP. It is uncertain whether this association can be generalized to other plasmid-encoded resistance genes, e.g., ESBL. If so, this could aid the translation of molecular data into clinically meaningful results. Further studies are warranted to analyse the association between ePCN and other plasmid-encoded resistance genes.

Conclusion

The present study applied sequencing depth to explore the association between MICs of beta-lactam antibiotics and an estimated plasmid copy number. The results show a significantly higher ePCN for the scaffolds containing a $bla_{\text{CMY-2}}$ gene in the isolates with high MIC values for CTX, CAZ and TZP compared to the isolates with low MIC

values. This method provides new insights for future studies on plasmid copy number and antibiotic resistance.

Acknowledgments

We are grateful to the members of the SoM Study Group for their contribution to the collection, culturing and whole-genome sequencing of ESBL-E isolates.

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Disclosures

JR consulted for IDbyDNA and ARES-genetics

Table S1. Summary of assembly characteristics of 20 ESBL-negative CMY-2-positive *E.coli* isolates. N50 = largest scaffold length N, such that 50% of the assembled genome size is contained in scaffolds with a length of at least N. A species-specific minimum and maximum reference genome size were based upon the assembled genomes used by Kluytmans *et al.* to create species-specific wgMLST schemes.

Isolate no	Sequencer	Number of Reads, *n*	Number of Matched Reads, *n*	Average Length of Reads (base count), *n*	Average Length of Reads (base count), *n*	Percentage reads matched, *%*	N50 (base count), *n*	Number of Scaffolds, *n*	Maximum Scaffold Length (base count), *n*	Total scaffolds base counts, *n*	Average Coverage (mean depth), *n*	Average Coverage Matched Reads, *n*	Percentage of expected min ref genome size, *%*	Percentage of expected max ref genome size, *%*
ref							≥15,000	≤1,000	≥50,000		≥20	≥20	>90%	<115%
1	HiSeq	2689989	2184501	97.63	98.23	81.21	92869	125	235223	5153917	50.96	41.64	113.05	98.32
2	HiSeq	3052370	2524911	94.85	95.71	82.72	169171	124	381463	5160328	56.10	46.83	113.19	98.44
3	HiSeq	2591258	2136036	95.95	96.56	82.43	188856	107	385860	4970841	50.02	41.49	109.03	94.83
4	MiSeq	1795657	1734949	151.45	153.90	96.62	74848	210	182193	5598647	48.57	47.69	122.81	106.80
5	HiSeq	2435466	1892845	97.17	97.53	77.72	43319	237	130321	5004132	47.29	36.89	109.77	95.46
6	MiSeq	2191597	2154474	210.21	212.20	98.31	257099	65	613545	5235212	88.00	87.33	114.83	99.87
7	MiSeq	1251167	1215241	140.71	141.04	97.13	86556	151	299258	4997853	35.23	34.29	109.63	95.34
8	MiSeq	1438343	1398268	168.84	170.24	97.21	82298	126	296509	4808959	50.50	49.50	105.48	91.74
9	MiSeq	1285931	1234773	114.97	117.77	96.02	36648	272	115045	5159584	28.65	28.18	113.17	98.43
10	HiSeq	3243522	2703474	96.07	96.64	83.35	142409	99	330072	4835565	64.44	54.03	106.07	92.25
11	MiSeq	750105	733057	232.06	234.93	97.73	265504	45	516628	4735908	36.76	36.36	103.88	90.35
12	HiSeq	3206359	2518175	95.48	96.23	78.54	60862	201	226117	5225597	58.59	46.37	114.62	99.69

Table S1. Continued.

Isolate no	Sequencer	Number of Reads, *n*	Number of Matched Reads, *n*	Average Length of Reads (base count), *n*	Average Length of Reads (base count), *n*	Percentage reads matched, %	N50 (base count), *n*	Number of Scaffolds, *n*	Maximum Scaffold Length (base count), *n*	Total scaffolds base counts, *n*	Average Coverage (mean depth), *n*	Average Coverage Matched Reads, *n*	Percentage of expected min ref genome size, %	Percentage of expected max ref genome size, %
13	MiSeq	1834592	1803247	165.65	166.44	98.29	177308	65	560195	5155241	58.95	58.22	113.08	98.35
14	MiSeq	1726652	1703412	219.40	221.06	98.65	400896	44	750637	4957492	76.42	75.96	108.74	94.57
15	MiSeq	1871506	1777645	184.39	190.94	94.98	119136	113	435121	5014037	68.82	67.69	109.98	95.65
16	HiSeq	2957635	2490566	95.10	96.12	84.21	123659	97	298455	5172753	54.38	46.28	113.46	98.68
17	HiSeq	4113007	3420987	95.76	96.50	83.17	138852	100	339284	5213313	75.55	63.32	114.35	99.45
18	MiSeq	2074454	2033574	166.78	168.33	98.03	229582	63	407247	5224271	66.23	65.52	114.59	99.66
19	HiSeq	3917452	3239752	95.41	96.22	82.70	292536	58	727187	5078058	73.60	61.39	111.39	96.87
20	HiSeq	3079895	2608397	95.73	96.52	84.69	139841	109	323752	4994505	59.03	50.41	109.55	95.28

Chapter 9

Summary and general discussion

Introduction

This thesis aims to investigate various aspects of AmpC-mediated antimicrobial resistance in Enterobacterales, with a particular focus on *Escherichia coli*. The first objective was to determine the prevalence of rectal colonization by AmpC-producing Enterobacterales in the Netherlands and analyse any potential trends in colonization rates over time. Another key aim was to optimize the detection of *ampC* genes in *E. coli* through the use of selective media and phenotypic characterization techniques. Additionally, this thesis examined the feasibility of accurately distinguishing related samples with AmpC-producing *E. coli* from unrelated ones based solely on plasmid sequencing data. Furthermore, it explored whether mutations occurring in the *ampC* promoter/attenuator region of *E. coli* are homoplastic and whether these homoplastic mutations are associated with cefotaxime resistance. Finally, the association between plasmid presence and the level of resistance to specific antibiotics was investigated. This involved comparing the sequencing depth between chromosomal household genes and plasmid-encoded scaffolds containing the $bla_{\text{CMY-2}}$ gene, while also utilizing the ratio as an estimated plasmid copy number. This thesis concludes with a comprehensive summary and discussion that synthesizes the main findings from the conducted studies and places them in a broader perspective.

