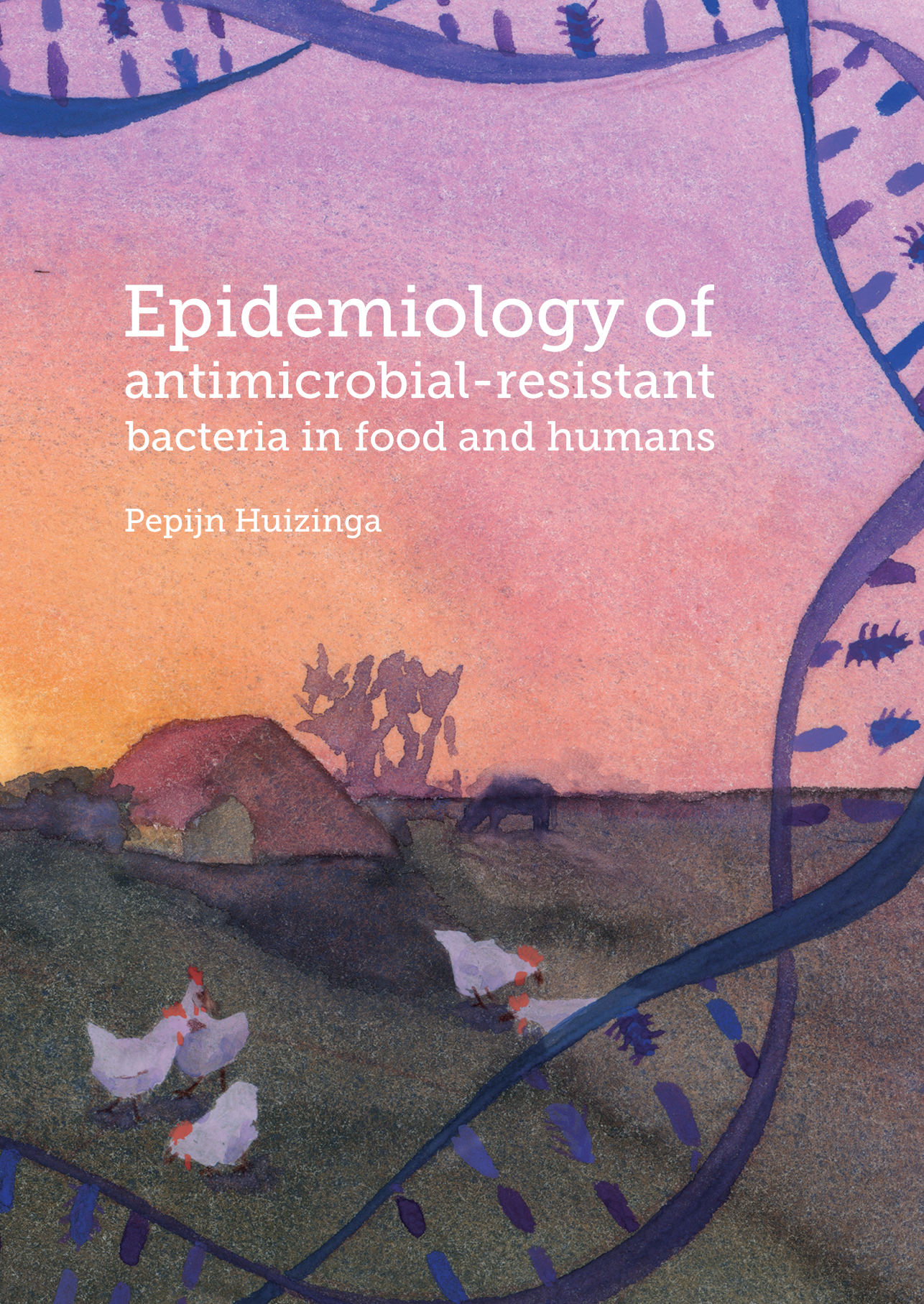
Epidemiology of antimicrobial-resistant bacteria in food and humans

Pepijn Huizinga



Epidemiology of antimicrobial-resistant bacteria in food and humans

Pepijn Huizinga

Epidemiology of antimicrobial-resistant bacteria in food and humans
PhD thesis, Utrecht University, the Netherlands

ISBN: 978-94-6416-716-0

Cover design and layout: © evelienjagtman.com
Printing: Ridderprint BV | Ridderprint.nl

Epidemiology of antimicrobial-resistant bacteria in food and humans

Epidemiologie van resistente bacteriën in voedsel en de mens
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de
Universiteit Utrecht
op gezag van
de rector magnificus, prof. dr. H.R.B.M. Kummeling,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op

dinsdag 21 september 2021 des middags te 4.15 uur

door

Pepijn Huizinga
geboren op 19 november 1985
te Maarssen

Promotor:
Prof. dr. J.A.J.W. Kluytmans

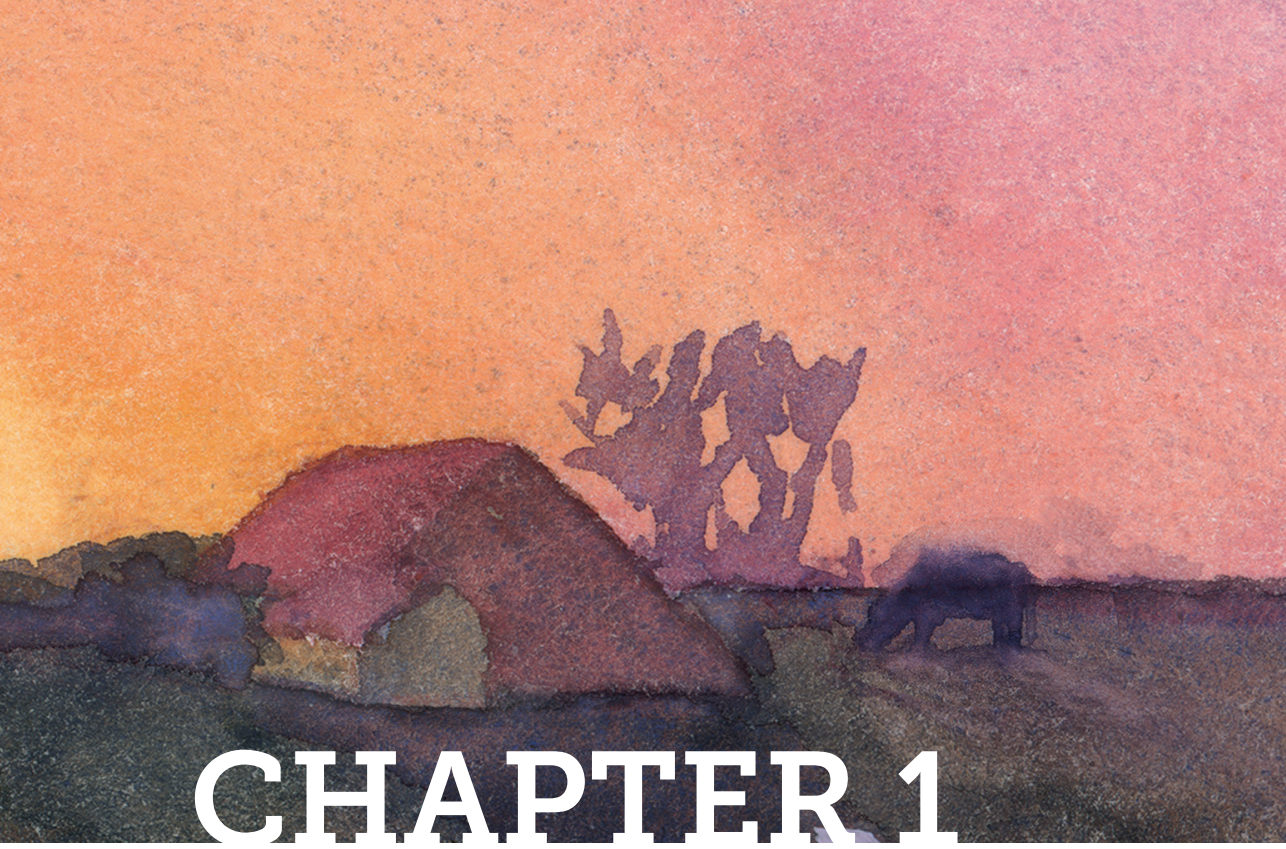
Beoordelingscommissie
Prof. dr. J.A. Wagenaar
Prof. dr. C.M. Vandenbroucke – Grauls
Prof. dr J.A. Stegeman
Prof. dr. J. Dewulf
Prof. dr. ir. D.J.J. Heederik

Paranimfen
Joep Stohr
Evert den Drijver

"What gets us into trouble
is not what we don't know.
It's what we know for sure
that just ain't so."

Mark Twain

TABLE OF CONTENTS



CHAPTER 1

General introduction

Pepijn Huizinga

GENERAL INTRODUCTION

Colonisation and infection

An often-heard figure is that there are 10 – 100 x more bacteria in the human body than there are human cells. This dates back to a relatively simple calculation performed in 1972 [1]. A more recent estimate is a ratio of 1:1, excluding any viruses or phages which likely outnumber bacteria and human cells combined [2]. This large number of bacteria is made up of around 1000 bacterial species which on average have 2000 genes per species, leading to an estimate of 2.000.000 bacterial genes. To place that in perspective the human genome has around 20.000 genes, bringing us back to the 1:100 ratio but now in the number of genes instead of actual cells [3,4].

A group of bacteria that often resides within the human gut as commensal flora is called the Enterobacteriaceae. Two of the most common species from this group are *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*). A person is called a carrier of a specific microorganism if they are colonised with that organism without active infection, also termed colonisation. Although usually harmless, these bacteria can cause infections such as urinary tract infections, pyelonephritis (infection of the kidney) or abdominal infections. These occasionally turn into more severe illnesses if the immune response to the infection is so strong that the immune response itself causes harm to the body. This state is called sepsis and is commonly accompanied by fever, low blood pressure, high heart rate and increased white blood cell count. Rapid treatment of the underlying illness, the bacterial infection, is the backbone to sepsis treatment, where time saves lives [5,6]. Source control, such as closing a perforated gut and antibiotic treatment of the infective organisms are of paramount importance [7–9].

What are antimicrobials

An antimicrobial is any substance that slows the growth of or kills microorganisms. An antibiotic is a relatively small molecule produced by a microorganism that inhibits or kills other microorganisms. As such, all antibiotics are antimicrobials, but antimicrobials are not always antibiotics. The terms are often used interchangeably. Ideally, an antimicrobial should be effective against the most common causes of infections, work quickly and cause little or no toxicity or other side effects.

The most frequently used class of antibiotics in hospitals are “beta-lactam” antibiotics, including penicillins and cephalosporins. All antibiotics in this group have a beta-lactam ring as their core structure, see Figure 1. The beta-lactam ring is a four-membered lactam, where a lactam is a cyclic (ringed) amide. The ‘beta’ part of the name indicates

the size of the ring, see Figure 1. The best-known example and first discovered beta-lactam antibiotic is penicillin, which derives its name from the fungus that produces it, *Penicillium,* as described by Sir Alexander Fleming [10].

Beta-lactam antibiotics work by inhibiting the synthesis of peptidoglycan, an essential component of the bacterial cell wall. More specifically it irreversibly binds and inactivates a protein, the penicillin-binding protein, that catalyses the final transpeptidation in forming the peptidoglycan layer. Because the penicillin-binding proteins no longer finalise cell wall formation the integrity of the cell wall is compromised, the bacterial multiplication stops and the high internal osmotic pressure of the bacterium ends up killing the bacterium. This process is shown in Figure 2a and 2b.

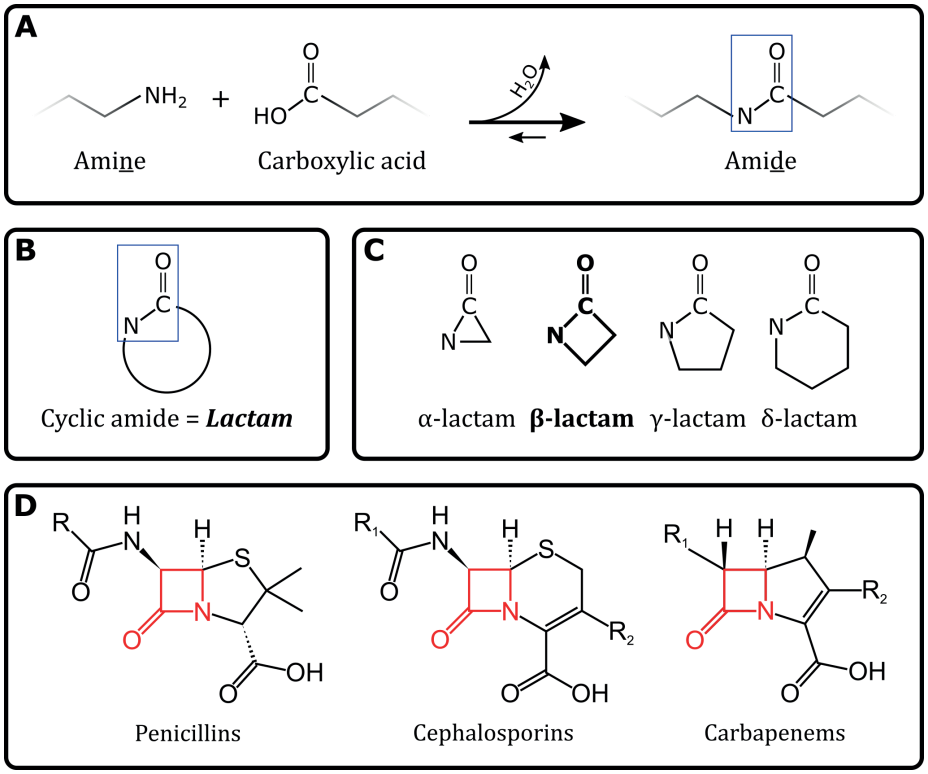


Figure 1. Explanation of the chemical structures of different beta-lactams. A) formation of an amide from an amine and carboxylic acid. B) Forming a cyclic bond from an amide to make a lactam. C) The lactam ring's size is shown in the name of the lactam. D) Examples of different types of beta-lactam antibiotics used in this thesis.

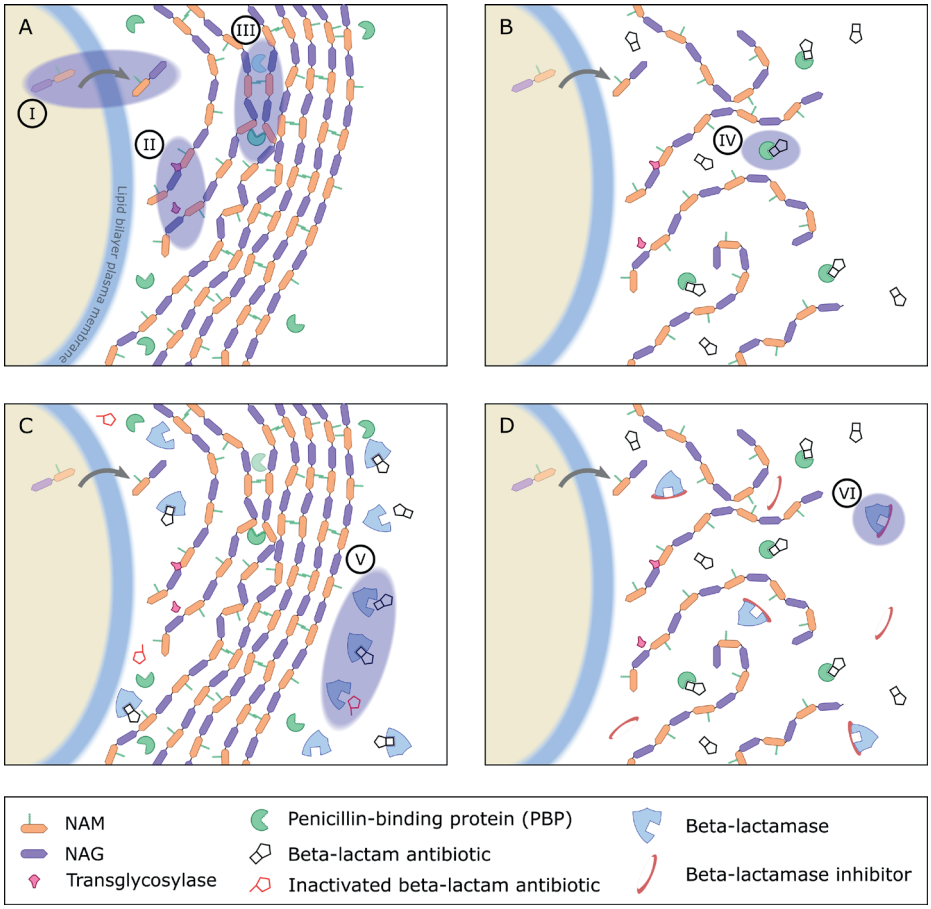


Figure 2. Formation of the peptidoglycan layer. A) Normal situation of peptidoglycan formation B) in the presence of a beta-lactam antibiotics inhibiting crosslinking C) in the presence of beta-lactamases produced by the bacteria that inhibit beta-lactam antibiotics D) with the addition of beta-lactamase inhibitors to beta-lactam antibiotics. The sub processes: I) subunits consist of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) with a short amino-acid side chain are synthesized in the bacterium and transported over the first lipid bilayer II) NAM-NAG units are connected forming chains III) the amino-acid side chains are cross linked by penicillin-binding proteins (PBPs), increasing the strength and finalizing peptidoglycan formation IV) in the presence of beta-lactam antibiotics, the PBPs are blocked and crosslinking cannot occur. The NAM-NAG chains remain loose and the peptidoglycan layer cannot be formed V) beta-lactam antibiotics are inactivated by beta-lactamases produced by bacteria VI) beta-lactamase inhibitors can be given together with beta-lactam antibiotics, these beta-lactamase inhibitors block the beta-lactamases allowing the beta-lactam antibiotics to effectively block PBPs.