Prevalence of AmpC-producing Enterobacterales in the Netherlands

The increasing global burden of antimicrobial resistance poses a significant and escalating threat. ESBL-producing Enterobacterales, in particular, show alarming upward trends worldwide, as evidenced by a tenfold increase in the global intestinal carriage rate of ESBL *E. coli* in the community, as reported in a recent review by Bezabih *et al.* (Bezabih *et al*. 2022). Despite relatively low prevalence in Western European countries, including the Netherlands (E. A. Reuland *et al*. 2016), the rise in global mobility has facilitated the spread of antimicrobial-resistant bacteria through travel (Paltansing *et al*. 2013; Lorme *et al*. 2018; Hu, Matsui, and W. Riley 2020; Raffelsberger *et al*. 2023). However, it is important to note that traveling is not the sole risk factor associated with the carriage of resistant Enterobacterales. A recent study by Meijs *et al.* has revealed an elevated prevalence of ESBL and plasmid-encoded AmpC producing *E. coli* and *K. pneumoniae* among Dutch veterinary healthcare workers when compared to the general

population. Additionally, other risk factors for intestinal carriage of ESBL-producing Enterobacterales, such as recent antibiotic or antacid use, have been identified (E. A. Reuland *et al*. 2016; Huizinga *et al*. 2017).

Although to a lesser extent than ESBL, the prevalence of plasmid-encoded AmpC-producing Enterobacterales in the Netherlands has been the subject of various studies. **Chapter 2** offers some valuable insights into the epidemiology of this resistance mechanism, specifically focusing on the situation in the Netherlands. Most of the studies have focused on the rectal or perineal colonization of humans, both in hospital and community settings. In the general population, the prevalence of plasmid AmpC-producing *E. coli* ranges between 0.2% and 1.3%, which indicates a relatively low prevalence compared to ESBL-producing *E. coli* (Van Hoek *et al*. 2015; E. Ascelijn Reuland *et al*. 2015; van den Bunt *et al*. 2017). In contrast, surveys conducted in countries across Asia and Africa have reported higher prevalences (Rodríguez-Guerrero *et al*. 2022). For instance, Huang *et al.* found that up to 16.1% of healthy adult participants in Taiwan were faecal carriers of plasmid-encoded AmpC-producing *E. coli* and/or *K. pneumoniae* (Y.-S. Huang *et al*. 2020). Bassyouni *et al.* found a prevalence of 5.0% plasmid-encoded AmpC-producing *E. coli* in stool samples from Egyptian healthcare workers, while Chirindze *et al.* reported a prevalence of 5.8% in stool samples from students in Mozambique (Bassyouni, Gaber, and Wegdan 2015; Chirindze *et al*. 2018). It's important to note that variations in study populations and screening methodologies significantly hinder direct comparisons between different regions.

While the prevalence of AmpC-producing Enterobacterales in the Netherlands has been the subject of limited research, comparing the results over time has been challenging due to the heterogeneity of study populations. In this thesis, **Chapter 3** describes a study on the prevalence of rectal carriage among hospitalized patients over a four-year period, providing an opportunity to analyse trends more effectively. Interestingly, our study did not observe a significant change in the prevalence of plasmid-encoded AmpC-producing *E. coli* during the study period. However, a decrease in chromosomal-encoded AmpC-producing *E. coli* was detected, indicating potential variations in the dynamics of different resistance mechanisms. Similarly, a study on ESBL carriage trends among hospitalized patients revealed overall stable ESBL prevalence, while specifically observing a decrease in CTX-M-1-like ESBL genes (Willemsen *et al*. 2015). These findings shed light on the complex dynamics of antimicrobial resistance patterns and highlight the need for further investigation into the underlying factors influencing the prevalence and persistence of different resistance mechanisms.

Optimization of the detection of AmpC-mediated resistance in *E. coli*

To ensure effective surveillance of antimicrobial resistance, accurate diagnostic tools play a crucial role. Detecting plasmid-encoded AmpC involves both phenotypic and genotypic testing. However, distinguishing between chromosome-encoded and plasmid-encoded ampC genes can be challenging due to their coexistence, particular in *E. coli*. While molecular confirmation tests are commonly used for screening plasmid-encoded AmpC-producing *E. coli*, they can be time-consuming and costly, making them less feasible in certain settings, especially those with limited resources.

In low-resource settings, it would be beneficial to have a practical algorithm that can distinguish between *ampC* genotypes in *E. coli* based on phenotype alone. This approach would help identify which isolates should undergo further confirmation with molecular testing. By quickly identifying plasmid-encoded AmpC isolates, infection control practices can be improved, unnecessary and costly isolation measures can be minimized, and appropriate treatment strategies can be implemented. **Chapter 4** provides a detailed description of the development of such a model, utilizing machine learning techniques. The algorithm utilizes the minimum inhibitory concentration (MIC) of cefotaxime to predict the presence of plasmid-encoded AmpC in cefoxitin-resistant (>8 mg/L) and ESBL-negative *E. coli*. Although the algorithm demonstrated high accuracy, it is important to note that the data used for model development and training primarily consisted of $bla_{\text{CMY-2}}$-containing *E. coli* and a limited number of other plasmid-encoded AmpC genotypes. While this reflects the Dutch epidemiological situation, it is worth considering that geographical variations may exist in other regions. Nevertheless, the application of machine learning techniques holds immense potential in optimizing screening algorithms within the field of microbiology. With well-curated datasets based on standardized phenotypical and genotypical testing, the possibilities for advancements in laboratory development and infection control methods are boundless.

The screening of antimicrobial resistance, particularly in Enterobacterales obtained from intestinal samples with a multitude of other gut bacteria, poses significant challenges. To improve detection rates, selective and differential media like MacConkey or Drigalski lactose agars are commonly employed, as they effectively suppress gram-positive bacteria. Furthermore, the use of highly selective media tailored for specific ESBL- or carbapenemase-producing Enterobacterales has shown enhanced detection capabilities for resistant isolates (Glupczynski *et al*. 2007; T. D. Huang *et al*. 2010; Göttig *et al*. 2020). However, limited attention has been given to the development of

selective media for AmpC-producing Enterobacterales. In **Chapter 5**, a comprehensive evaluation is presented, aiming to assess the performance of various agars containing cefotaxime and ceftazidime. This evaluation takes into consideration the specificity and sensitivity of these agars in detecting both chromosomal AmpC-hyperproducing and plasmid AmpC-harbouring *Escherichia coli* strains, in comparison to ESBL-producing *E. coli* and those without ESBL, pAmpC, or cAmpC hyperproduction. Furthermore, the impact of incorporating cefoxitin into these agars is examined to improve the detection of both chromosomal AmpC-hyperproducing and plasmid AmpC-harbouring *E. coli* strains.

The addition of cefoxitin showed little influence on sensitivity but increased the specificity of AmpC-producing *E. coli* detection. However, the agar used did not differentiate between plasmid-encoded and chromosomal AmpC-producing *E. coli* strains. It is worth noting that the screening set primarily consisted of bla_{CMY}-type-containing and chromosomal AmpC-producing *E. coli* strains, which warrants further studies to evaluate an agar containing cefotaxime or ceftazidime with cefoxitin in a clinical setting. Nevertheless, the screening agars are expected to be feasible for AmpC rectal carriage screening when employing a pre-enrichment step, similar to current ESBL screening agar strategies (M. F.Q. Kluytmans-Van Den Bergh *et al*. 2015).

Sources and transmission routes of AmpC-mediated resistance

Understanding the sources and transmission routes of antimicrobial resistance vectors is crucial to prevent further increases in antibiotic resistance. Plasmids, as mobile genetic elements, play a significant role in the dissemination of antimicrobial resistance genes, harbouring various resistance mechanisms. The prevalent AmpC resistance gene, bla_{CMY-2}, is frequently linked to specific plasmid families, such as IncA/C, IncB/O/K, and IncI.

In **Chapter 6**, the focus is on exploring the relationship between bla_{CMY-2}-containing IncI1-pST12 plasmids in epidemiologically linked and unrelated Enterobacteriaceae isolates from both humans and livestock. The objective was to assess the feasibility of accurately distinguishing between related and unrelated samples based solely on plasmid sequencing data. By investigating this relationship, valuable insights can be gained into the potential transmission dynamics and relatedness of antimicrobial resistance strains.

Although the study was conducted using a limited sample size, the findings clearly demonstrate the similarity of plasmid sequences, as indicated by the low number of single nucleotide polymorphism (SNP) differences and a high number of shared

genes. Furthermore, the range of SNPs overlapped among plasmids with different epidemiological links, suggesting that distinguishing between epidemiologically related and unrelated plasmids based solely on plasmid sequence is unlikely. Previous studies have shown the conserved sequences of IncI1-pST12 plasmids; however, these studies utilized either gene presence/absence-based analysis or SNP-based analysis, potentially missing subtle differences between various plasmid (Roer *et al*. 2019; Shirakawa *et al*. 2020; Castellanos *et al*. 2019). The current analysis, which combines long-read and short-read sequence data for a comprehensive plasmid analysis, including the identification of rearrangements, demonstrates the added value of this approach. Nevertheless, further studies with larger sample sizes are necessary to validate these results.

As discussed in the thesis introduction, plasmid-encoded *ampC* genes are not the sole mechanism of AmpC resistance in *E. coli*. Another mechanism involves the occurrence of mutations in the promoter region of the *ampC* beta-lactamase gene, leading to hyperproduction of the chromosomally-encoded beta-lactamase. These promoter mutations are associated with various degrees of resistance against beta-lactam antibiotics (Tracz *et al*. 2007). While previous research has predominantly focused on the chromosomal AmpC resistance mechanism and its impact on AmpC hyperproduction, there is a knowledge gap regarding the evolutionary origin of these promoter/attenuator variants.

Chapter 7 aims to address this gap by conducting an investigation into the homoplasy and association of the *ampC* promoter/attenuator region in *E. coli* with cefotaxime resistance. The study involves a genome-wide analysis of homoplasy, which examines the occurrence of identical mutations in different lineages, combined with a comprehensive examination of polymorphisms associated with cefotaxime resistance. By analysing the mutations occurring in the reference chromosome and determining their distribution within the phylogeny, the study reveals a remarkable level of homoplasy at the −42 position of the *ampC* promoter. Specifically, the presence of a T nucleotide at the −42 position, instead of the wild-type C nucleotide, was found in 24 instances, resulting from 18 independent C>T mutations across five phylogroups. These findings shed light on the complex evolutionary dynamics and genetic diversity within the *ampC* promoter region and its association with cefotaxime resistance in *E. coli*.

Complexity of AmpC-Mediated Resistance Mechanisms

The diversity of mechanisms and phenotypic variations associated with AmpC-mediated resistance is an intriguing characteristic. Different types of ampC genes, such as bla_{CMY},

bla$_{DHA}$, or *bla*$_{ACC}$, appear to have varying impacts on the hydrolysis of different beta-lactam antibiotics (Philippon, Arlet, and Jacoby 2002). Additionally, mutations in the *ampC* promoter/attenuator region can lead to varying levels of resistance, and studies have even identified mutations in the *ampC* gene sequence that result in changes in the active site and higher resistance levels (Tracz *et al*. 2007; Nordmann, Poirel, and Nordmann 2007). Furthermore, other mechanisms affecting membrane permeability, such as efflux pumps and porins, have been observed in conjunction with AmpC-mediated resistance, leading to more extensive resistance patterns (Goessens *et al*. 2013). Variations in gene and/or plasmid copy number have also been proposed as contributing factors to differences in phenotypes (Kurpiel and Hanson 2012).