Abbreviations: NAM, N-acetylglucosamine; NAG, N-acetylglucosamine; PBP, penicillin-binding protein.

Importance of antimicrobials in medicine

Antimicrobials play an essential role in medicine in its current form. Not only is it vital for treating community-acquired infections, but it is also just as important in treating infectious complications of other medical treatments. This can be anything from pneumonia caused by invasive ventilation on the intensive care to infections in stem cell transplantations, to treating an infected hip prosthesis. Many clinical interventions are possible because we can treat most of the infectious complications and in doing so, make the inherent risks of these procedures acceptable. If this were not possible, many relatively simple procedures would not be performed due to the risk of an untreatable infection potentially waiting around the corner.

As an indication of the value given to antimicrobials by society, multiple Nobel prizes have been awarded for work on antimicrobials [11].

- 1939 Gerhard Domagk "for the discovery of antibacterial effects of prontosil."
- 1945 to Sir Alexander Fleming, Ernst Boris Chain and Sir Howard Walter Florey "for the discovery of penicillin and its curative effect in various infectious diseases"
- 1952 to Selman Abraham Waksman "for his discovery of streptomycin, the first antibiotic effective against tuberculosis."
- 2009 to Venkatraman Ramakrishnan, Thomas A. Steitz and Ada E. Yonath "for studies of the structure and function of the ribosome" which was important for the production of antibiotics.

To understand the impact of resistance to antimicrobials later in this thesis, it is important to state that the group of antimicrobials called "cephalosporins" are frequently used in hospitals. Suppose a patient is treated in a hospital for abdominal sepsis, urosepsis or sepsis of unknown origin, in the Netherlands. In that case, the patient will most likely receive a second- or third-generation cephalosporin. As such, resistance to these antimicrobials directly impacts how physicians can effectively treat patients in hospitals.

What is antimicrobial resistance and "ESBL" and how can this spread?

Bacteria are resistant if they can withstand concentrations of an antimicrobial that would normally kill them and make treatment with that antimicrobial useless. In this thesis, we will mainly be looking at extended-spectrum beta-lactamases, abbreviated as "ESBLs". These are enzymes that break down antimicrobials, rendering them inactive. A wide range of enzymes falls under the ESBLs, which all have the general characteristics that they can hydrolyse most penicillins and cephalosporins but not carbapenems [12]. The penicillins and cephalosporins are frequently used in medicine in the Netherlands. Carbapenems are seen as our 'reserve' or 'last defence' antibiotics in case all else fails.

Molecules exist that inhibit ESBL enzymes, such as clavulanic acid, returning the beta-lactam antibiotic's efficacy by inhibiting the beta-lactamase enzyme. Some of these inhibitors are only effective in the laboratory, whilst others have clinical use, such as ceftazidime-avibactam [13].

Although there are many different ESBL enzymes, the most clinically relevant enzymes can be arranged into three groups. The currently most frequently encountered group in humans are the "CTX-M" enzymes. The name is derived from the abbreviated form of cefotaxime, CTX, the third-generation cephalosporin which the enzyme breaks down and the "M" from Munich where it was first described in 1990. The enzymes also break down other third-generation cephalosporins such as ceftazidime, but less efficiently [14]. The two other important groups are SHV, for "sulfhydryl reagent variable" and TEM from "Temoneira", the latter being the name of the patient from whom the *E. coli* was cultured from which the enzyme was first detected [15]. The TEM and SHV enzyme groups mainly contain "normal" beta-lactamases that cannot break down third-generation cephalosporins. Some variants have been observed that are effective in breaking down third-generation cephalosporins, making them "extended-spectrum" beta-lactamases.

The blueprints for ESBL enzymes are located on bacterial DNA where they are called ESBL-genes. The enzymes are described for instance as "CTX-M-1", a protein composed of amino acids. The genes that contain the genetic code for the specific proteins are written as "$bla_{\text{CTX-M-1}}$". "*bla*" for "beta-lactamase" and "CTX-M" for the group. The number at the end specifies different variants in nucleotide sequences that encode for the proteins. These numbers are arbitrary, and numbers close together are not more or less related to numbers further away.

Bacteria have all of the DNA needed for normal survival on one circular chromosome. Suppose the gene that encodes for the resistance enzyme is located on this chromosome. In that case, the simplest form of spread for that resistance gene – called *clonal transfer* – is if the bacterium duplicates itself and the new bacterium simply spreads to a different place. In addition to the chromosome, bacteria often possess extra DNA with particular functionalities such as antimicrobial resistance genes on smaller circular pieces of DNA called plasmids. These plasmids can contain multiple genes that convey resistance to different types of antimicrobials or other functionalities. Plasmids can be passed between different kinds of bacteria and even spread between different species of bacteria. Spreading of plasmids between bacteria – *horizontal gene transfer* – is an important tool for bacteria to share functionalities when needed, such as under antimicrobial pressure.

In short, bacteria produce penicillin-binding proteins (PBPs) to create and maintain their cell wall. We treat infections with antimicrobials that inhibit the bacterial penicillin-binding proteins. In turn, bacteria produce enzymes that break down antimicrobials, also known as antimicrobial resistance. In response to this resistance, specific medication can inhibit the enzymes that break down the antimicrobials. This can be seen as a right 'arms race' between medicine and rapidly responding bacteria.

Are antimicrobial-resistant microorganisms a problem?

To answer the central question of whether antimicrobial resistance (AMR) is a problem, multiple questions have to be considered. Firstly, how frequently do infections with AMR microorganisms occur? Secondly, when an infection with an AMR microorganism occurs, can it still be treated? In other words, are there sufficient alternative treatments? And finally, do infections with AMR microorganisms lead to more mortality than infections with their susceptible counterparts? In short, does AMR cause attributable morbidity and mortality?

To start with the first question, how frequent are infections with AMR microorganisms? There are considerable differences in the prevalence of AMR microorganisms throughout Europe, and the prevalence of the different AMR microorganisms also change over time. To give an impression, the percentage of third-generation cephalosporin resistance in invasive (blood cultures and cerebrospinal fluids) *E. coli* isolates in Europe in 2002 and 2019 is shown in Figure 3a and 3b. Resistance levels to carbapenems (our 'reserve' antibiotics) in *K. pneumoniae* in 2006 and 2019 are shown in Figure 3c and 3d. Countries with higher rates of third-generation cephalosporin resistance in 2002 are roughly the same countries with the highest carbapenem resistance rates in 2019. This is not a coincidence. It is likely that factors contributing to higher resistance levels to third-generation cephalosporins also select for and drive carbapenem resistance. When ESBL-producing Enterobacteriaceae causes more infections, it becomes more common to treat these with carbapenems, the next and final step up in beta-lactam treatment ladder. This increased use of carbapenems increases the selection pressure on carbapenemases, enzymes that break down carbapenems. The cycle repeats itself, but now not only have we exhausted one of the treatment options, an alternative treatment option, for instance, colistin, is not as effective and has more side effects. This is the answer to the second question: are there alternative treatment options? For infections with ESBL-producing Enterobacteriaceae, the answer is clearly yes. For carbapenemase-producing Enterobacteriaceae, the answer is often still yes, but not as good as some of the beta-lactams [16]. For Enterobacteriaceae resistant to carbapenems and colistin, there are other options depending on co-resistance mechanisms.

However, treating infections with increasingly resistant microorganisms becomes more difficult very quickly. Infections with 'pan-resistant' organisms, organisms to which no effective treatment exist for all practical purposes are increasing.

Another factor complicating the problem even further is that no truly new antibiotics classes have been discovered in the past 30 years, and none are expected any time soon [17].

This leaves the third question: do infections with AMR microorganisms cause attributable mortality? The definition of attributable mortality of AMR microorganisms is the extra mortality caused by the AMR microorganisms compared to the infection with its susceptible counterpart. Unfortunately, this question does not have a simple yes or no answer. It is difficult to determine what part of morbidity or mortality of infections caused by AMR microorganisms are attributable to the resistance and not to the infection per se. A lot of factors play a role and depend on the health system in place. In the Netherlands, the frequency of infections with AMR microorganisms is relatively low and the infections with AMR microorganisms that do occur have enough treatment alternatives. Also, the infrastructure of rapid detection of resistance in the microbiological laboratory with prompt feedback to the clinic that an alternative treatment is warranted keeps the negative effects of AMR to a minimum. In a recent study that addressed the precise question of attributable mortality for Gram-negative infections in the Netherlands, the attributable mortality was found to be close to zero [18,19]. Changes to the factors mentioned before could drastically change the attributable mortality. Although beyond the scope of this introduction, one can imagine that in areas of the world where many of these prerequisites are not in place the effect of resistance to widely available antimicrobials and lack of access to intravenous antibiotics or antimicrobial susceptibility testing can have a large impact on the attributable mortality of infections caused by AMR microorganisms.

In conclusion: there are large differences in the prevalence of antimicrobial resistance depending on geographical location, even within Europe. The Netherlands has relatively low levels of antimicrobial resistance. The antimicrobial resistance that we find in the Netherlands can almost always be treated with different antimicrobials. The speed of the diagnostics in the Netherlands decreases the time that inadequate empirical therapy is given. In the current setting, the attributable mortality and morbidity in the Netherlands are small. In different areas of the world, some or all of these factors are different, causing problems on a completely different scale.

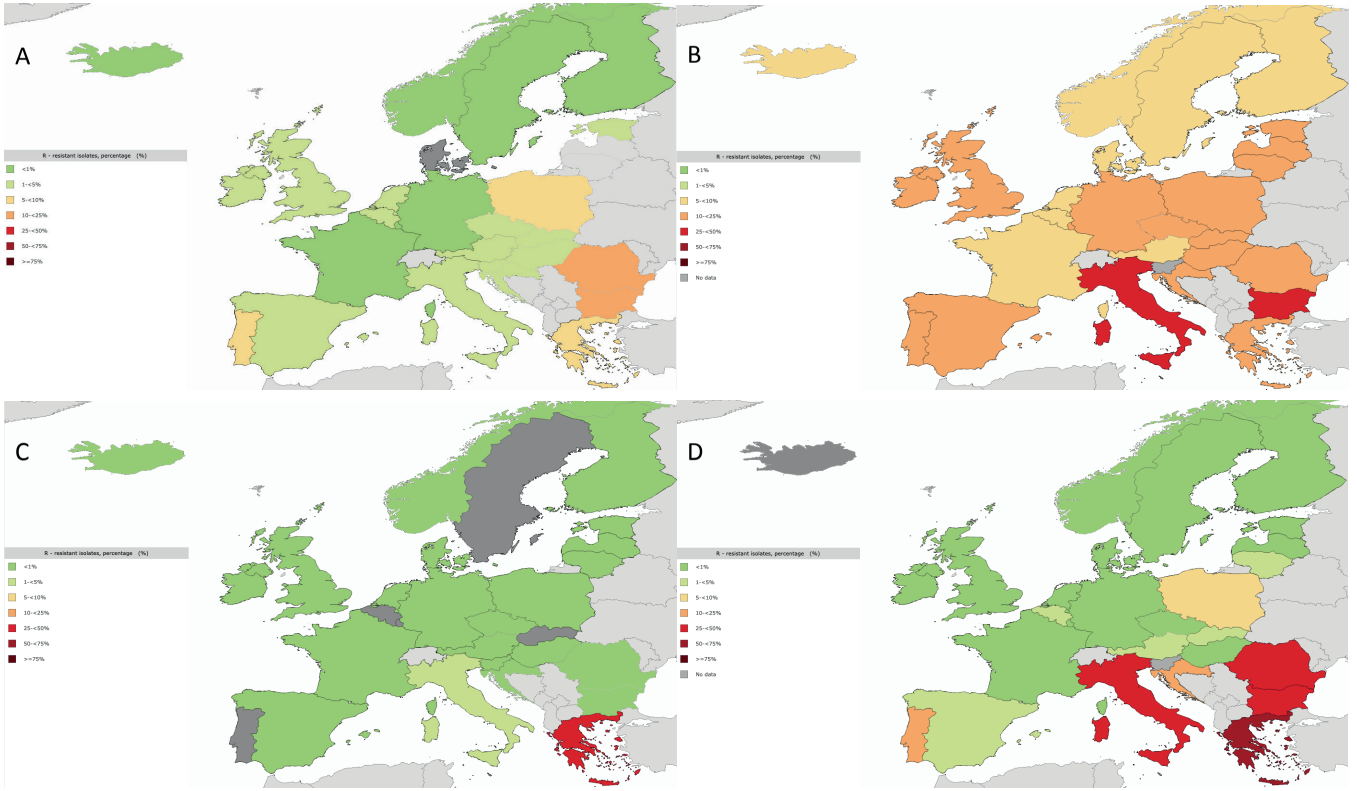


Figure 3. Percentage of invasive *E. coli* isolates with resistance to third-generation cephalosporins per country in EU/EEA in (a) 2002 and (b) 2019. Percentage of invasive *K. pneumoniae* isolates with resistance to carbapenems per country in the EU/EEA in (c) 2006 and (d) 2019. Source: Dataset provided by ECDC (European Centre for Disease prevention and Control) based on data provided by WHO and Ministries of Health from the affected countries.

ESBL epidemiology in the Netherlands

In the previous paragraphs AMR microorganisms in Europe and the Netherlands were mentioned. Here we would like to focus specifically on the epidemiology of ESBL-producing Enterobacteriaceae in the Netherlands. Before the turn of the century, resistance to third-generation cephalosporins in Enterobacteriaceae was scarce. This was followed by an increase in resistance rates until 2010 when it stabilized, see Figure 4, which shows the percentage of resistance to third-generation cephalosporins in *E. coli*, isolated from invasive infections in the Netherlands since the year 2000.

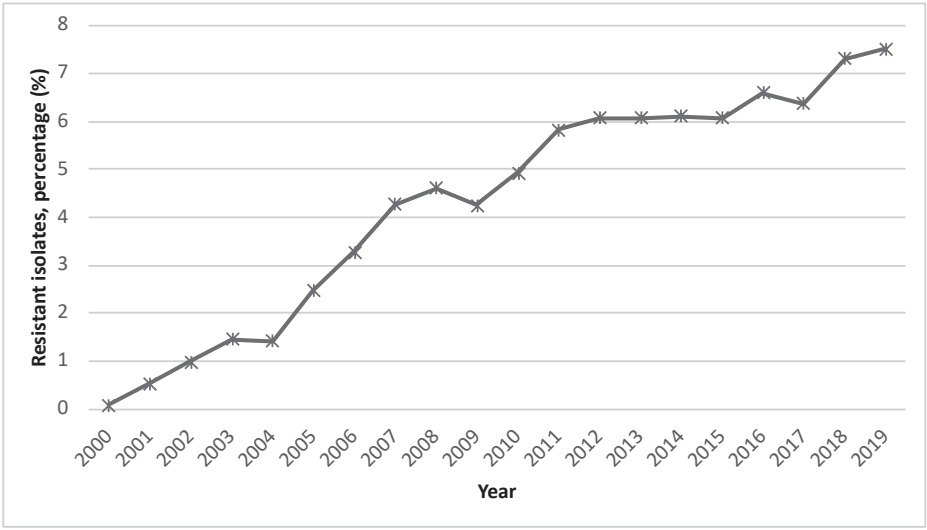


Figure 4. Percentage of *E. coli* isolates resistant to third-generation cephalosporins isolated from invasive isolates in the Netherlands between 2001 and 2019. Source: Dataset provided by ECDC (European Centre for Disease prevention and Control) based on data provided by WHO and Ministries of Health from the affected countries.