In **Chapter 8** of this thesis, the sequencing depth of chromosomal housekeeping genes is compared to plasmid-encoded scaffolds carrying the *bla*$_{CMY-2}$ gene. The ratio between these two components serves as an estimate of the plasmid copy number. Interestingly, isolates with elevated minimal inhibitory concentrations for cefotaxime, ceftazidime, and piperacillin-tazobactam exhibited higher estimated plasmid copy numbers. This finding suggests a potential association between plasmid copy number and increased resistance rates to beta-lactamase inhibitor combinations. However, further research is necessary to validate and expand upon these initial findings.

In summary, the emergence of AmpC-mediated resistance represents a significant challenge in combating antibiotic resistance. The investigation conducted in Chapter 8 sheds light on the potential role of plasmid copy number in influencing resistance rates to beta-lactamase inhibitor combinations. Additional studies are needed to corroborate these findings and enhance our understanding of the underlying mechanisms involved.

Future perspectives

The exploration of AmpC-mediated resistance in this thesis has provided valuable insights into its intricacies. However, the depth of this topic reveals a multitude of variants and mechanisms, leading to intriguing questions and avenues for future research.

One important aspect to consider is the clinical relevance of AmpC-mediated resistance and its lower prevalence compared to ESBL-mediated resistance in humans. The prevalence of CTX-M types, such as *bla*$_{CTX-M}$, is significantly higher than that of CMY- or DHA-type *ampC* genes. Further investigation is needed to understand the reasons behind this disparity, such as the potential role of specific vectors in the

dissemination of certain ESBL genes or the occurrence of fewer nosocomial outbreaks associated with AmpC-mediated resistance (David M. Livermore *et al*. 2007; Bevan, Jones, and Hawkey 2017). Potential factors, such as the association of certain ESBL genes, like $bla_{\text{CTX-M}}$ types, with clonal strains that exhibit enhanced human-to-human transmission, as observed in ST131 *E. coli*, might be one of the causes of this difference (David M. Livermore *et al*. 2007; Bevan, Jones, and Hawkey 2017). Alternatively, the acquisition of plasmid-encoded *ampC* genes may exert a greater fitness cost on bacteria, compared to ESBL genes on mobile elements (San Millan and MacLean 2017). The co-occurrence of plasmid-encoded *ampC* genes and ESBL genes within the same isolate is seem to be quite uncommon in most prevalence studies(Alvarez et al. 2004), as is the presence of plasmid-encoded *ampC* genes in isolates harbouring mutations in the *ampC* promoter/attenuator region associated with AmpC hyperproduction. While it seems plausible that possessing multiple resistance mechanisms could be impractical for microorganisms, comprehensive studies exploring this phenomenon are limited.

Another crucial consideration is the difficulty in detecting plasmid-encoded AmpC (pAmpC) genes, particularly in low-resource settings or low- and middle-income countries where access to molecular confirmation tests may be limited. Developing effective surveillance strategies for pAmpC detection in these settings is essential for understanding the true burden of antibiotic resistance.

Furthermore, multiple surveillance strategies should be explored to optimize the detection of AmpC-mediated resistance. This includes evaluating the use of non-specific agars versus specific agars in different settings. For instance, in an intensive care unit (ICU) with selective digestive tract decontamination (SDD) practices, the screening approach may differ from that used in the general population or community settings. Understanding the context-specific strategies for AmpC detection is crucial for implementing appropriate control measures and treatment strategies.

Looking ahead, further investigations are necessary to address these knowledge gaps and unravel the complexities underlying AmpC-mediated resistance. By delving deeper into these areas of inquiry, we can enhance our understanding of the interplay between different resistance mechanisms and their implications for treatment strategies and control measures. In the near future, advancements in our knowledge, such as the ability to predict phenotypical traits based on sequence data in combination with machine learning, may pave the way for more precise and effective treatment strategies.

Closing pages

Nederlandse samenvatting

Inleiding

Dit proefschrift heeft tot doel verschillende aspecten van AmpC-gemedieerde antimicrobiële resistentie bij Enterobacterales te onderzoeken, met name bij *Escherichia coli*. Het eerste doel was het bepalen van de prevalentie van rectaal dragerschap met AmpC-producerende Enterobacterales in Nederland en het analyseren van eventuele trends in dragerschap in de loop van de tijd. Een ander belangrijk doel was het optimaliseren van de detectie van *ampC*-genen in *E. coli* met behulp van selectieve media en fenotypische testen. Daarnaast onderzocht deze scriptie de haalbaarheid van het nauwkeurig onderscheiden van verwante monsters met AmpC-producerende *E. coli* en niet-verwante monsters op basis van plasmide-sequentiedata. Verder werd onderzocht of mutaties die optreden in het *ampC*-promoter/attenuator regio van *E. coli* onderhevig zijn aan homoplasie zijn en of deze homoplastische mutaties geassocieerd zijn met cefotaxim resistentie. Ten slotte werd de associatie tussen de aanwezigheid van plasmiden en het niveau van resistentie tegen specifieke antibiotica onderzocht. Dit omvatte het vergelijken van de sequentiediepte tussen chromosomaal gecodeerde huishoudelijke genen en plasmide-gecodeerde "scaffolds" die het $bla_{\text{CMY-2}}$-gen bevatten, waarbij de verhouding werd gebruikt als geschat aantal plasmidekopieën. Deze scriptie wordt afgesloten met een uitgebreide samenvatting en discussie, waarin de belangrijkste bevindingen van de uitgevoerde studies onder elkaar worden gezet en in een breder perspectief worden geplaatst.