Initially, SHV and TEM ESBL enzymes caused most of the resistance to third-generation cephalosporins and the acquisition of resistance was usually associated with healthcare contact. The epidemiology changed considerably over a short time interval. The most prevalent ESBL enzymes changed from TEM and SHV to the CTX-M group [20]. Also, healthcare contact remains a risk factor for ESBL-E carriage, but ESBL has spread throughout the population and is also endemic in population groups without any healthcare contact [21,22]. From spreading through the healthcare system to the endemic presence in the general population, this change is an essential difference. While for the former hygiene in the hospital

and waste management should contain much of the AMR microorganisms, in a situation where acquisition occurs in the general population the sources of AMR microorganisms are much broader and considerably more challenging to contain.

What are sources of ESBL-producing Enterobacteriaceae transmission to humans?
There is a large variety of sources from which ESBL-producing Enterobacteriaceae have been cultured. Untangling the impact of individual sources on the total burden of ESBL-producing Enterobacteriaceae colonization and disease is complex. The sources from where transmission can occur can be divided into several categories; these are human sources, animal sources, environmental sources and food-related sources.

The human-to-human spread is probably the most crucial factor for ESBL-producing Enterobacteriaceae presence in the open community [23]. Human-to-human transmission can occur in different settings. This can be in clinical settings such as hospitals or care facilities and the open community such as within households [23–26]. When looking at introduction into the Dutch population, human-to-human transmission related to international travel is likely an important factor. Especially travelers returning from 'high' ESBL endemic areas such as Asia and northern Africa are at risk of carrying ESBL [27–29]. Most of the carriage is lost again in the first months after travel, but some will become long term carriers, 10% >1 year [27]. Onward transmission from these travel-acquired ESBL-E carriers does occur in 12% of cases.

Animal-to-human transmission occurs from both farm animals and pets [30–34]. And ESBL-producing Enterobacteriaceae acquisition occurs directly from the environment; such as swimming in fresh surface water or the sea [21,35,36].

A final source of ESBL-E is the food chain. An entity to which the entire population is exposed and of which we know that a variety of products is contaminated with ESBL-E, such as different vegetables, different meat products or in certain areas drinking water [37–44]. One food item has attracted much attention because of the high contamination rates: chicken meat [38,40]. The questions if ESBL-producing Enterobacteriaceae contamination of food lead to colonization in humans and how extensive this share is in the total burden of ESBL-producing Enterobacteriaceae in humans remains a topic of debate and will be discussed in this thesis for retail chicken meat.

Retail chicken meat as a potential source of AMR in humans in the Netherlands
Many types of reservoirs have been found to contain ESBL-E, including food products [45]. One food product associated with high ESBL-E positivity rates in the Netherlands, which is frequently consumed by the public is poultry meat [38–40,46].

Typing of the ESBL genes and other conventional typing techniques suggested overlap between ESBL-E derived from retail chicken meat and ESBL-E derived from human carriage and disease [39,40]. Based on the available data and the fact that in Netherlands human antimicrobial use was low and veterinary antimicrobial use was high, programs were initiated to decrease the use of veterinary antimicrobials, which reduced veterinary antibiotic use between 2009 and 2017 with 63%; for broiler farms, this was 72% [47–50].

With advancements in whole-genome sequencing techniques, more in-depth analyses can be performed compared to older typing techniques; increasing the resolution of the typing methods. The study's primary aim is to investigate if ESBL-EC derived from human carriage and disease could have its origin from ESBL-EC from retail chicken meat based on the genetic composition. As a secondary aim, we try to quantify how large the contribution of ESBL-EC with potential chicken meat origin is in the human domain and compare the findings from this study with other studies that used whole-genome sequencing for similar questions.

Human medicine was and remains restrictive in its antimicrobial use in the Netherlands. Compared to other European countries, the Netherlands is among the most restrictive users of antimicrobials of all countries both in and outside the hospital [51]. This was in stark contrast with veterinary antimicrobial use where the Netherlands ranked among the highest users compared to other European countries between 2005 and 2009 [52]. High antimicrobial use in livestock was a likely factor for the high prevalence of AMR microorganisms in livestock in the Netherlands. This can lead to frequently contaminated retail meat products and a potential source for AMR transmission from livestock, through the food chain, to humans.

Research around 2010 showed considerable overlap in ESBL genes in humans and chicken meat, suggesting potential transmission from chicken meat to humans through the food chain [38,40]. The combination of rising rates of ESBL-producing Enterobacteriaceae in human carriage and disease, a potential source in livestock and meat products and growing public awareness of these problems led to rapid political action. In April 2010, the first targets were set to decrease veterinary antibiotic use with 20% in 2011 and 50% in 2013, compared to 2009 [53]. Through a well thought out strategy and close collaboration between veterinary professionals, government and stakeholders within the livestock sector these targets were achieved ultimately leading to a decrease in antibiotic sales of 70% comparing 2009 to 2019 [54,55]. The general reduction in antimicrobial use in the

veterinary sector and specific targeted interventions, such as ceasing administering third-generation cephalosporins to eggs, was followed by a rapid decline in the presence of third-generation cephalosporin resistance in poultry faeces around 2011 – 2013 and beyond, see Figure 5.

The impact of these measures on and the frequency of contamination of retail chicken meat with ESBL-E was not clear at the time and are discussed in this thesis.

Finally, it is important to note that the genetic typing methods for typing bacterial isolates have drastically changed over the past five to ten years due to the emergence of whole-genome sequencing. Where previously genetic codes of a few hundred base pairs were investigated, this increased to >5 million base pairs for an *E. coli* isolate. This transformation also brought many challenges interpreting the vast amounts of data and has also led to reevaluating certain previously drawn conclusions.

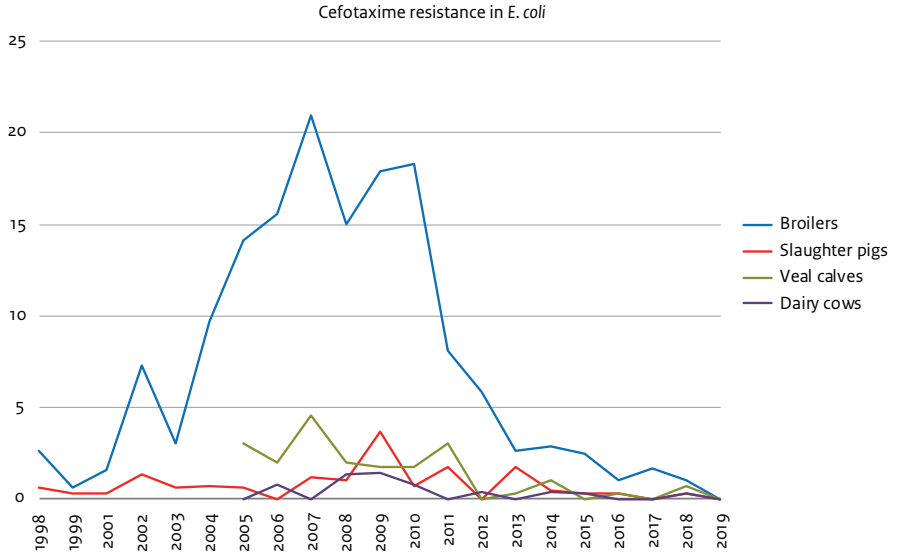


Figure 5. Trends in cefotaxime resistance (%) of *E. coli* randomly isolated from faecal samples of broilers, slaughter pigs, veal calves and dairy cows. The Figure was taken from MARAN 2020 [55].

Plasmid-mediated colistin resistance

Colistin, also called polymyxin E, is an antimicrobial that was discovered in 1949. It was not frequently used in humans as there were problems with nephrotoxicity (kidney problems caused by the antimicrobial). With increasing resistance to cephalosporins

and carbapenems in Enterobacteriaceae, colistin regained interest as a last-resort antimicrobial. Until 2015, all known resistance mechanisms to colistin were chromosomally mediated, and there were little worries that this would become a large issue [56]. However, in 2015, Liu et al. published a report describing the first plasmid-mediated colistin resistance gene, mobile colistin resistance one or *mcr-1* [57]. The fundamental difference between chromosomal resistance and plasmid-mediated resistance is the speed with which the gene can spread. Plasmid-mediated resistance can spread much faster because the resistance plasmid can easily be shared between an extensive range of Gram-negative bacilli. If this were to be the case, Enterobacteriaceae resistant to carbapenems and colistin could increase in numbers rapidly leaving us with little or no treatment options where previously colistin was the last-resort antimicrobial.

Aims of this thesis

This thesis looks into different topics around the central theory that antimicrobial-resistant microorganisms (AMR) could be a source of AMR in humans.

Different questions are addressed:

1. The first aim was to quantify the frequency of contamination of different food items with ESBL-E and/or plasmid-mediated colistin resistance and describe their genetic characteristics.
2. The second aim was to investigate if proton pump inhibitor use could be a risk factor for carriage of ESBL-producing Enterobacteriaceae.
3. The third aim was to investigate if based on genetic characteristics ESBL-producing Enterobacteriaceae cultured from humans and retail chicken meat share genetic characteristics and if there is overlap to what extent human-derived ESBL-EC could have their origin from retail chicken meat.

Outline of this thesis

In the first chapters, the aim has been to quantify the frequency of contamination of different food items with ESBL-E or plasmid-mediated colistin resistance. **Chapter 2** describes the decreasing prevalence of ESBL-E in retail chicken meat in the Netherlands between December 2013 and August 2015. **Chapter 3** describes the prevalence of contamination of retail bean sprouts with ESBL-E in the Netherlands in 2015. In both these papers, the genetic characteristics of the cultured ESBL-E are discussed.

If food is a potential source of ESBL-E in humans, the low pH in the gastric environment can be seen as a barrier of the ESBL-E that is consumed via food. It was hypothesised that if proton pump inhibitors increased the gastric pH, this could increase the risk of becoming colonised with ESBL-E. This hypothesis is investigated in **Chapter 4.**

In **Chapter 5**, the genetic characteristics of ESBL-E isolates cultured from human blood cultures and perianal swabs and isolates from retail chicken meat are compared to investigate whether there is genetic overlap between the human and the food domain.

Chapter 6 looks at the presence of *mcr*-1, a gene conferring resistance to colistin, mainly in ESBL-producing Enterobacteriaceae isolates cultured from retail chicken meat and humans. **Chapter 7** describes the prevalence of *mcr-1* in retail chicken meat in the Netherlands in 2015.

Disclaimer ECDC with Figure 3: The views and opinions of the authors expressed herein do not necessarily state or reflect those of the ECDC. The accuracy of the authors' statistical analysis and the findings they report are not the responsibility of ECDC. ECDC is not responsible for conclusions or opinions drawn from the data provided. ECDC is not responsible for the correctness of the data and data management, data merging and data collation after the provision of the data. ECDC shall not be held liable for improper or incorrect use of the data.

REFERENCES:

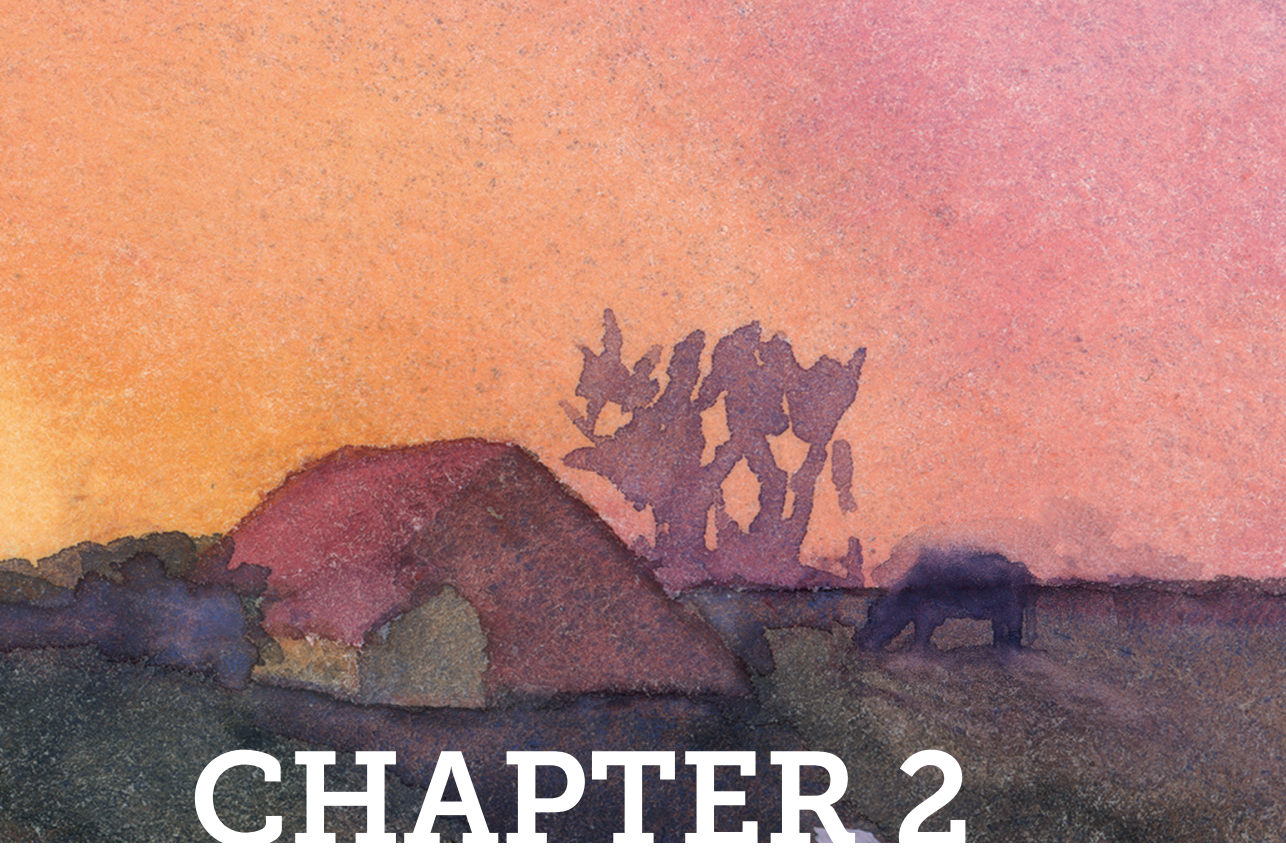
1. Luckey TD. Introduction to intestinal microecology. Am J Clin Nutr. 1972;25: 1292–4. doi:10.1093/ajcn/25.12.1292
2. Sender R, Fuchs S, Milo R. Are We Really Vastly Outnumbered? Revisiting the Ratio of Bacterial to Host Cells in Humans. Cell. Elsevier Inc.; 2016;164: 337–340. doi:10.1016/j.cell.2016.01.013
3. Turnbaugh PJ, Ley RE, Hamady M, Fraser-liggett C, Knight R, Gordon JI. The human microbiome project: exploring the microbial part of ourselves in a changing world. Nature. 2007;449: 804–810. doi:10.1038/nature06244.The
4. Gilbert JA, Blaser MJ, Caporaso JG, Jansson JK, Lynch S V, Knight R. Current understanding of the human microbiome. Nat Med. 2018;24: 392–400. doi:10.1038/nm.4517
5. Howell MD, Davis AM. Management of sepsis and septic shock. JAMA - J Am Med Assoc. 2017;317: 847–848. doi:10.1001/jama.2017.0131
6. Rhodes A, Evans LE, Alhazzani W, Levy MM, Antonelli M, Ferrer R, et al. Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016. Intensive Care Med. Springer Berlin Heidelberg; 2017;43: 304–377. doi:10.1007/s00134-017-4683-6
7. Kumar A, Roberts D, Wood KE, Light B, Parrillo JE, Sharma S, et al. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. Crit Care Med. 2006;34: 1589–1596. doi:10.1097/01.CCM.0000217961.75225.E9
8. Zhang D, Micek ST, Kollef MH. Time to appropriate antibiotic therapy is an independent determinant of postinfection ICU and hospital lengths of stay in patients with sepsis. Crit Care Med. 2015;43: 2133–2140. doi:10.1097/CCM.0000000000001140
9. Ferrer R, Martin-Loeches I, Phillips G, Osborn TM, Townsend S, Dellinger RP, et al. Empiric antibiotic treatment reduces mortality in severe sepsis and septic shock from the first hour: Results from a guideline-based performance improvement program. Crit Care Med. 2014;42: 1749–1755. doi:10.1097/CCM.0000000000000330
10. Fleming A. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B. influenzae. Br J Exp Pathol. 1929;10: 226–236.
11. Ergonul O, Yalcin CE, Erkent MA, Demirci M, Uysal SP, Ay NZ, et al. Who can get the next Nobel Prize in infectious diseases? Int J Infect Dis. International Society for Infectious Diseases; 2016;45: 88–91. doi:10.1016/j.ijid.2016.02.022
12. European Committee on Antimicrobial Susceptibility Testing. Guidelines for detection of resistance mechanisms and specific resistance of clinical and/or epidemiological importance. Version 2.0 [Internet]. 2017. Available: www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_170711.pdf
13. Shirley M. Ceftazidime-Avibactam: A Review in the Treatment of Serious Gram-Negative Bacterial Infections. Drugs. Springer International Publishing; 2018;78: 675–692. doi:10.1007/s40265-018-0902-x
14. Bauernfeind A, Grimm H, Schweighart S. A new plasmidic cefotaximase in a clinical isolate of Escherichia coli. Infection. 1990;18: 294–8. doi:10.1007/BF01647010
15. Turner PJ. Extended-spectrum beta-lactamases. Clin Infect Dis. 2005;41 Suppl 4: S273-5. doi:10.1086/430789