Prevalentie van AmpC-producerende Enterobacterales in Nederland

De wereldwijde toename van antimicrobiële resistentie vormt een aanzienlijke bedreiging voor de volksgezondheid. Met name ESBL-producerende Enterobacterales laten aanzienlijke stijgende trends wereldwijd zien, zoals blijkt uit een recente review van Bezabih *et al.* waarin een tienvoudige toename aan dragerschapsgraad van ESBL *E. coli* in de gemeenschap wordt gerapporteerd (Bezabih *et al.* 2022). Ondanks een relatief lage prevalentie in West-Europese landen, waaronder Nederland (E. A. Reuland *et al.* 2016), heeft de toename van de wereldwijde mobiliteit de verspreiding van antimicrobiële resistente bacteriën versterkt (Paltansing *et al.* 2013; Lorme *et al.* 2018; Hu, Matsui, and W. Riley 2020; Raffelsberger *et al.* 2023). Het is echter belangrijk op te merken dat reizen niet het enige risicofactor is die verband houdt met dragerschap van multiresistente Enterobacterales. Een recent onderzoek door Meijs *et al.* heeft een verhoogde prevalentie van ESBL- en plasmide-gecodeerde AmpC-producerende *E. coli* en *K. pneumoniae*

aangetoond onder Nederlandse dierenartsen in vergelijking met de algemene bevolking (Meijs *et al.* 2021). Bovendien zijn andere risicofactoren voor intestinale dragerschap van ESBL-producerende Enterobacterales geïdentificeerd, zoals recent antibioticagebruik of gebruik van maagzuurremmers (E. A. Reuland *et al.* 2016; Huizinga *et al.* 2017).

Hoewel in mindere mate dan ESBL, is de prevalentie van plasmide-gecodeerde AmpC-producerende Enterobacterales in Nederland onderwerp geweest van verschillende studies. **Hoofdstuk 2** biedt inzichten in de epidemiologie van dit resistentiemechanisme, met name gericht op de situatie in Nederland. De meeste studies hebben zich gericht op rectaal of perineaal dragerschaap in zowelziekenhuizen als onder de algemene bevolking. In de algemene bevolking varieert de prevalentie van plasmide-gecodeerde AmpC-producerende *E. coli* tussen de 0,2% en 1,3%, wat wijst op een relatief lage prevalentie in vergelijking met ESBL-producerende *E. coli* (Van Hoek *et al.* 2015; E. Ascelijn Reuland *et al.* 2015; van den Bunt *et al.* 2017). Daarentegen hebben studies uitgevoerd in landen in Azië en Afrika hogere prevalenties gerapporteerd (Rodríguez-Guerrero *et al.* 2022). Zo detecteerden Huang *et al.* dat tot 16,1% van de gezonde volwassen deelnemers in Taiwan fecale dragers waren van plasmide-gecodeerde AmpC-producerende *E. coli* en/of *K. pneumoniae* (Y.-S. Huang *et al.* 2020). Bassyouni *et al.* vonden een prevalentie van 5,0% plasmide-gecodeerde AmpC-producerende *E. coli* in ontlastingsmonsters van Egyptische zorgmedewerkers, terwijl Chirindze *et al.* een prevalentie van 5,8% rapporteerden in ontlastingsmonsters van studenten in Mozambique (Bassyouni, Gaber, and Wegdan 2015; Chirindze *et al.* 2018). Het is belangrijk op te merken dat variaties in onderzoekspopulaties en screeningsmethodologieën directe vergelijkingen tussen verschillende regio's aanzienlijk bemoeilijken.

Hoewel de prevalentie van AmpC-producerende Enterobacterales in Nederland beperkt onderzocht is, is het vergelijken van de resultaten in de loop van de tijd uitdagend gebleken vanwege de heterogeniteit van de onderzoekspopulaties. In dit proefschrift beschrijft **Hoofdstuk 3** een onderzoek naar de prevalentie van rectaal dragerschap bij opgenomen patiënten over een periode van vier jaar, wat de mogelijkheid biedt om trends effectiever te analyseren. Interessant genoeg werd in ons onderzoek geen significante verandering in de prevalentie van plasmide-gecodeerde AmpC-producerende *E. coli* waargenomen gedurende de onderzoeksperiode. Er werd echter een afname van chromosoom-gecodeerde AmpC-producerende *E. coli* gedetecteerd, wat wijst op mogelijke variaties in de dynamiek van verschillende resistentiemechanismen. Op vergelijkbare wijze toonde een onderzoek naar trends in ESBL-dragerschap bij opgenomen patiënten over het algemeen een stabiele ESBL-prevalentie aan, waarbij specifiek een afname van CTX-M-1-achtige ESBL-genen werd waargenomen

(Willemsen et al. 2015). Deze bevindingen werpen licht op de complexe dynamiek van antimicrobiële resistentiepatronen en benadrukken de noodzaak van verder onderzoek naar de onderliggende factoren die van invloed zijn op de prevalentie van verschillende resistentiemechanismen.

Optimalisatie van de detectie van AmpC-gemedieerde resistentie in E. coli

Om effectieve surveillance van antimicrobiële resistentie te waarborgen, spelen nauwkeurige diagnostische testen een cruciale rol. Het detecteren van plasmide-gecodeerde AmpC omvat zowel fenotypische als genotypische technieken. Het onderscheiden van chromosoom-gecodeerde en plasmide-gecodeerde *ampC*-genen kan uitdagend zijn vanwege hun co-existentie, met name bij *E. coli*. Hoewel moleculaire bevestigingstests vaak worden gebruikt voor het screenen van plasmide-gecodeerde AmpC-producerende *E. coli*, kunnen ze tijdrovend en kostbaar zijn, waardoor ze minder haalbaar zijn in bepaalde situaties, vooral indien slechts beperkte middelen beschikbaar zijn.

In deze situaties zou het nuttig zijn om een praktisch algoritme te hebben dat op basis van alleen het fenotype onderscheid kan maken tussen ampC-genotypen in *E. coli*. Deze aanpak zou helpen identificeren welke isolaten verder moeten worden bevestigd met moleculaire testen. Door snel plasmide-gecodeerde AmpC-isolaten te identificeren, kunnen infectiepreventiemaatregelen worden verbeterd, onnodige en kostbare isolatiemaatregelen worden beperkt en kunnen passende behandelstrategieën worden geïmplementeerd. **Hoofdstuk 4** biedt een gedetailleerde beschrijving van de ontwikkeling van een dergelijk model, waarbij gebruik wordt gemaakt van machine learning-technieken. Het algoritme maakt gebruik van de minimale remmende concentratie (MIC) van cefotaxime om de aanwezigheid van plasmide-gecodeerde AmpC te voorspellen in cefoxitin-resistente (>8 mg/L) en ESBL-negatieve *E. coli*. Hoewel het algoritme een hoge nauwkeurigheid heeft aangetoond, is het belangrijk op te merken dat de gegevens die zijn gebruikt voor modelontwikkeling en training voornamelijk bestonden uit *E. coli* met $bla_{\text{CMY-2}}$ en een beperkt aantal andere plasmide-gecodeerde AmpC-genotypen. Hoewel dit de epidemiologische situatie in Nederland weerspiegelt, is het de moeite waard om te overwegen dat geografische variaties kunnen bestaan in andere regio's. Desalniettemin heeft het gebruik van machine learning-technieken enorm potentieel om screeningsalgoritmen binnen het vakgebied van de microbiologie te optimaliseren. Met goed samengestelde datasets op basis van gestandaardiseerde fenotypische en genotypische tests zijn de mogelijkheden voor vooruitgang in laboratoriumontwikkeling en infectiepreventiemethoden grenzeloos.