16. Rodríguez-Baño J, Navarro MD, Retamar P, Picón E, Pascual Á. ??-Lactam/??-lactam inhibitor combinations for the treatment of bacteremia due to extended-spectrum ??-lactamase-producing Escherichia coli: A post hoc analysis of prospective cohorts. Clin Infect Dis. 2012; doi:10.1093/cid/cir790
17. WHO. Antimicrobial resistance - gobal report on surveillance 2014. 2014.
18. de Kraker MEA, Davey PG, Grundmann H. Mortality and hospital stay associated with resistant Staphylococcus aureus and Escherichia coli bacteremia: estimating the burden of antibiotic resistance in Europe. PLoS Med. 2011;8: e1001104. doi:10.1371/journal.pmed.1001104
19. Rottier WC, Deelen JWT, Caruana G, Buiting AGM, Dorigo-Zetsma JW, Kluytmans JAJW, et al. Attributable mortality of antibiotic resistance in gram-negative infections in the Netherlands: a parallel matched cohort study. Clin Microbiol Infect. 2020; doi:10.1016/j.cmi.2020.07.014
20. Livermore DM, Canton R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, et al. CTX-M: Changing the face of ESBLs in Europe [Internet]. Journal of Antimicrobial Chemotherapy. 2007. pp. 165–174. doi:10.1093/jac/dkl483
21. Bunt van den G, Pelt van W, Hidalgo L, Scharringa J, Greeff SC de, Schürch AC, et al. Prevalence, risk factors and genetic characterization of Extended-Spectrum Beta-Lactamase and Carbapenemase-producing Enterobacteriaceae (ESBL-E and CPE): a community-based repeated cross-sectional study in the Netherlands from 2014 to 2016. Eurosurveillance (forthcoming). 2019; doi:eurosurveillance-D-18-00594R1
22. Rodríguez-Baño J, Navarro MD, Romero L, Martínez-Martínez L, Muniain MA, Perea EJ, et al. Epidemiology and clinical features of infections caused by extended-spectrum beta-lactamase-producing Escherichia coli in nonhospitalized patients. J Clin Microbiol. 2004;42: 1089–94. doi:10.1128/jcm.42.3.1089-1094.2004
23. Mughini-Gras L, Dorado-García A, van Duijkeren E, van den Bunt G, Dierikx CM, Bonten MJM, et al. Attributable sources of community-acquired carriage of Escherichia coli containing β-lactam antibiotic resistance genes: a population-based modelling study. Lancet Planet Heal. 2019;3: e357–e369. doi:10.1016/S2542-5196(19)30130-5
24. Shitrit P, Reisfeld S, Paitan Y, Gottesman BS, Katzir M, Paul M, et al. Extended-spectrum beta-lactamase-producing Enterobacteriaceae carriage upon hospital admission: Prevalence and risk factors. J Hosp Infect. Elsevier Ltd; 2013;85: 230–232. doi:10.1016/j.jhin.2013.07.014
25. Overdevest I, Haverkate M, Veenemans J, Hendriks Y, Verhulst C, Mulders A, et al. Prolonged colonisation with escherichia coli O25:ST131 versus other extended-spectrum beta-lactamase-producing E. coli in a long-term care facility with high endemic level of rectal colonisation, the Netherlands, 2013 to 2014. Eurosurveillance. 2016;21. doi:10.2807/1560-7917.ES.2016.21.42.30376
26. Haverkate MR, Platteel TN, Fluit AC, Cohen Stuart JW, Leverstein-van Hall MA, Thijsen SFT, et al. Quantifying within-household transmission of extended-spectrum β-lactamase-producing bacteria. Clin Microbiol Infect. Elsevier Ltd; 2017;23: 46.e1-46.e7. doi:10.1016/j.cmi.2016.08.021
27. Arcilla MS, Hattem JM van, Haverkate MR, Bootsma MCJ, Genderen PJJ van, Goorhuis A, et al. Import and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae by international travellers (COMBAT study): a prospective, multicentre cohort study. Lancet Infect Dis. 2017;17: 78–85. doi:10.1016/S1473-3099(16)30319-X
28. Östholm-Balkhed Å, Tärnberg M, Nilsson M, Nilsson LE, Hanberger H, Hällgren A. Travel-associated faecal colonization with esbl-producing enterobacteriaceae: Incidence and risk factors. J Antimicrob Chemother. 2013;68: 2144–2153. doi:10.1093/jac/dkt167

29. Barreto Miranda IDM, Ignatius RPDM, Pfuller RDM, Friedrich-Janicke BDM, Steiner FDM, Paland MDM, et al. High carriage rate of ESBL-producing Enterobacteriaceae at presentation and follow-up among travellers with gastrointestinal complaints returning from India and Southeast Asia. J Travel Med. 2016;23: 1–7. doi:10.1093/jtm/tav024
30. Nguyen VT, Jamrozy D, Matamoros S, Carrique-Mas JJ, Ho HM, Thai QH, et al. Limited contribution of non-intensive chicken farming to ESBL-producing *Escherichia coli* colonization in humans in Vietnam: an epidemiological and genomic analysis. J Antimicrob Chemother. 2019; doi:10.1093/jac/dky506
31. Fischer J, Hille K, Ruddat I, Mellmann A, Köck R, Kreienbrock L. Simultaneous occurrence of MRSA and ESBL-producing Enterobacteriaceae on pig farms and in nasal and stool samples from farmers. Vet Microbiol. Elsevier B.V.; 2017;200: 107–113. doi:10.1016/j.vetmic.2016.05.021
32. Dahms C, Hubner N-O, Kossow A, Mellmann A, Dittmann K, Kramer A. Occurrence of ESBL-Producing Escherichia coli in Livestock and Farm Workers in Mecklenburg-Western Pomerania, Germany. PLoS One. United States; 2015;10: e0143326. doi:10.1371/journal.pone.0143326
33. Dierikx C, van der Goot J, Fabri T, van Essen-Zandbergen A, Smith H, Mevius D. Extended-spectrum-β-lactamase- and AmpC-β-lactamase-producing Escherichia coli in Dutch broilers and broiler farmers. J Antimicrob Chemother. 2013;68: 60–67. doi:10.1093/jac/dks349
34. Meyer E, Gastmeier P, Kola A, Schwab F. Pet animals and foreign travel are risk factors for colonisation with extended-spectrum beta-lactamase-producing Escherichia coli. Infection. 2012;40: 685–687. doi:10.1007/s15010-012-0324-8
35. Egervärn M, Englund S, Ljunge M, Wiberg C, Finn M, Lindblad M, et al. Unexpected common occurrence of transferable extended spectrum cephalosporinase-producing Escherichia coli in Swedish surface waters used for drinking water supply. Sci Total Environ. 2017;587–588: 466–472. doi:10.1016/j.scitotenv.2017.02.157
36. Søraas A, Sundsfjord A, Sandven I, Brunborg C, Jenum PA. Risk Factors for Community-Acquired Urinary Tract Infections Caused by ESBL-Producing Enterobacteriaceae -A Case-Control Study in a Low Prevalence Country. PLoS One. 2013;8: 1–7. doi:10.1371/journal.pone.0069581
37. de Boeck H, Miwanda B, Lunguya-Metila O, Muyembe-Tamfum JJ, Stobberingh E, Glupczynski Y, et al. ESBL-positive enterobacteria isolates in drinking water. Emerg Infect Dis. 2012;18: 1019–1020. doi:10.3201/eid1806.111214
38. Overdevest I, Willemsen I, Rijnsburger M, Eustace A, Xu L, Hawkey P, et al. Extended-spectrum β-lactamase genes of Escherichia coli in chicken meat and humans, the Netherlands. Emerg Infect Dis. 2011;17: 1216–1222. doi:10.3201/eid1707.110209
39. Kluytmans JAJW, Overdevest ITMA, Willemsen I, Bergh MFQK Den, Zwaluw K Van Der, Heck M, et al. Extended-Spectrum β -Lactamase – Producing Escherichia coli From Retail Chicken Meat and Humans : Comparison of Strains , Plasmids , Resistance Genes , and Virulence Factors. 2013;56: 478–487. doi:10.1093/cid/cis929
40. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Voets GM, van den Munckhof MP, van Essen-Zandbergen A, et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect. 2011;17: 873–880. doi:10.1111/j.1469-0691.2011.03497.x
41. van Hoek AHAM, Veenman C, van Overbeek WM, Lynch G, de Roda Husman AM, Blaak H. Prevalence and characterization of ESBL- and AmpC-producing Enterobacteriaceae on retail vegetables. Int J Food Microbiol. 2015;204: 1–8. doi:10.1016/j.ijfoodmicro.2015.03.014

42. Reuland EA, Al Naiemi N, Raadsen SA, Savelkoul PHM, Kluytmans JAJW, Vandenbroucke-Grauls CMJE. Prevalence of ESBL-producing Enterobacteriaceae in raw vegetables. Eur J Clin Microbiol Infect Dis. 2014;33: 1843–6. doi:10.1007/s10096-014-2142-7
43. Huizinga P, Schrauwen E, García-Cobos S, Willemsen I, Verhulst C, Friedrich AW, et al. Extended-spectrum beta-lactamase producing Enterobacteriaceae (ESBL-E) isolated from bean sprouts in the Netherlands. PLoS One. 2018;13: e0203338. doi:10.1371/journal.pone.0203338
44. Kim HS, Chon JW, Kim YJ, Kim DH, Kim M sang, Seo KH. Prevalence and characterization of extended-spectrum-beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in ready-to-eat vegetables. Int J Food Microbiol. Elsevier B.V.; 2015;207: 83–86. doi:10.1016/j.ijfoodmicro.2015.04.049
45. Woolhouse M, Ward M, van Bunnik B, Farrar J. Antimicrobial resistance in humans, livestock and the wider environment. Philos Trans R Soc Lond B Biol Sci. 2015;370: 20140083. doi:10.1098/rstb.2014.0083
46. Cohen Stuart J, van den Munckhof T, Voets G, Scharringa J, Fluit A, Hall ML Van. Comparison of ESBL contamination in organic and conventional retail chicken meat. Int J Food Microbiol. Elsevier B.V.; 2012;154: 212–214. doi:10.1016/j.ijfoodmicro.2011.12.034
47. Veldman K, Mevius D. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2017 (MARAN 2018) [Internet]. 2018. Available: https://www.wur.nl/en/show/Maran-rapport-2018.htm
48. Bos MEH, Taverne FJ, van Geijlswijk IM, Mouton JW, Mevius DJ, Heederik DJJ. Consumption of Antimicrobials in Pigs, Veal Calves, and Broilers in The Netherlands: Quantitative Results of Nationwide Collection of Data in 2011. PLoS One. 2013;8. doi:10.1371/journal.pone.0077525
49. Bos MEH, Mevius DJ, Wagenaar J a., van Geijlswijk IM, Mouton JW, Heederik DJJ. Antimicrobial prescription patterns of veterinarians: introduction of a benchmarking approach. J Antimicrob Chemother. 2015; 1–3. doi:10.1093/jac/dkv104
50. Avined. Antibioticumgebruik pluimveesector in 2017 [Internet]. 2018. Available: https://www.avined.nl/sites/www.avined.nl/files/antibioticagebruik_-_sectorrapportage_2017.pdf
51. European Centre for Disease Prevention and Control. Antimicrobial consumption in the EU / EEA, annual epidemiological report for 2018. Stockholm; 2019.
52. European Medicines Agency. Trends in the sales of veterinary antimicrobial agents in nine European countries. Report period 2005-2009. 2011; 77. Available: http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2011/09/news_detail_001336.jsp&murl=menus/news_and_events/news_and_events.jsp&mid=WC0b01ac058004d5c1
53. Bleker H, Schippers EI. Kamerbrief betreft: antibiotica in de veehouderij, 245727. 2011.
54. Speksnijder DC, Mevius DJ, Bruschke CJM, Wagenaar JA. Reduction of veterinary antimicrobial use in the Netherlands. The dutch success model. Zoonoses Public Health. 2015;62: 79–87. doi:10.1111/zph.12167
55. Veldman KT, Wit B, Franz E, Wullings B, Heederik D, Mevius DJ. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2019 (MARAN 2020) [Internet]. 2020. Available: https://www.rivm.nl/publicaties/nethmap-2020-consumption-of-antimicrobial-agents
56. Giani T, Arena F, Vaggelli G, Conte V, Chiarelli A, Henrici De Angelis L, et al. Large Nosocomial Outbreak of Colistin-Resistant, Carbapenemase-Producing Klebsiella pneumoniae Traced to Clonal Expansion of an mgrB Deletion Mutant. J Clin Microbiol. 2015;53: 3341–4. doi:10.1128/JCM.01017-15

57. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. Lancet Infect Dis. Elsevier Ltd; 2016;16: 161–168. doi:10.1016/S1473-3099(15)00424-7



CHAPTER 2

Decreasing prevalence of contamination with extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E) in retail chicken meat in the Netherlands

Pepijn Huizinga, Marjolein Kluytmans-van den Bergh, John W. Rossen, Ina Willemsen, Carlo Verhulst, Paul H.M. Savelkoul, Alexander W. Friedrich, Silvia García-Cobos, Jan Kluytmans

Adapted from: PLoS ONE 14(12): e0226828

ABSTRACT

Retail chicken meat is a potential source of extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E). In the past decade, vast national efforts were undertaken to decrease the antibiotic use in the veterinary sector, resulting in a 58% decrease in antibiotic sales in the sector between 2009 and 2014. This decrease in antibiotic use was followed by a decrease in ESBL-E prevalence in broilers. The current study investigates the prevalence of contamination with ESBL-E in retail chicken meat purchased in the Netherlands between December 2013 and August 2015. It looks at associations between the prevalence of contamination with ESBL-E and sample characteristics such as method of farming (free-range or conventional), supermarket chain of purchase and year of purchase.

In the current study, 352 chicken meat samples were investigated for the presence of ESBL-E using selective culture methods. Six samples were excluded due to missing isolates or problems obtaining a good quality sequence leaving 346 samples for further analyses. Of these 346 samples, 188 (54.3%) were positive for ESBL-E, yielding 216 ESBL-E isolates (*Escherichia coli* (n=204), *Klebsiella pneumoniae* (n=11) and *Escherichia fergusonii* (n=1)). All ESBL-E isolates were analysed using whole-genome sequencing. The prevalence of contamination with ESBL-E in retail chicken meat decreased from 68.3% in 2014 to 44.6% in 2015, absolute risk difference 23.7% (95% confidence interval (CI): 12.6% - 34.1%). The ESBL-E prevalence was lower in free-range chicken meat (36.4%) compared with conventional chicken meat (61.5%), absolute risk difference 25.2% (95% CI: 12.9% - 36.5%). The prevalence of contamination with ESBL-E varied between supermarket chains, the highest prevalence of contamination was found in supermarket chain 4 (76.5%) and the lowest in supermarket chain 1 (37.8%). Pairwise isolate comparisons using whole-genome multilocus sequence typing (wgMLST) showed that clustering of isolates occurs more frequently within supermarket chains than between supermarket chains. In conclusion, the prevalence of contamination with ESBL-E in retail chicken in the Netherlands decreased over time; nevertheless, it remains substantial and as such a potential source for ESBL-E in humans.

INTRODUCTION

Infections with extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E) are associated with substantial morbidity, mortality and increased costs compared to infections with their susceptible counterparts [1–4]. Carriage with ESBL-E is often a prerequisite and a predictor for infections with ESBL-E [5–7]. In 2015, about 5% of invasive *E. coli* isolates (blood- and cerebrospinal-fluid cultures) were resistant to third generation cephalosporins in the Netherlands [8]. This is lower than the European population-weighted mean of 13%. Nevertheless, it is more than a five-fold increase since the turn of the century [9].

Originally ESBL-E infections were mainly a hospital-related problem with acquisition in hospitals or related to healthcare contact. This has changed in the past two decades with people that have had no healthcare contact also being rectal carriers of ESBL-E [10,11]. Research efforts have focussed on uncovering routes of transmission and reservoirs of antimicrobial resistant microorganisms and resistance genes by using a one-health approach that includes humans, animals and the environment as an interconnected entity.