Het screenen op antimicrobiële resistentie, met name bij Enterobacterales verkregen uit rectale of perineale monsters met een veelheid aan andere darmbacteriën, brengt aanzienlijke uitdagingen met zich mee. Om de detectie te verbeteren, worden selectieve en differentiële media zoals MacConkey of Drigalski-lactose-agar doorgaans gebruikt, omdat ze grampositieve bacteriën effectief onderdrukken. Bovendien heeft het gebruik van zeer selectieve media die zijn afgestemd op specifieke ESBL- of carbapenemase-producerende Enterobacterales aangetoond dat ze verbeterde detectiemogelijkheden hebben voor resistente isolaten (Glupczynski *et al.* 2007; T. D. Huang *et al.* 2010; Göttig *et al.* 2020). Er is echter beperkte aandacht besteed aan de ontwikkeling van selectieve media voor AmpC-producerende Enterobacterales. In **Hoofdstuk 5** wordt een uitgebreide evaluatie gepresenteerd, met als doel de prestaties van verschillende media met cefotaxim en ceftazidim te beoordelen. Deze evaluatie houdt rekening met de specificiteit en gevoeligheid van deze media bij het detecteren van zowel chromosoom-gecodeerde AmpC-hyperproducerende als plasmide-gecodeerde AmpC-dragende *E. coli*-stammen, in vergelijking met ESBL-producerende *E. coli* en die zonder ESBL, pAmpC of cAmpC-hyperproductie. Bovendien wordt onderzocht wat het effect is van het toevoegen van cefoxitin aan deze media om de detectie van zowel chromosoom-gecodeerde AmpC-hyperproducerende als plasmide-gecodeerde AmpC-dragende *E. coli*-stammen te verbeteren.

Het toevoegen van cefoxitin had weinig invloed op de gevoeligheid, maar verhoogde de specificiteit van de detectie van AmpC-producerende *E. coli*. Het gebruikte agar maakte echter geen onderscheid tussen plasmide-gecodeerde en chromosoom-gecodeerde AmpC-producerende *E. coli*-stammen. Het is vermeldenswaardig dat de screeningset voornamelijk bestond uit bla_{CMY}-type-bevattende en chromosoom-gecodeerde AmpC-producerende *E. coli*-stammen, wat verder onderzoek rechtvaardigt naar een agar met cefotaxim of ceftazidim met cefoxitin in een klinische setting. Desalniettemin wordt verwacht dat de screeningsmedia geschikt zijn voor screeningsonderzoek naar AmpC-dragerschap in de darm wanneer een voorverrijkingsstap wordt toegepast, vergelijkbaar met de huidige screeningsstrategieën voor ESBL (M. F.Q. Kluytmans-Van Den Bergh et al. 2015).

Bronnen en transmissieroutes van AmpC-gemedieerde resistentie

Het begrijpen van de bronnen en transmissieroutes van antimicrobiële resistentievectoren is cruciaal om verdere toenames in antibioticumresistentie te voorkomen. Plasmiden, als mobiele genetische elementen, spelen een belangrijke rol in de verspreiding van antimicrobiële resistentiegenen en dragen verschillende resistentiemechanismen.

Het veelvoorkomende AmpC-resistentiegen, *bla*$_{CMY-2}$, wordt vaak geassocieerd met specifieke plasmidenfamilies, zoals IncA/C, IncB/O/K en IncI.

In **Hoofdstuk 6** ligt de focus op het onderzoeken van de relatie tussen *bla*$_{CMY-2}$-bevattende IncI1-pST12-plasmiden in epidemiologisch gerelateerde en niet-gerelateerde Enterobacteriaceae-isolaten van zowel mensen als dieren. Het doel was om de haalbaarheid te beoordelen van het nauwkeurig onderscheiden van verwante en niet-verwante monsters op basis van plasmide-sequentiedata. Door deze relatie te onderzoeken, kunnen waardevolle inzichten worden verkregen in de potentiële transmissiedynamiek en verwantschap van antimicrobiële resistentiestammen.

Hoewel het onderzoek is uitgevoerd in een beperkte omvang, tonen de bevindingen duidelijk de gelijkenis van plasmidesequenties aan, zoals aangegeven door het lage aantal enkelvoudige nucleotidepolymorfismen (SNP's) en het hoge aantal gedeelde genen. Bovendien overlapten de reeksen SNP's tussen plasmiden met verschillende epidemiologische achtergronden, wat suggereert dat het uitsluitend op plasmidesequentie gebaseerd onderscheid maken tussen epidemiologisch gerelateerde en niet-verwante plasmiden onwaarschijnlijk is. Eerdere studies hebben de geconserveerde sequenties van IncI1-pST12-plasmiden aangetoond; echter, deze studies maakten gebruik van analyse gebaseerd op genen aanwezigheid/afwezigheid of SNP-analyse, waarbij mogelijk subtiele verschillen tussen verschillende plasmiden werden gemist (Roer *et al.* 2019; Shirakawa *et al.* 2020; Castellanos *et al.* 2019). De huidige analyse, die long-read en short-read sequentiedata combineert voor een uitgebreide plasmideanalyse, inclusief de identificatie van herordeningen, toont de toegevoegde waarde van deze benadering aan. Desalniettemin zijn er verdere studies met grotere steekproefomvang nodig om deze resultaten te valideren.