Contaminated food has been suggested as a potential source for ESBL-E. ESBL-E contamination rates up to 80% were reported for retail chicken meat in the Netherlands between 2008 and 2010 [12–14]. Exchange of bacteria or genetic material between animals and humans has been suggested, for instance between farmers and their animals, where the epidemiological link is relatively concrete [15]. Transfer of ESBL-E isolates or plasmids carrying resistance genes from bacteria on retail chicken meat to humans in the general community is more difficult to prove due to larger spatial-temporal differences. However, it was recently described that poultry meat can act as a vehicle for exposure and infection with a specific ST131 sublineage [16]. Overlap in genetic content between animal and human domains has clearly been shown, albeit without directionality of possible transmission [13,14,17–19].

Based on the hypothesis of spread of antimicrobial resistant bacteria from chicken meat to humans, the Dutch government set goals to decrease the antibiotic use in Dutch livestock. This initiative resulted in a decrease in antibiotic sales in veterinary medicine of 63.4% between 2009 and 2017 with little to no impact on the production or economic results in the sector [20,21]. Although a causal relation is difficult to prove, the decrease in antibiotic use was followed by a subsequent decrease in isolation of ESBL-E from livestock in the Netherlands. The level of cefotaxime resistance in randomly picked *E. coli* isolates from broiler faeces decreased from 15 – 20% in 2007 to

1.7% in 2017 [20]. The current study focusses on fresh retail chicken meat from common supermarket chains in the Netherlands. Poultry meat makes up 29% (22kg) of the total meat consumption of the average citizen of the Netherlands and more than half of this consists of chicken breast fillet [22].

The aim of this study is to describe the ESBL-E prevalence in Dutch retail chicken meat over time and in relation to the method of farming (free-range or conventional), and the supermarket chain where the meat was purchased. In addition, the genetic constitution of the isolated ESBL-E is described.

METHODS

Sample collection

A convenience sample of chicken meat samples were collected from December 2013 until October 2014 and will be referred to as "period 2014", and from June 2015 until August 2015 this will be referred to as "period 2015". Only unprocessed, raw, conventional or free-range farming chicken-breast fillet was used for this study. Organic chicken meat was not sampled for this study. Only one sample per supermarket chain per day with the same method of farming and/or batch number was included.

The following information was noted for each sample: date of purchase, best before date, supermarket chain and method of farming. Two supermarket chains, which were already part of the same group of supermarkets, merged during the study period and were analysed together as one supermarket chain as we assumed overlapping suppliers already before the official merger. The combined market share of the sampled supermarket chains in the Netherlands is around 70% [23].

The sample size of the second period was calculated after the first collection period. To detect a decline of 15% in ESBL-E prevalence with a power of 80% and an alpha of 0.05 with 142 samples in the first period and an ESBL-E prevalence of around 68% in that first period, 199 samples had to be collected in the second sampling period.

Microbiological methods

Twelve grams of chicken meat per sample was pre-enriched in 15mL tryptic soy broth (TSB). After overnight incubation, 100 µL of the TSB was transferred to 5mL of selective TSB, containing vancomycin (8 mg/L) and cefotaxime (0.25 mg/L) (TSB-VC). After a second overnight incubation, 10 µL of the TSB-VC was subcultured on an

ESBL screening agar, EbSA (AlphaOmega, 's-Gravenhage, the Netherlands), consisting of a split McConkey agar plate containing cloxacillin (400 mg/L), vancomycin (64 mg/L) and on one half cefotaxime (1 mg/L) and the other half ceftazidime (1 mg/L). Species identification (VITEK-MS, bioMérieux, Marcy l'Etoile, France) and antibiotic susceptibility testing (VITEK2, bioMérieux, Marcy l'Etoile, France) were performed for all oxidase-negative Gram-negative isolates that grew on the EbSA agar plate with different morphology. Minimal inhibitory concentrations (MIC) are given in mg/L. The production of ESBL was phenotypically confirmed with the combination disk diffusion method using cefotaxime (30 µg), ceftazidime (30 µg) and cefepime (30 µg) disks, with and without clavulanic acid (10 µg) (Rosco, Taastrup, Denmark). Test results were considered positive if the diameter of the inhibition zone was ≥5 mm larger for the disk with clavulanic acid as compared with the disk without clavulanic acid [24,25]. Antimicrobial susceptibility testing results were interpreted using EUCAST clinical breakpoints (v 7.1) [26].

Whole-genome sequencing and quality control

All isolates for which ESBL production was phenotypically confirmed were sequenced. Genomic DNA was prepared using the Nextera XT library preparation kit (Illumina, San Diego, United States). The libraries were sequenced on a MiSeq sequencer (Illumina, San Diego, United States) generating 250- to 300-bp paired-end reads using the MiSeq reagent kit v2 or v3 respectively. Quality trimming and *de novo* assembly was performed using CLC Genomics Workbench version 11.0 (Qiagen, Hilden, Germany) as previously described [27]. The following quality control parameters were considered to assess assembly quality: coverage ≥ 20; number of scaffolds ≤1000; N50 ≥ 15,000 bases and maximum scaffold length ≥ 50,000 bases. If one or more of the criteria was not met, the assembly was excluded from the analyses. In addition, isolates for which the genotypic genus identification did not match the phenotypic (MALDI-TOF) identification were excluded from the analysis.

Definition of ESBL-E positive samples and isolate selection

Samples were classified as ESBL-E positive when one or more isolates from a sample had a sequence satisfying the quality control criteria and an ESBL gene was located in the sequence data. Samples containing only isolates phenotypically suspected for ESBL production with a good quality sequence where no ESBL gene was identified were reported as ESBL-E negative. Samples were excluded when the only isolate from that sample was phenotypically suspected for ESBL production but sequence data did not satisfy the quality control criteria and hence, no conclusion could be drawn on the on the presence or absence of the ESBL gene.

If samples contained multiple isolates and these clustered according to whole genome multilocus sequence typing and the ESBL gene(s) were identical, only one of the isolates was kept for further analyses.

Bioinformatics analyses of whole genome sequence data: species identification, resistance gene detection, plasmid replicon detection, multilocus sequence typing (MLST) and whole-genome MLST (wgMLST)

Assembled genomes were analysed using the bacterial analysis pipeline-batch upload mode from Center for Genomic Epidemiology (accessed week 52 of 2017) (https://cge.cbs.dtu.dk/services/cge/, DTU, Copenhagen) with KmerFinder-2.1 for species identification, ResFinder-2.1 for detection of acquired resistance genes and PlasmidFinder-1.2 for detection of plasmid replicons [28–31]. If multiple plasmid replicons from the same family were detected in one isolate, the plasmid replicon family was counted once for that isolate.

MLST sequence type (ST)(Achtman) was determined using the bioinformatics tool "mlst" by T. Seemann v2.16.1 (https://github.com/tseemann/mlst) [32,33]. For *E. coli* isolates with unknown STs or problems in determining the ST, the raw FASTQ files were submitted to the EnteroBase website to assign new STs (https://enterobase.warwick.ac.uk/species/ecoli v1.1.2) [34].

The phylogroups as described by Clermont *et al* were determined using the ClermonTyping tool v1.0.0 (https://github.com/A-BN/ClermonTyping) which uses a method with different *in-silico* PCR assays and a method using the Mash genome clustering tool [35,36]. When discrepancies between the *in-silico* PCR assay method and the Mash genome-clustering tool method were observed, the phylogroup was reported as "undetermined". The ClermonTyping tool also discriminates between *E. coli* and *E. fergusonii* on the basis of the *citP* gene. If the ClermonTyper identified an isolate as *E. fergusonii* that was previously identified as an *E. coli (*MALDI-TOF and Kmer-Finder 1.2)*,* the species was changed to *E. fergusonii.*

wgMLST analysis was performed for all *E. coli* isolates using Ridom SeqSphere + v5.1.0 (Ridom, Münster, Germany) applying the *E. coli* scheme and clonality threshold according to Kluytmans-van den Bergh *et al.* [27]. Pairwise genetic distances were determined by calculating the proportion of allele differences between isolates. Only good targets present in both sequences were used, ignoring missing values. The threshold used for clonality for *E. coli* was 0.0095 [27]. As a sensitivity analysis the threshold for clonality was doubled to 0.019. Another option to make the criteria for clonality less stringent was taking the core-genome MLST scheme. It was

chosen to maximize discriminatory power and work with the originally proposed cut-off for the wgMLST scheme. A neighbour-joining tree was constructed in Ridom SeqSphere + v5.1.0 using the pairwise genetic distances and metadata were added in the webtool "Interactive Tree of Life" v4.4.2 (https://itol.embl.de)[37–39].

Statistical analyses

Confidence Intervals (CIs) of proportions were calculated using the adjusted Wald method [40]. All analyses on the ESBL-E prevalence data were performed using Statistical Package for Social Science software (IBM SPSS Statistics 25.0, Armonk, NY). Relative risks for ESBL-E contamination of meat samples were estimated using univariable and multivariable generalized linear models (GLM) with a Poisson distribution, log link and robust error estimation, with year of purchase, supermarket chain and method of farming as independent variables. Associations were measured using relative risks (RR) for a more appropriate interpretation, the high ESBL-E prevalence would lead to high odds ratios overestimating the actual RR [41–43].

Relative risks for clonality were estimated using univariable and multivariable GLM with a binomial distribution, a log link and robust error estimation, with time interval between dates of purchase, supermarket chain (within or between) and farming method (within or between) as independent variables. Due to the non-linear effect of time between isolates related to the frequency of clonality of the pairwise isolate comparisons, it was not suitable as a continuous variable in the logistic regression analyses. As such, time between isolates was taken as a categorical variable with three groups: 0 – 6 months, 6 – 12 months and >12 months. The categories were chosen to coincide with changing frequency of clonality, and as such were based on the observed results. As these choices were made with prior knowledge of the data, two alternative models were made excluding the time variable and using shorter time intervals in the first year.

Accession number

Raw sequencing reads were submitted to the European Nucleotide Archive of the European Bioinformatics Institute and are available under the study accession number PRJEB33495.

RESULTS

ESBL-E prevalence survey of retail chicken meat

Of 352 cultures of retail chicken meat six were excluded from further analyses, leaving 346 samples for further analyses, Figure 1. The number of samples taken per month, per supermarket chain and per method of farming is shown in S1 table. Of the 346 samples, 188 (54.3%) were positive for ESBL-E. Year of purchase, supermarket chain and method of farming were independently associated with the prevalence of contamination with ESBL-E, Table 1. The prevalence of contamination with ESBL-E decreased from 68.3% in the period 2014 to 44.6% in the period 2015, absolute risk difference 23.7% (95% CI: 12.6% - 34.1%) or adjusted relative risk of 0.69 (95% CI: 0.58 – 0.83) and is shown in more detail in S1 Figure. The prevalence of contamination with ESBL-E was lower in free-range chicken meat (36.4%) compared with conventional chicken meat (61.5%), absolute risk difference 25.2% (95% CI: 12.9% - 36.5%) or adjusted relative risk of 0.60 (95% CI 0.46 – 0.78), Table 1 and Figure 2. The prevalence of contamination with ESBL-E varied between supermarket chains; the highest ESBL-E prevalence was found in supermarket chain 4 (76.5%) and the lowest in supermarket chain 1 (37.8%).

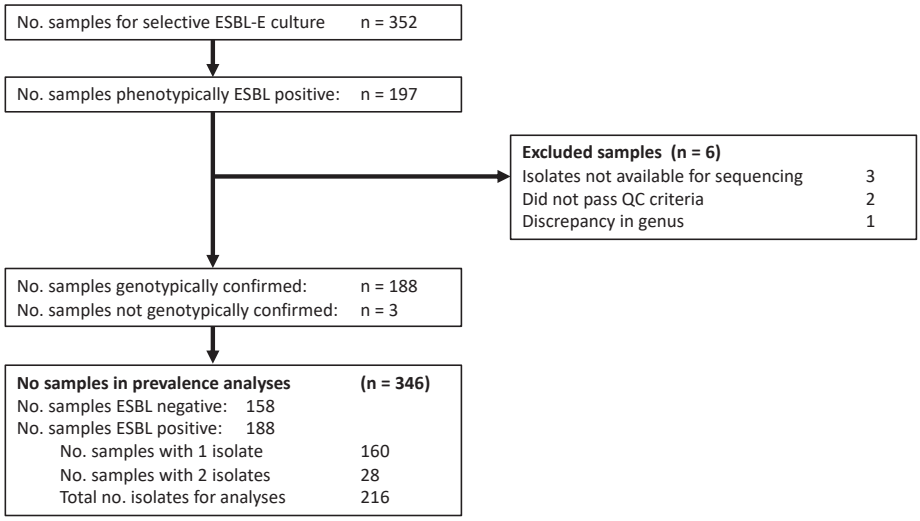


Figure 1. Flowchart showing the number of chicken meat samples in the study.

Phenotypic and genetic characterization of ESBL-E isolates from retail chicken meat

A total of 240 isolates were selected for sequencing, 24 were excluded from further analyses for the following reasons: they were not available for sequencing (n=4), the assembled genomes did not pass quality control requirements (n=4), a discrepancy in the genetically determined genus compared with the genus as determined with MALDI-TOF (n=1), they were clonal isolates compared with a second isolate from the same sample (n=8) and no ESBL gene was detected in the isolate (n=7). This resulted in 216 isolates from 346 cultured samples for further analyses: 204 (94.4%) were *E. coli*, 11 (5.1%) were *K. pneumoniae* and one (0.5%) isolate was *E. fergusonii.* Regarding antimicrobial resistance, 51 (23.6%) isolates were phenotypically resistant to ciprofloxacin, 108 (50.0%) to norfloxacin, 111 (51.4%) to trimethoprim-sulfamethoxazole, 12 (5.6%) to tobramycin, 14 (6.5%) to gentamicin, 1 (0.5%) to piperacillin-tazobactam, and 18 (8.3%) to amoxicillin-clavulanic acid. No isolates were phenotypically resistant to meropenem, imipenem or colistin.

Table 1. Prevalence of contamination with ESBL-E in retail chicken meat in the Netherlands according to year of purchase of the sample, supermarket chain of purchase and method of farming.

	Number of samples	ESBL-E Positive (n=188)		GLM - Poisson (REE) univariable			GLM - Poisson (REE) multivariable		
	n = 346	n	%	RR	95% CI		RR	95% CI	
Period of purchase									
2014	142	97	68.3	Ref			Ref		
2015	204	91	44.6	0.65	0.54	0.79	0.69	0.58	0.83
Method of farming									
Conventional	247	152	61.5	Ref			Ref		
Free range	99	36	36.4	0.59	0.45	0.78	0.60	0.46	0.78
Supermarket chain									
SC1	82	31	37.8	Ref			Ref		
SC2	83	37	44.6	1.18	0.82	1.70	1.22	0.87	1.73
SC3	100	58	58.0	1.53	1.11	2.12	1.41	1.04	1.91
SC4	81	62	76.5	2.03	1.50	2.74	2.12	1.60	2.81

Abbreviations: ESBL-E, extended-spectrum beta-lactamase-producing Enterobacteriaceae; GLM, generalized linear model; REE, robust error estimation; RR, relative risk; n, number; CI, confidence interval; Ref, reference

STs and phylogroups were determined for the 204 *E. coli* isolates. The most common STs were: ST117 (16.2%), ST10 (8.8%), ST602 (7.4%), ST88 (4.4%) and ST57 (3.9%), see Table 2. Frequencies of *E. coli* phylogroups were as follows: 61 (29.9%) isolates belonged to phylogroup A, 44 (21.6%) to B1, 41 (20.1%) to F, 17 (8.3%) to D, 16 (7.8%) to E, 13 (6.4%) to C, 2 (1.0%) to Clade I, 1 (0.5%) to B2 (non-ST131) and for 9 isolates (4.4%) the phylogroup was undetermined. Isolates within the same ST always had the same phylogroup, except for one isolate of ST10 where the phylogroup was undetermined. For all individual isolates the detected STs and corresponding phylogroups are given in S2 Table.

Table 2. Frequency distribution of the *E. coli* sequence types (ST) and the corresponding phylogroups cultured from retail chicken meat in the Netherlands. *13 STs were found in two isolates each and 38 STs were found only once.

Sequence Type	No. Isolates (n = 204)	(%)	Phylogroup
117	33	16.2	F
10	18	8.8	A
602	15	7.4	B1
88	9	4.4	C
57	8	3.9	E
58	6	2.9	B1
69	6	2.9	D
752	6	2.9	A
1158	4	2.0	Undetermined
1818	4	2.0	A
3778	4	2.0	F
38	4	2.0	D
665	4	2.0	A
115	3	1.5	D
155	3	1.5	B1
162	3	1.5	B1
189	3	1.5	A
5183	3	1.5	A
93	3	1.5	A
pairs*	26	12.7	-
singletons*	38	18.6	-
undetermined	1	0.5	E

Among *K. pneumoniae* isolates (n=11) the following STs were detected: ST231 (n=3, 27.3%), ST1530 (n=2, 18.2%) and one isolate (9.1%) of ST15, ST280, ST307, ST607, ST2176 and ST3161. The *E. fergusonii* isolate was determined as ST8330 with the *E. coli* scheme from Enterobase [**https://enterobase.warwick.ac.uk/species/ecoli**].

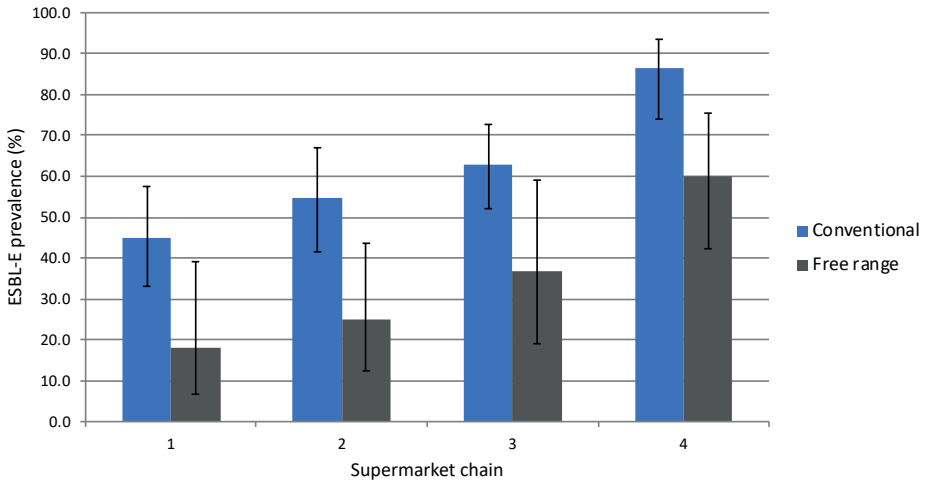


Figure 2. The prevalence of contamination with ESBL-E according to method of farming and supermarket chain. Error bars show the 95% confidence intervals.

A total of 220 ESBL genes were detected in 216 isolates. The most common ESBL genes were $bla_{\text{CTX-M-1}}$ (n=88, 40.0%) and $bla_{\text{SHV-12}}$ (n=70, 31.8%). The $bla_{\text{CTX-M-15}}$ gene was found in five isolates (2.3%). Four isolates (*E. coli* n=3 and *K. pneumoniae* n=1) contained more than one ESBL gene: two isolates with $bla_{\text{CTX-M-1}}$ and $bla_{\text{SHV-12}}$, one isolate with $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-2}}$ and one *K. pneumoniae* isolate contained $bla_{\text{TEM-52B}}$ and $bla_{\text{SHV-27}}$. The frequency distribution of all detected ESBL genes is given in Table 3.

Investigating all antimicrobial resistance genes from the ResFinder database resulted in hits with 62 different genes. For genes and percentage of isolates the genes were detected in, see S2 Figure. The most common antimicrobial resistance genes detected besides the aforementioned ESBL genes were: *sul*2 (n=119, 55.1%) and *sul*1 (n=62, 28.7%) conferring resistance to sulphonamides; *tet*(A) (n=101, 46.8%), conferring resistance to tetracyclines; *aad*A1 (n=81, 37,5%), conferring resistance to spectinomycin and streptomycin; and *str*A (n=66, 30,6%) and *str*B (n=65, 30,1%), conferring resistance to streptomycin. The presence or absence of all individual antimicrobial resistance genes for all individual isolates is shown in S2 Table.

IncFIB, Col, IncI and IncFII were the most abundant plasmid replicon families with a frequency of 80.1%, 77.3%, 69.0% and 55.6%, respectively. The detected plasmid replicon families and the number of isolates in which they were detected are shown in S3 Table.

Table 3. Frequency distribution of detected ESBL genes in ESBL-E isolates cultured from retail chicken meat in the Netherlands. 220 detected ESBL genes from 216 ESBL-E isolates.

ESBL gene	Frequency n=220 (%)	*E. coli*	*K. pneumoniae*	*E. fergusonii*
$bla_{CTX\text{-}M\text{-}1}$	88 (40.0)	88		
$bla_{SHV\text{-}12}$	70 (31.8)	70		
$bla_{TEM\text{-}52C}$	23 (10.5)	23		
$bla_{TEM\text{-}52B}$	18 (8.2)	16	1	1
$bla_{SHV\text{-}2}$	9 (4.1)	2	7	
$bla_{CTX\text{-}M\text{-}15}$	5 (2.3)	4	1	
$bla_{SHV\text{-}5}$	2 (0.9)		2	
$bla_{CTX\text{-}M\text{-}2}$	2 (0.9)	2		
$bla_{TEM\text{-}15}$	1 (0.5)	1		
$bla_{CTX\text{-}M\text{-}32}$	1 (0.5)	1		
$bla_{SHV\text{-}27}$	1 (0.5)		1	

Investigation of clonality using whole-genome MLST

Clonality within the *E. coli* isolates was investigated using wgMLST, a neighbour-joining tree of the data is shown in S3 Figure. A total of 20,706 pairwise comparisons were made of which 148 (0.7%) were within the threshold of clonality. As a sensitivity analysis the cut-off value for clonality of the wgMLST was doubled to 0.019, this increased the percentage of clonality from 0.7% to 0.8%.

Most of the isolates from which the pairwise isolate comparisons indicated clonality belonged to a limited number of sequence types (ST): ST602 (n=87, 58,8%), ST117 (n=24, 16,2%), ST10 (n=8, 5.4%), ST69 (n=6, 4,1%), ST57 (n=4, 2,7%) and ST1158, ST58 and ST88 (each with n=3, 2.0%). There were ten other pairs of clonally related isolates, all with their own sequence type; one in which the wgMLST clonally related pair of isolates consisted of two different conventional ST, ST45 and ST8567. The frequency of clonality within sequence types was 4.5% and 5.2% for ST117 and ST10, respectively, whereas it was 82.9% for ST602, S4 Table. The median number of days between time of purchase of the samples that the clonally related isolates were cultured from was longer in ST602 (median 94 days, range 0 – 226 days), compared to ST117 (median 6.5 days, range 0 – 346 days) and ST10 (median 8 days, range 0 – 21 days), see S4 Table.

The general trend in frequency of clonality shows a decrease in clonality with increasing time interval between isolates as is shown in Table 4 and in more detail in S4 Figure. However, the first months show an increase in clonality with up to five months between the isolates showing the highest rates of clonality. All clonally related isolates with 3 – 5 months between the isolates belong to ST602, see S5 Table for frequency of clonality per month per ST. No clonal relatedness was found in isolates more than 13 months apart. The frequency of clonality within supermarket chains was higher than the frequency of clonality between supermarket chains, see Table 4. This holds true with the exception of supermarket chain 3 and supermarket chain 4, for which the between supermarket chain frequency of clonality was higher than most other within supermarket chain comparisons. See Table 4 for all individual supermarket chain comparisons. No effect on the frequency of clonality within or between methods of farming was observed. In the multivariable analyses within supermarket chain comparisons were twice as likely to be clonally related compared with between supermarket chain comparisons, adjusted RR of 2.0 with 95% CI 1.5 – 2.8. As time intervals were chosen with knowledge of the data, different models were tested, see S6 Table. Decreased time intervals of four months between the isolates in the first year showed higher clonality with 5 – 8 months between isolates compared to 1-4 months between the isolates. This was however followed by the expected decrease in clonality. Also, the time component was removed from the multivariable analyses. These changes to the model had little impact on the point estimates for supermarket chain or method of farming.

Table 4. Frequency of clonality of the pairwise isolate comparisons and the univariable and multivariable regression analyses on the different epidemiological relations.

	No. clonally related comparisons	No. comparisons	% clonally related
Time between isolates			
0 - 6 months	123	8973	1.37
6 - 12 months	24	6144	0.39
> 12 months	1	5589	0.02
Method of farming			
Between	35	6308	0.55
Within	113	14398	0.78
Supermarket chain			
Between	83	15161	0.55
Within	65	5545	1.17
Individual supermarket chain comparisons			
SC3	29	1891	1.53
SC3/SC4	57	4278	1.33
SC4	26	2346	1.11
SC1	5	528	0.95
SC2	5	780	0.64
SC2/SC3	10	2480	0.40
SC2/SC4	9	2760	0.33
SC1/SC2	2	1320	0.15
SC1/SC4	3	2277	0.13
SC1/SC3	2	2046	0.10

Abbreviations: No., number of; GLM, generalized linear model; REE, robust error estimation; RR, relative risk; ARR, adjusted relative risk; CI, confidence interval.

	GLM – binomial (REE) univariable		GLM – binomial (REE) multivariable	
	RR	95% CI	ARR	95% CI
	ref		ref	
	0.29	0.18 – 0.44	0.29	0.19 – 0.45
	0.01	0.00 – 0.09	0.01	0.00 – 0.10
	ref		ref	
	1.41	0.97 – 2.06	1.40	0.96 – 2.03
	ref		ref	
	2.14	1.55 – 2.96	2.02	1.47 – 2.79

DISCUSSION

In the current study the prevalence of contamination with ESBL-E in retail chicken meat was investigated over a period of two years in the Netherlands. First, a decrease in prevalence of contamination with ESBL-E was seen over time. Second, the method of farming was associated with the prevalence of contamination with ESBL-E; free-range chicken meat had a lower ESBL-E prevalence compared with conventional chicken meat. Third, the ESBL-E prevalence in retail chicken meat differed between supermarket chains. These three factors were all independently associated with the prevalence of contamination with ESBL-E in a multivariable model.

Two datasets have been described in peer-reviewed literature on the presence of ESBL-E in retail chicken meat in the Netherlands. Cohen Stuart *et al.* and Leverstein van Hall *et al.* reported an ESBL-E prevalence of 94% (tested samples: 98) in chicken meat purchased in 2010 [12,13]; and Overdevest *et al.* reported a prevalence of 79.9% (tested samples: 89) in randomly chosen packages of retail chicken meat purchased in 2009 [14]. Yearly updates on antimicrobial use and resistance data in the veterinary sector are published in the Netherlands in the "Monitoring of Antimicrobial Resistance and Antibiotic usage in Animals in the Netherlands (MARAN)" reports [20,44–46]. These reports also describe the ESBL- and/or AmpC- (ESBL/AmpC) producing Enterobacteriaceae prevalence in retail chicken meat. The reported results by MARAN are not directly comparable with the current study as the culture methods are different. Also, besides ESBL-E, AmpC-producing Enterobacteriaceae are included in the reported numbers. Despite these differences, the decrease in ESBL-E prevalence in retail chicken meat is similar in the MARAN reports compared to the current study. Confirmed ESBL/AmpC-producing Enterobacteriaceae were present in 73% and 83% of tested samples in 2012 and 2013, respectively [46,47]. This was followed by a decrease in 2014 and 2015, with the lowest prevalence (24%) of ESBL/AmpC producing *E. coli* reported in fresh chicken meat in 2016; which increased again in 2017 to 31.6% [20,44]. A decreasing ESBL-E prevalence was also reported from broiler faeces, both in selective cultures for ESBL/AmpC producing *E. coli* and in the proportion of cefotaxime resistance in non-selectively cultured *E. coli* isolates [44].

Different articles have reported on the effect of farming practices on antimicrobial resistant microorganisms in meat products. Cohen Stuart *et al.* found high ESBL-E prevalence both in conventional, 100% (95%CI 92.8% - 100.0%) and organic chicken meat, 81.6% (95%CI 66.3 – 91.1%) [12]. In a study by Miranda *et al.* that looked at resistance rates to eight different types of antibiotics in randomly picked *E. coli* isolates, the resistance rates were higher in conventional chicken meat compared

to organic chicken meat [48]. Looking at resistance rates in randomly selected *E. coli* isolates to 12 types of antibiotics, under which three cephalosporin's, Davis *et al.* found differences in resistance rates in turkey meat with different antibiotic use claims, but found that in chicken meat the brand of the meat had a larger effect than the antibiotic use claim [49]. The current study finds effects of both the supermarket chain and the method of farming used. Free-range chickens receive less antibiotics compared with conventionally farmed chickens, which could be a factor related to this observed difference [50].

Comparing the ESBL-E genes detected on retail chicken meat from the current study with previously published data shows broadly similar results with $bla_{CTX\text{-}M\text{-}1}$ being the dominant gene [18,51]. Other genes frequently present are $bla_{SHV\text{-}12}$, $bla_{TEM\text{-}52B}$ and $bla_{TEM\text{-}52C}$. In the current study the frequency of $bla_{SHV\text{-}12}$ is higher compared to the numbers found in retail chicken meat as described in the aforementioned study [18,51]. This may be due to differences in culture techniques (MARAN does not use selective plates with ceftazidime in addition to selective plates with cefotaxime) or to differences in sampling, for instance from a supermarket chain not included in the current study. Another difference is the high frequency of $bla_{CTX\text{-}M\text{-}2}$ in meat samples in 2014 described by MARAN, which in that report was comparable to the frequency of $bla_{CTX\text{-}M\text{-}1}$ [45]. In the same year the current study did not detect any $bla_{CTX\text{-}M\text{-}2}$ and it was only sporadically detected in June 2015. We currently have no explanation for this difference. The $bla_{CTX\text{-}M\text{-}15}$ gene, which is the most frequently detected ESBL gene in human infections in the Netherlands, was detected in 2.3% of the isolates [14,18,52].

The most abundant STs from the current study, ST117 and ST10 are in concordance with previously published data from chicken meat in the Netherlands. [13,14] The third most common sequence type, ST602 has not been described in Dutch poultry to the best of our knowledge, but has frequently been described in poultry in other countries such as Sweden, Japan, England and Tunisia[18,53–56].

Clonal relatedness of the *E. coli* isolates from the current study was investigated using a cut-off for clonal relatedness that was set to determine clonal spread within a hospital setting in a timeframe of 30 days [27]. The current study has a different setting, with potential epidemiological relations more distant compared to that for which the cut-off was set, thus less stringent cut-off values were considered. Doubling the cut-off value for the wgMLST only had a small effect on the frequency of clonality. As such, the original cut-off value was used.

We were surprised by the relation of time between the isolates and the frequency of clonality. We expected a decrease over time, but found an increase in frequency of clonality up to five months between the isolates. After this increase in frequency of clonality it declines rapidly with the maximum time between clonally related isolates being 13 months. The increase in the frequency of clonality in the first months is almost solely caused by ST602. This highly clonal cluster stands out and the clonally related isolates have a longer median time between isolates compared to other clusters (ST10 and ST117). A possible explanation could be relatively low genetic variability in the sequence type. However, continued introduction to the food chain from a point source or temporary storage of a contaminated batch are other possibilities to explain the observation. Different options to cope with this time observation were tried in multivariable models that also looked at the effect of the supermarket chain and the method of farming on the frequency of clonality. In the different models the effect sizes of the latter two factors remained stable but the effect size of time between the isolates fluctuated with the different categorical options for time. We believe the key message on time between the isolates and clonality is that almost no clonality is seen in samples more than 12 months apart.

The second message from the clonality analysis is that isolates are twice as likely to be clonally related when the isolates are from within one supermarket chain, compared to isolates from different supermarket chains. This may be explained by overlapping production chains that give rise to more epidemiological relations between the isolates. Such relations could be isolates from chicken meat from the same farm, or possible contaminations from a common source in the processing of the meat. The higher frequency of clonality between supermarket chain 3 and supermarket chain 4 suggests a common source somewhere in the production chains. Clustering isolates closely matched in time could be due to batch contamination during processing of the meat, transmission between chickens or the chickens acquired the isolates from a common source. Clonal isolates cultured from samples collected months apart could have a wide range of possible sources of contamination. We could not verify hypotheses of where contamination or transfer may have occurred, as the production chain of the individual chicken meat samples was not accessible to us. However, combining this type of high-resolution typing data with precise knowledge of the flow of the products through the production chain and the possibility to go back and sample through that production chain could create the possibility to eliminate steps where contamination of meat products occurs.

Strengths of the study are the focus on a specific and frequently used product, raw chicken breast fillet. Choosing one specific type of product allowed investigations into differences between free-range and conventional chicken meat and differences between supermarket chains. Carefully performed sampling, including only one

sample per supermarket chain per day (or with different batch numbers), to minimize possible effects of batch contaminations on the prevalence of contamination with ESBL-E and relative gene abundance. A selective pre-enrichment step and a well-tested ESBL-E screening agar was used to ensure a high sensitivity in detecting ESBL-E in the samples [57,58]. The use of WGS enabled molecular detection of all currently known genes responsible for an ESBL phenotype. It also allowed for genetic screening of resistance genes other than ESBL, ST identification and phylotyping. In addition, WGS will allow future genetic evaluations as time passes and future comparisons with other strain collections.