Zoals besproken in de inleiding van de scriptie, zijn plasmide-gecodeerde *ampC*-genen niet het enige mechanisme van AmpC-resistentie in *E. coli*. Een ander mechanisme omvat het optreden van mutaties in het promotorgebied van het *ampC*-beta-lactamas-gen, wat leidt tot hyperproductie van de chromosoom-gecodeerde beta-lactamase. Deze promotormutaties zijn geassocieerd met verschillende mate van resistentie tegen beta-lactam-antibiotica (Tracz *et al.* 2007). Hoewel eerdere onderzoeken zich voornamelijk hebben gericht op het chromosomale AmpC-resistentiemechanisme en de invloed ervan op AmpC-hyperproductie, is er met over de evolutionaire oorsprong van deze promotoraanpassingen nog beperkte kennis.

Hoofdstuk 7 heeft tot doel onderzoek te doen naar de homoplasie en associatie van het ampC-promotor/verzwakkingsgebied in *E. coli* met cefotaxim resistentie. Het onderzoek omvat een genoomwijde analyse in combinatie met methoden om

de aanwezige homoplasie aan te tonen, waarbij wordt gekeken naar het voorkomen van identieke mutaties in verschillende fylogenetische lijnen, gecombineerd met een uitgebreide analyse van polymorfismen die geassocieerd zijn met cefotaxim resistentie. Door de mutaties in het referentiechromosoom te analyseren en hun verdeling binnen de fylogenie te bepalen, onthult het onderzoek een opmerkelijk niveau van homoplasie op de -42-positie van de ampC-promotor. Specifiek werd in 24 gevallen een T-nucleotide aangetroffen op de -42-positie, in plaats van het wildtype C-nucleotide, als gevolg van 18 onafhankelijke C>T-mutaties over vijf fylogroepen. Deze bevindingen werpen licht op de complexe evolutionaire dynamiek en genetische diversiteit binnen het *ampC*-promotorgebied en de associatie ervan met cefotaximeresistentie in *E. coli*.

Complexiteit van AmpC-gemedieerde resistentiemechanismen

De diversiteit aan mechanismen en fenotypische variaties geassocieerd met AmpC-gemedieerde resistentie is een intrigerend fenomeen. Verschillende typen plasmide-gecodeerde *ampC*-genen, zoals bla_{CMY}, bla_{DHA} of bla_{ACC}, lijken verschillende effecten te hebben op de hydrolyse van verschillende bètalactam-antibiotica (Philippon, Arlet, and Jacoby 2002). Bovendien kunnen mutaties in het *ampC*-promoter/attenuator leiden tot verschillende niveaus van resistentie, en studies hebben zelfs mutaties in de *ampC*-gensequentie geïdentificeerd die leiden tot veranderingen in de actieve site en hogere resistentieniveaus (Tracz *et al.* 2007; Nordmann, Poirel, and Nordmann 2007). Bovendien zijn er andere mechanismen die de membraanpermeabiliteit beïnvloeden, zoals effluxpompen en porines, waargenomen in combinatie met AmpC-gemedieerde resistentie, wat leidt tot uitgebreidere resistentiepatronen (Goessens *et al.* 2013). Variaties in het gen- en/of plasmidekopienummer zijn ook voorgesteld als bijdragende factoren aan verschillen in fenotypen (Kurpiel and Hanson 2012).

In **Hoofdstuk 8** van deze scriptie wordt de sequentiediepte van chromosomale huishoudelijke genen vergeleken met plasmide-gecodeerde “scaffolds” die het bla_{CMY-2}-gen dragen. De verhouding tussen deze twee dient als schatting van het plasmidekopienummer. Interessant is dat isolaten met verhoogde minimale remmende concentraties voor cefotaxim, ceftazidim en piperacilline-tazobactam hogere geschatte plasmidekopiënummers vertoonden. Deze bevinding suggereert een mogelijke associatie tussen plasmidekopienummers en verhoogde resistentieniveaus tegen combinaties van bètalactamase-remmers. Desalniettemin is verder onderzoek nodig om deze initiële bevindingen te valideren en uit te breiden.

Samengevat vertegenwoordigt het opkomen van AmpC-gemedieerde resistentie een aanzienlijke uitdaging in de strijd tegen antibioticaresistentie. Het onderzoek uitgevoerd

in **Hoofdstuk 8** werpt licht op de mogelijke rol van plasmidekopienummers bij het beïnvloeden van de resistentieniveaus tegen combinaties van bètalactamase-remmers. Aanvullende studies zijn nodig om deze bevindingen te bevestigen en ons begrip van de onderliggende mechanismen verder te vergroten.

Toekomstperspectieven

Het onderzoek naar AmpC-gemedieerde resistentie in deze scriptie heeft waardevolle inzichten opgeleverd in de complexiteit ervan. Echter, dit onderwerp onthult een veelvoud aan varianten en mechanismen, wat leidt tot nieuwe vragen en mogelijkheden voor toekomstig onderzoek.

Een belangrijk aspect is de klinische relevantie van AmpC-gemedieerde resistentie en de lagere prevalentie ervan vergeleken met ESBL-gemedieerde resistentie. De prevalentie van CTX-M-typen, zoals $bla_{\text{CTX-M}}$, is aanzienlijk hoger dan die van ampC-genen van het CMY- of DHA-type. Verder onderzoek is nodig om de redenen achter dit verschil te begrijpen, zoals de mogelijke rol van specifieke vectoren bij de verspreiding van bepaalde ESBL-genen of het voorkomen van minder nosocomiale uitbraken in verband met AmpC-gemedieerde resistentie (David M. Livermore *et al.* 2007; Bevan, Jones, and Hawkey 2017). Potentiële factoren, zoals de associatie van bepaalde ESBL-genen, zoals $bla_{\text{CTX-M}}$-typen, met clonale stammen die snellere overdracht van mens op mens vertonen, zoals waargenomen bij ST131 *E. coli*, zouden een van de oorzaken van dit verschil kunnen zijn (David M. Livermore *et al*. 2007; Bevan, Jones, and Hawkey 2017). Aan de andere kant kan de verwerving van plasmide-gecodeerde *ampC*-genen een grotere fitnesskost met zich meebrengen voor bacteriën in vergelijking met ESBL-genen (San Millan and MacLean 2017)(San Millan and MacLean 2017). Het voorkomen van plasmide-gecodeerde *ampC*-genen en ESBL-genen binnen hetzelfde isolaat lijkt vrij zeldzaam te zijn in de meeste prevalentiestudies (Alvarez *et al.* 2004), evenals het voorkomen van plasmide-gecodeerde *ampC*-genen in isolaten met mutaties in de *ampC*-promoter/attenuator regio die geassocieerd zijn met AmpC-hyperproductie. Hoewel het plausibel lijkt dat het bezitten van meerdere resistentiemechanismen tegennatuurlijk zou kunnen zijn voor micro-organismen, zijn uitgebreide studies die dit fenomeen onderzoeken beperkt.