The current study gives a precise genetic overview of the ESBL genes and isolates found in chicken breast fillet, a broader selection of chicken products may have increased the variability of the gene content. Secondly, although care was taken during sampling to obtain a good representation of chicken breast fillet over time, it would have been preferable to have had a more continuous sampling strategy instead of periods with higher intensity sampling and different periods, including the last four months of 2015, with no samples being taken. A third limitation of the study is the fact that no quantitative cultures were performed on the chicken meat. Therefore, we cannot conclude on the bacterial (ESBL-E) load per sample over time. A final point of caution is the fact that the prevalence of ESBL-E has been known to vary over time and the timeframe in the current study is relatively short. However, the measured decrease in ESBL-E prevalence is considerable and the prevalence of ESBL/AmpC-producing Enterobacteriaceae has been shown to remain low. The MARAN reports show rates of contamination of retail chicken meat of 24% and 31.6% in 2016 and 2017 respectively which supports that our findings indicate a sustainable reduction of ESBL-E in retail chicken meat [20,44].

Chicken meat is a frequently consumed product and is known to often be contaminated with ESBL-E. Combining these facts, retail chicken meat is a potential source of ESBL-E for humans. Better understanding of factors that describe the prevalence of contamination with ESBL-E creates opportunities for concrete control measures and allows for a more in-depth analysis of production chains. What also makes the data from the current study relevant is that much effort was made to decrease antibiotic use in veterinary sector starting from 2009. The total decrease in antibiotic sales for the complete veterinary sector was 58% from 2009 to 2014 [20]. The antibiotic sales were relatively stable during the time frame of the sample collection for the current study [20]. Although it is interesting that in the years after a large decrease in antibiotic use in the veterinary sector the prevalence of contamination with ESBL-E on retail chicken meat subsequently decreased, no conclusions on the possible causality

of these observations can be drawn. The study design was not intended to look at this relation and there are too many unknown factors that could also influence the ESBL-E prevalence on retail chicken meat such as changes in the slaughter process, changes in packaging practices and differences in the origin of the meat sold in the supermarkets.

Concluding, the current study describes a decreasing prevalence of contamination with ESBL-E in retail chicken meat in the Netherlands from December 2013 until August 2015. The prevalence of contamination with ESBL-E was lower in free-range chicken meat compared with conventional chicken meat and also varied between supermarket chains. In pairwise isolate comparisons, clustering occurs more often within supermarket chains than between supermarket chains and clustering was not found in isolates cultured more than 13 months apart.

FUNDING

Part of the sequence data were generated and reused from the Dutch Food & Nutrition Delta Programme 2013 project PathoDetect.

This study was also partially funded by the Amphia Hospital and the medical specialist company Amphia (MSB-A), Breda, the Netherlands.

This study was also partly supported by the Interreg IVa-funded projects “EurSafety Health-Net - EURegional network for patient safety and protection against infections” and “SafeGuard”, as part of a Dutch–German cross-border network supported by the European Commission, German Ministry of Economic Affairs, Energy and Industry of the State of North Rhine- Westphalia, and German Ministry for Economic Affairs, Labour, Transport and Digitalisation of the State of Lower Saxony, and the Dutch Provinces of Overijssel, Gelderland, and Limburg.

ACKNOWLEDGMENTS

This publication made use of the PubMLST website (https://pubmlst.org/) developed by Keith Jolley and sited at the University of Oxford [59]. The development of that website was funded by the Wellcome Trust.

REFERENCES

1. Melzer M, Petersen I. Mortality following bacteraemic infection caused by extended spectrum beta-lactamase (ESBL) producing E. coli compared to non-ESBL producing E. coli. J Infect. 2007;55: 254–259. doi:10.1016/j.jinf.2007.04.007
2. Rottier WC, Ammerlaan HSM, Bonten MJM. Effects of confounders and intermediates on the association of bacteraemia caused by extended-spectrum b -lactamase-producing Enterobacteriaceae and patient outcome : a meta-analysis. 2012; 1311–1320. doi:10.1093/jac/dks065
3. Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect Dis. 2018;3099: 1–11. doi:10.1016/S1473-3099(18)30605-4
4. Tumbarello M, Spanu T, Di Bidino R, Marchetti M, Ruggeri M, Trecarichi EM, et al. Costs of bloodstream infections caused by Escherichia coli and influence of extended-spectrum-beta-lactamase production and inadequate initial antibiotic therapy. Antimicrob Agents Chemother. 2010;54: 4085–4091. doi:10.1128/AAC.00143-10
5. Rottier WC, van Werkhoven CH, Bamberg YRP, Dorigo-Zetsma JW, van de Garde EM, van Hees BC, et al. Development of diagnostic prediction tools for bacteraemia caused by third-generation cephalosporin-resistant enterobacteria in suspected bacterial infections: a nested case-control study. Clin Microbiol Infect. Elsevier Ltd; 2018;24: 1315–1321. doi:10.1016/j.cmi.2018.03.023
6. Rottier WC, Bamberg YRP, Dorigo-Zetsma JW, van der Linden PD, Ammerlaan HSM, Bonten MJM. Predictive value of prior colonization and antibiotic use for third-generation cephalosporin-resistant enterobacteriaceae bacteremia in patients with sepsis. Clin Infect Dis. 2015;60: 1622–30. doi:10.1093/cid/civ121
7. Denis B, Lafaurie M, Donay J-L, Fontaine J-P, Oksenhendler E, Raffoux E, et al. Prevalence, risk factors, and impact on clinical outcome of extended-spectrum beta-lactamase-producing Escherichia coli bacteraemia: a five-year study. Int J Infect Dis. 2015;39: 1–6. doi:10.1016/j.ijid.2015.07.010
8. European Centre for Disease Prevention and Control (ECDC). Antimicrobial resistance surveillance in Europe 2014. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm: ECDC; 2015 [Internet]. 2015. doi:10.2900/23549
9. Stobberingh EE, Arends J, Hoogkamp-Korstanje JAA, Goessens WHF, Visser MR, Buiting AGM, et al. Occurrence of extended-spectrum beta-lactamases (ESBL) in Dutch hospitals. Infection. 1999;27: 348–354. doi:10.1007/s150100050041
10. Livermore DM, Canton R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, et al. CTX-M: Changing the face of ESBLs in Europe [Internet]. Journal of Antimicrobial Chemotherapy. 2007. pp. 165–174. doi:10.1093/jac/dkl483
11. Bunt van den G, Pelt van W, Hidalgo L, Scharringa J, Greeff SC de, Schürch AC, et al. Prevalence, risk factors and genetic characterization of Extended-Spectrum Beta-Lactamase and Carbapenemase-producing Enterobacteriaceae (ESBL-E and CPE): a community-based repeated cross-sectional study in the Netherlands from 2014 to 2016. Eurosurveillance (forthcoming). 2019; doi:eurosurveillance-D-18-00594R1

12. Cohen Stuart J, van den Munckhof T, Voets G, Scharringa J, Fluit A, Hall ML Van. Comparison of ESBL contamination in organic and conventional retail chicken meat. Int J Food Microbiol. Elsevier B.V.; 2012;154: 212–214. doi:10.1016/j.ijfoodmicro.2011.12.034
13. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Voets GM, van den Munckhof MP, van Essen-Zandbergen A, et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect. 2011;17: 873–880. doi:10.1111/j.1469-0691.2011.03497.x
14. Overdevest I, Willemsen I, Rijnsburger M, Eustace A, Xu L, Hawkey P, et al. Extended-spectrum β-lactamase genes of Escherichia coli in chicken meat and humans, the Netherlands. Emerg Infect Dis. 2011;17: 1216–1222. doi:10.3201/eid1707.110209
15. Dierikx C, van der Goot J, Fabri T, van Essen-Zandbergen A, Smith H, Mevius D. Extended-spectrum-β-lactamase- and AmpC-β-lactamase-producing Escherichia coli in Dutch broilers and broiler farmers. J Antimicrob Chemother. 2013;68: 60–67. doi:10.1093/jac/dks349
16. Liu CM, Stegger M, Aziz M, Johnson TJ, Waits K, Nordstrom L, et al. Escherichia coli ST131-H22 as a Foodborne Uropathogen. MBio. 2018;9: 1–11. doi:10.1128/mBio.00470-18
17. Muloi D, Ward MJ, Pedersen AB, Fèvre EM, Woolhouse MEJ, van Bunnik BAD. Are Food Animals Responsible for Transfer of Antimicrobial-Resistant Escherichia coli or Their Resistance Determinants to Human Populations? A Systematic Review. Foodborne Pathog Dis. 2018;15: 467–474. doi:10.1089/fpd.2017.2411
18. Dorado-García A, Smid JH, van Pelt W, Bonten MJM, Fluit AC, van den Bunt G, et al. Molecular relatedness of ESBL/AmpC-producing Escherichia coli from humans, animals, food and the environment: a pooled analysis. J Antimicrob Chemother. 2018;73: 339–347. doi:10.1093/jac/dkx397
19. Kluytmans JAJW, Overdevest ITMA, Willemsen I, Bergh MFQK Den, Zwaluw K Van Der, Heck M, et al. Extended-Spectrum β -Lactamase – Producing Escherichia coli From Retail Chicken Meat and Humans : Comparison of Strains , Plasmids , Resistance Genes , and Virulence Factors. 2013;56: 478–487. doi:10.1093/cid/cis929
20. Veldman K, Mevius D. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2017 (MARAN 2018) [Internet]. 2018. Available: https://www.wur.nl/en/show/Maran-rapport-2018.htm
21. Bergevoet R, van Asseldonk M, Bondt N, van Horne P, Hoste R, de Lauwere C, et al. Economics of antibiotic usage on Dutch farms - The impact of antibiotic reduction on economic results of pig and broiler farms in the Netherlands [Internet]. 2019. Available: https://edepot.wur.nl/475403
22. Terluin I, Verhoog D, Dagevos H, van Horne P, Hoste R. Vleesconsumptie per hoofd van de bevolking in Nederland, 2005-2016 [Internet]. Wageningen; 2017. doi:10.18174/424550
23. Nielsen. Markets and Finances. In: Markets and Finances [Internet]. 2017 [cited 14 Oct 2019]. Available: https://www.nielsen.com/nl/nl/insights/article/2017/nielsen-release-market-shares/
24. Netherlands Society for Medical Microbiology. NVMM Guideline Laboratory detection of highly resistant microorganisms, version 2.0, 2012. Leeuwarden, Netherlands. 2012; Available: http://www.nvmm.nl/system/files/2012.11.15 richtlijn BRMO %2528version 2.0%2529 - RICHTLIJN.pdf

25. European Committee on Antimicrobial Susceptibility Testing. Guidelines for detection of resistance mechanisms and specific resistance of clinical and/or epidemiological importance. Version 2.0 [Internet]. 2017. Available: www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_170711.pdf
26. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 7.1 [Internet]. 2017. Available: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf
27. Kluytmans-van den Bergh MFQ, Rossen JWA, Bruijning-Verhagen PCJ, Bonten MJM, Friedrich AW, Vandenbroucke-Grauls CMJE, et al. Whole genome multilocus sequence typing of extended-spectrum beta-lactamase-producing Enterobacteriaceae. J Clin Microbiol. 2016;54: 2919–2927. doi:10.1128/JCM.01648-16
28. Larsen M V., Cosentino S, Lukjancenko O, Saputra D, Rasmussen S, Hasman H, et al. Benchmarking of methods for genomic taxonomy. J Clin Microbiol. 2014;52: 1529–1539. doi:10.1128/JCM.02981-13
29. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012;67: 2640–2644. doi:10.1093/jac/dks261
30. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother. 2014;58: 3895–903. doi:10.1128/AAC.02412-14
31. Thomsen MCF, Ahrenfeldt J, Cisneros JLB, Jurtz V, Larsen MV, Hasman H, et al. A Bacterial Analysis Platform: An Integrated System for Analysing Bacterial Whole Genome Sequencing Data for Clinical Diagnostics and Surveillance. PLoS One. 2016;11: e0157718. doi:10.1371/journal.pone.0157718
32. Seemann T. mlst. In: https://github.com/tseemann/mlst.
33. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in Escherichia coli: An evolutionary perspective. Mol Microbiol. 2006;60: 1136–1151. doi:10.1111/j.1365-2958.2006.05172.x
34. Alikhan NF, Zhou Z, Sergeant MJ, Achtman M. A genomic overview of the population structure of Salmonella. PLoS Genet. 2018;14: 1–13. doi:10.1371/journal.pgen.1007261
35. Beghain J, Bridier-Nahmias A, Le Nagard H, Denamur E, Clermont O. ClermonTyping: an easy-to-use and accurate in silico method for Escherichia genus strain phylotyping. Microb genomics. 2018;4. doi:10.1099/mgen.0.000192
36. Clermont O, Christenson JK, Denamur E, Gordon DM. The Clermont Escherichia coli phylo-typing method revisited: Improvement of specificity and detection of new phylo-groups. Environ Microbiol Rep. 2013; doi:10.1111/1758-2229.12019
37. Letunic I, Bork P. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics. 2007;23: 127–8. doi:10.1093/bioinformatics/btl529
38. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. Oxford University Press; 2019;47: W256–W259. doi:10.1093/nar/gkz239
39. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4: 406–25. Available: http://www.ncbi.nlm.nih.gov/pubmed/3447015
40. Agresti A, Coull BA. Approximate is better than "Exact" for interval estimation of binomial proportions. Am Stat. 1998;52: 119–126. doi:10.1080/00031305.1998.10480550

41. Knol MJ, Le Cessie S, Algra A, Vandenbroucke JP, Groenwold RHH. Overestimation of risk ratios by odds ratios in trials and cohort studies: Alternatives to logistic regression. Cmaj. 2012;184: 895–899. doi:10.1503/cmaj.101715
42. Zhang J, Yu KF. What's the relative risk? A method of correcting the odds ratio in cohort studies of common outcomes. JAMA. 1998;280: 1690–1. Available: http://www.ncbi.nlm.nih.gov/pubmed/9832001
43. Schmidt CO, Kohlmann T. When to use the odds ratio or the relative risk? Relative Risk and odds ratio in epidemiology. Int J Public Heal. 2008;5308: 165–167. doi:10.1007/s000
44. Veldman K, Mevius D. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2016 (MARAN 2017) [Internet]. 2017. Available: https://www.wur.nl/upload_mm/c/8/4/50343a1f-a2ad-4389-8208-d0e595b9a946_Maran report 2017.pdf
45. Veldman K, Dierikx C, Mevius D. Monitoring of antimicrobial resistance and antibiotic asage in animals in the Netherlands in 2014 (MARAN 2015) [Internet]. 2015. Available: https://www.wur.nl/upload_mm/1/3/3/53df1fe1-ca3d-4050-a4ad-a1fde4ed158b_NethmapMaran2015.pdf
46. Mevius D, Dierikx C, Wit B, van Pelt W, Heederik D. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2013 (MARAN 2014) [Internet]. 2014. Available: https://www.wur.nl/upload_mm/4/a/4/46c626bf-5ccb-4996-a294-3e9ef4843355_NethMap-MARAN2014.pdf
47. Veldman K, Kant A, Dierikx C, van Essen-Zandbergen A, Wit B, Mevius D. Enterobacteriaceae resistant to third-generation cephalosporins and quinolones in fresh culinary herbs imported from Southeast Asia. Int J Food Microbiol. Elsevier B.V.; 2014;177: 72–77. doi:10.1016/j.ijfoodmicro.2014.02.014
48. Miranda JM, Guarddon M, Vázquez BI, Fente CA, Barros-Velázquez J, Cepeda A, et al. Antimicrobial resistance in Enterobacteriaceae strains isolated from organic chicken, conventional chicken and conventional turkey meat: A comparative survey. Food Control. 2008;19: 412–416. doi:10.1016/j.foodcont.2007.05.002
49. Davis GS, Waits K, Nordstrom L, Grande H, Weaver B, Papp K, et al. Antibiotic-resistant Escherichia coli from retail poultry meat with different antibiotic use claims. BMC Microbiol. 2018;18: 174. doi:10.1186/s12866-018-1322-5
50. Ellen H, Leenstra F, van Emous R, Groenestein K, van Ham J, van Horne P, et al. Vleeskuikenproductiesystemen in Nederland [Internet]. Livestock Research. Lelystad, the Netherland; 2012. Available: https://www.wageningenur.nl/nl/Publicatie-details.htm?publicationId=publication-way-343237323531
51. Ceccarelli D, Kant A, van Essen-Zandbergen A, Dierikx C, Hordijk J, Wit B, et al. Diversity of Plasmids and Genes Encoding Resistance to Extended Spectrum Cephalosporins in Commensal Escherichia coli From Dutch Livestock in 2007–2017. Front Microbiol. 2019;10: 1–9. doi:10.3389/fmicb.2019.00076
52. Van Der Bij AK, Peirano G, Goessens WHF, Van Der Vorm ER, Van Westreenen M, Pitout JDD. Clinical and molecular characteristics of extended-spectrum-beta-lactamase-producing Escherichia coli causing bacteremia in the Rotterdam Area, Netherlands. Antimicrob Agents Chemother. 2011;55: 3576–3578. doi:10.1128/AAC.00074-11
53. Börjesson S, Bengtsson B, Jernberg C, Englund S. Spread of extended-spectrum beta-lactamase producing Escherichia coli isolates in Swedish broilers mediated by an incl plasmid carrying bla(CTX-M-1). Acta Vet Scand. 2013;55: 3. doi:10.1186/1751-0147-55-3