Een andere belangrijke overweging is de moeilijkheid bij het detecteren van plasmide-gecodeerde *ampC*-genen, met name in gebieden met beperkte middelen of in landen met een laag of gemiddeld inkomen waar toegang tot moleculaire bevestigingstests mogelijk beperkt is. Het ontwikkelen van effectieve surveillancestrategieën voor

pAmpC-detectie in deze omgevingen is essentieel voor het begrijpen van de werkelijke last van antibioticaresistentie.

Bovendien moeten meerdere surveillancestrategieën worden onderzocht om de detectie van AmpC-gemedieerde resistentie te optimaliseren. Dit omvat het evalueren van het gebruik van niet-specifieke agars versus specifieke agars in verschillende omgevingen. Bijvoorbeeld, in een intensive care unit (ICU) met praktijken voor selectieve darmdecontaminatie (SDD), kan de screeningaanpak verschillen van die in de algemene bevolking. Het begrijpen van de contextspecifieke strategieën voor AmpC-detectie is cruciaal voor het implementeren van passende beheersmaatregelen en behandelstrategieën.

Vooruitkijkend zijn verdere onderzoeken nodig om deze kennishiaten aan te pakken en de complexiteit van AmpC-gemedieerde resistentie te ontrafelen. Door dieper in te gaan op deze onderzoeksvragen kunnen we ons begrip vergroten van de wisselwerking tussen verschillende resistentiemechanismen en de implicaties daarvan voor behandelstrategieën en beheersmaatregelen. In de nabije toekomst kunnen vooruitgangen in onze kennis, zoals het vermogen om fenotypische kenmerken te voorspellen op basis van sequentiegegevens in combinatie met machine learning, de weg effenen voor nauwkeurigere en effectievere behandelstrategieën.

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List of publications

Den Drijver, Evert P.M., Jaco J. Verweij and Jan A.J.W. Kluytmans. 2023. 'AmpC beta-lactamases: epidemiology, infection control and treatment'. *Tijdschrift voor Infectieziekten*, 18(1)

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About the author

Evert den Drijver was born on January 17th, 1987, in Gorinchem, Netherlands. He completed his education at Gymnasium Camphasianum in Gorinchem. Following his graduation, he initially pursued studies in molecular sciences at Radboud University Nijmegen. However, in 2007, he transitioned to a medical training program.

In 2013, Evert successfully obtained his medical degree and gained practical experience as a resident in geriatrics at Catharina Hospital, Eindhoven, for one year. In 2015, he embarked on his training as a clinical microbiologist, which took place at Elisabeth TweeSteden Hospital in Tilburg and Amphia Hospital in Breda. During this training, he conducted research projects that contributed to the development of this thesis under the guidance of Prof. Dr. J.A.J.W. Kluytmans and dr. Jaco J. Verweij.

In 2020, Evert completed his training as a clinical microbiologist and worked as one for the “Maatschap Medische Micrologie en Immunologie Gelderland” for nearly two and a half years. Subsequently, he made the decision to pursue a one-year Master’s degree in Public Health at Umeå University in Sweden.

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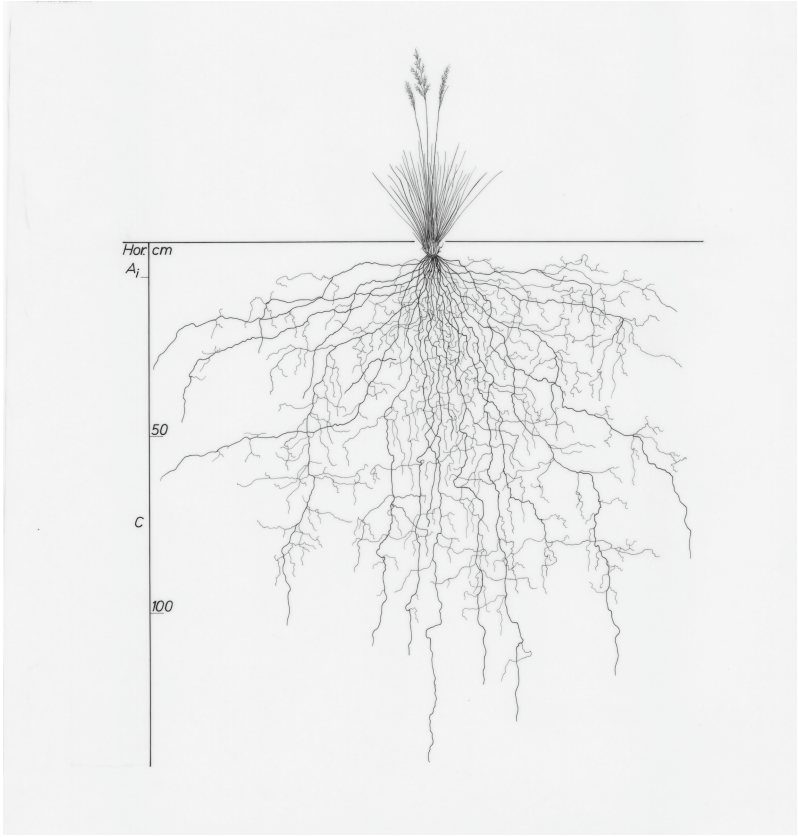
Hier eindigt dit proefschrift, en begint een nieuwe uitdaging



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