54. Ludden C, Moradigaravand D, Jamrozy D, Gouliouris T, Blane B, Naydenova P, et al. A One Health study of the genetic relatedness of Klebsiella pneumoniae and their mobile elements in the East of England. Clin Infect Dis. 2019; 1–28. doi:10.1093/cid/ciz174
55. Hayashi W, Ohsaki Y, Taniguchi Y, Koide S, Kawamura K, Suzuki M, et al. High prevalence of blaCTX-M-14among genetically diverse Escherichia coli recovered from retail raw chicken meat portions in Japan. Int J Food Microbiol. Elsevier; 2018;284: 98–104. doi:10.1016/j.ijfoodmicro.2018.08.003
56. Jouini A, Slama K Ben, Klibi N, Sallem R Ben, Estepa V, Vinué L, et al. Lineages and virulence gene content among extended-spectrum β-lactamase-producing Escherichia coli strains of food origin in Tunisia. J Food Prot. 2013;76: 323–7. doi:10.4315/0362-028X.JFP-12-251
57. Kluytmans-van den Bergh MFQ, Verhulst C, Willemsen LE, Verkade E, Bonten MJM, Kluytmans JAJW. Rectal Carriage of Extended-Spectrum-Beta-Lactamase-Producing Enterobacteriaceae in Hospitalized Patients: Selective Preenrichment Increases Yield of Screening. J Clin Microbiol. 2015;53: 2709–12. doi:10.1128/JCM.01251-15
58. Overdevest ITMA, Willemsen I, Elberts S, Verhulst C, Kluytmans JAJW. Laboratory detection of extended-spectrum-beta-lactamase-producing Enterobacteriaceae: evaluation of two screening agar plates and two confirmation techniques. J Clin Microbiol. 2011;49: 519–22. doi:10.1128/JCM.01953-10
59. Jolley KA, Maiden MCJ. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics. 2010;11: 595. doi:10.1186/1471-2105-11-595

SUPPLEMENTARY MATERIALS

S1 Table. Number of samples taken per month, per supermarket chain and per method of farming.

		SC1		SC2	
Period	Month	Conv	Free	Conv	Free
Period 14	1			3	
	2	8	3	1	5
	3				
	4				
	5			2	1
	6	2		2	
	7	2	1	3	3
	8	3		4	1
	9	7	2	5	1
	10	2	1		
Period 15	18	1		7	2
	19	19	8	28	15
	20	16	7		
	Total period 14	24	7	20	11
	Total period 15	36	15	35	17
	Total period 14 + 15	60	22	55	28
	Total per SC	82		83	

Abbreviation: SC, supermarket Chain; Conv, conventional; Free, free range

SC3		SC4		
Conv	Free	Conv	Free	Samples per month
5		2		10
8		1	2	28
1				1
3				3
3		1	1	8
6		3		13
		2		11
12	1	4	2	27
6		6	4	31
3	1	2	1	10
6	2	2	1	21
27	13	22	16	148
1	2	6	3	35
47	2	21	10	142
34	17	30	20	204
81	19	51	30	346
100		81		

S2 Table. Table showing species, multilocus sequence type, *E. coli* phylogroup, day of sampling after start of study, anonymized supermarket chain and the presence and or absence of the different resistance genes and results of phenotypic antimicrobial susceptibility testing for all isolates in the study. Download table online: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0226828

S3 Table. Detected plasmid replicon families and the number of ESBL-E isolates from retail chicken meat they were detected in.

Plasmid	Frequency n=974	Prevalence in ESBL-E isolates (%)
IncFIB	173	80.1
Col	167	77.3
IncI1	149	69.0
IncFII	120	55.6
p0111	68	31.5
IncFIC(FII)	63	29.2
IncB/O/K/Z	45	20.8
IncX1	38	17.6
IncFIA	26	12.0
IncX3	21	9.7
IncI2	19	8.8
IncQ1	13	6.0
IncX4	13	6.0
IncY	10	4.6
IncA/C2	8	3.7
IncHI1B	8	3.7
IncHI2	7	3.2
IncHI2A	7	3.2
TrfA	7	3.2
IncR	5	2.3
IncHI1A	4	1.9
IncN	3	1.4

S4 Table. Frequency of clonality within the three most common multilocus sequence types with the median, minimum and maximum time between isolates for related and unrelated isolates within the ST.

	No. Comparisons	Clonally related	% Clonally related	Clonally related comparisons			Non-clonally related comparisons		
ST				Med t	Min t	Max t	Med t	Min t	Max t
ST 117	528	24	4.5	6.5	0	346	276	0	535
ST 10	153	8	5.2	8	0	21	286	1	558
ST 602	105	87	82.9	94	0	226	374	0	530

Abbreviations: ST, sequence type; No, number of; t, time between isolates; med, median; min, minimum; max, maximum

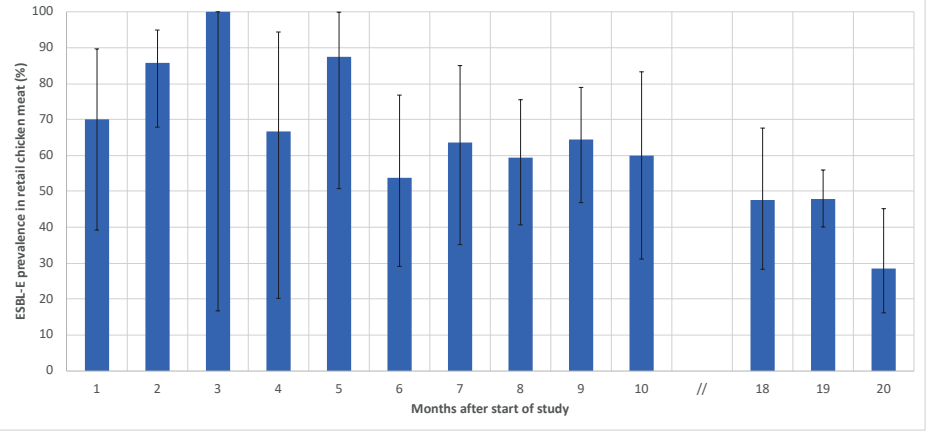
S5 Table. Number of clonally related isolate comparisons per sequence type (ST) with increasing time between isolates, in months. Within the pairwise comparisons all ST were the same except for an isolate with ST43 which was clonally related to an isolate of ST8567.

	Months between isolates													
ST	1	2	3	4	5	6	7	8	9	10	11	12	13	Total
602	16	15	10	9	17	8		12						87
117	17	1								1	2	3		24
10	8													8
69	6													6
57	2											2		4
1158	1						2							3
58	1	2												3
88	3													3
1072	1													1
1304													1	1
1818	1													1
354		1												1
3778						1								1
43 - 8567*								1						1
4663	1													1
48	1													1
5183	1													1
752											1			1
Total	59	19	10	9	17	9	2	13	0	1	3	5	1	148

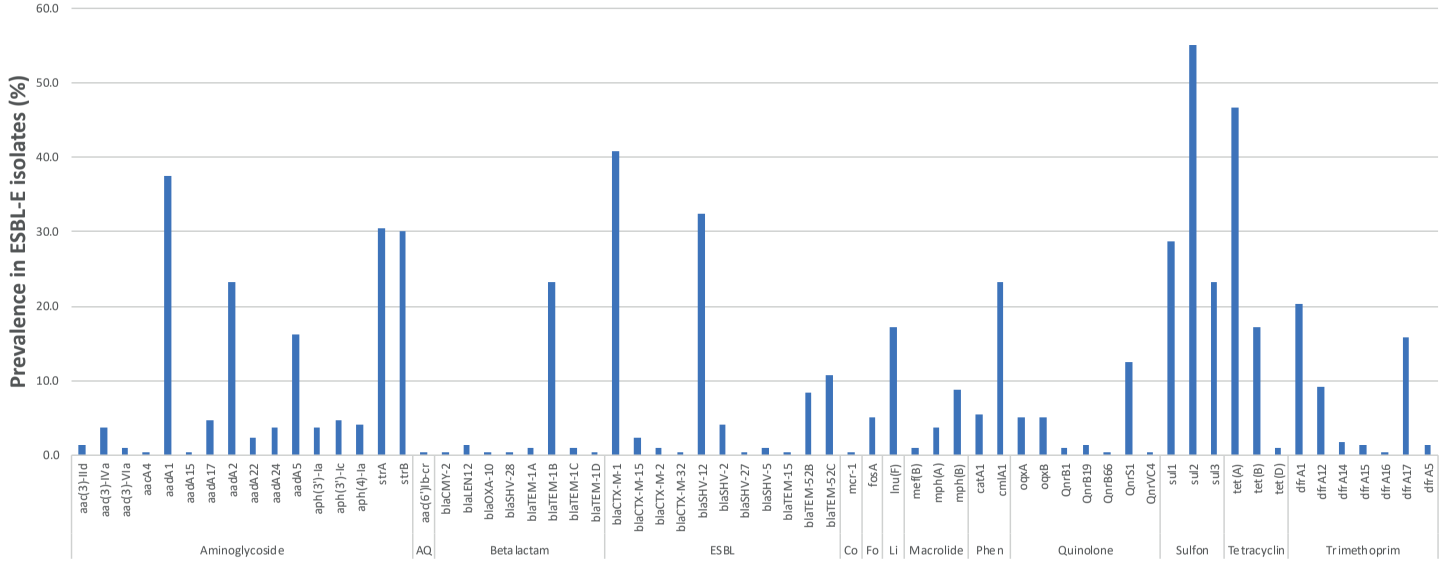
S6 Table. Alternate multivariable models of frequency of clonality using wgMLST, excluding the time variable in alternative model 1 and using shorter time periods in the first year in alternative model 2.

	Generalized linear model – Binomial – Robust error estimation			
	Alternate multivariable model 1		Alternate multivariable model 2	
	ARR	95% CI	ARR	95% CI
Method of farming				
Between	ref		ref	
Within	1.41	0.97 - 2.05	1.34	0.92 - 1.95
Supermarket chain				
Between	ref			
Within	2.14	1.55 - 2.95	2.04	1.48 - 2.81
Time between isolates				
Month 1-4			ref	
Month 5-8			1.61	1.10 - 2.38
Month 9-12			0.73	0.42 - 1.25
Months >=12			0.08	0.04 - 0.16

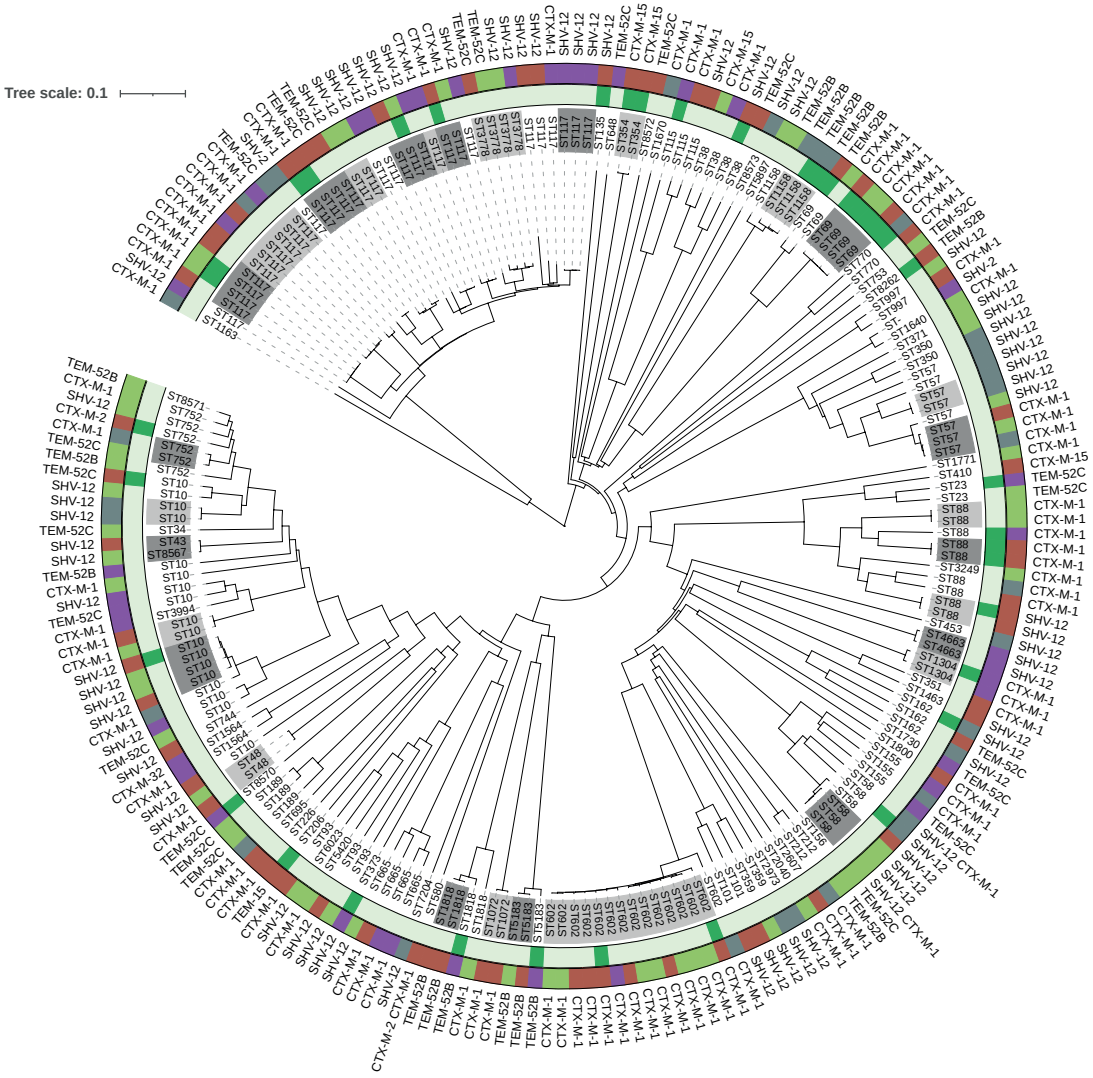
Abbreviations: ARR, adjusted relative risk; CI, confidence interval.



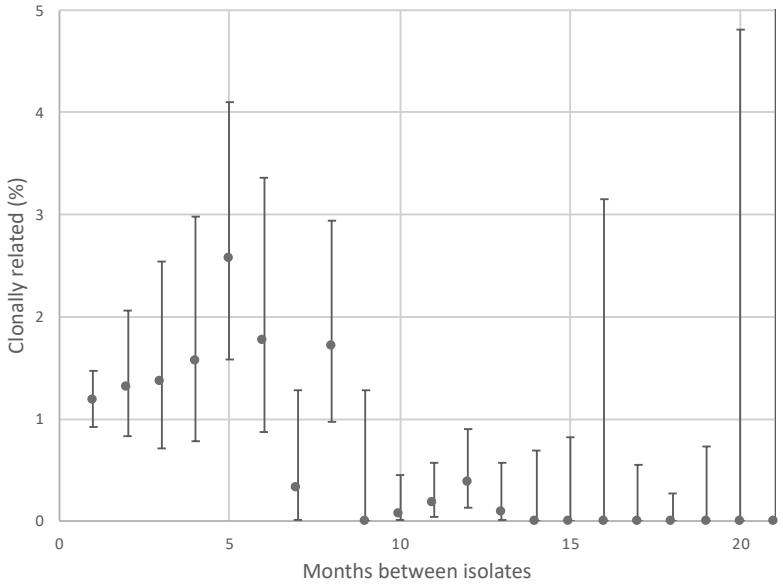
S1 Figure. Prevalence of contamination with ESBL-E in retail chicken meat in the Netherlands after start of the study in December 2013. X-axis shows time in months after start of the study, error bars show 95% confidence intervals.



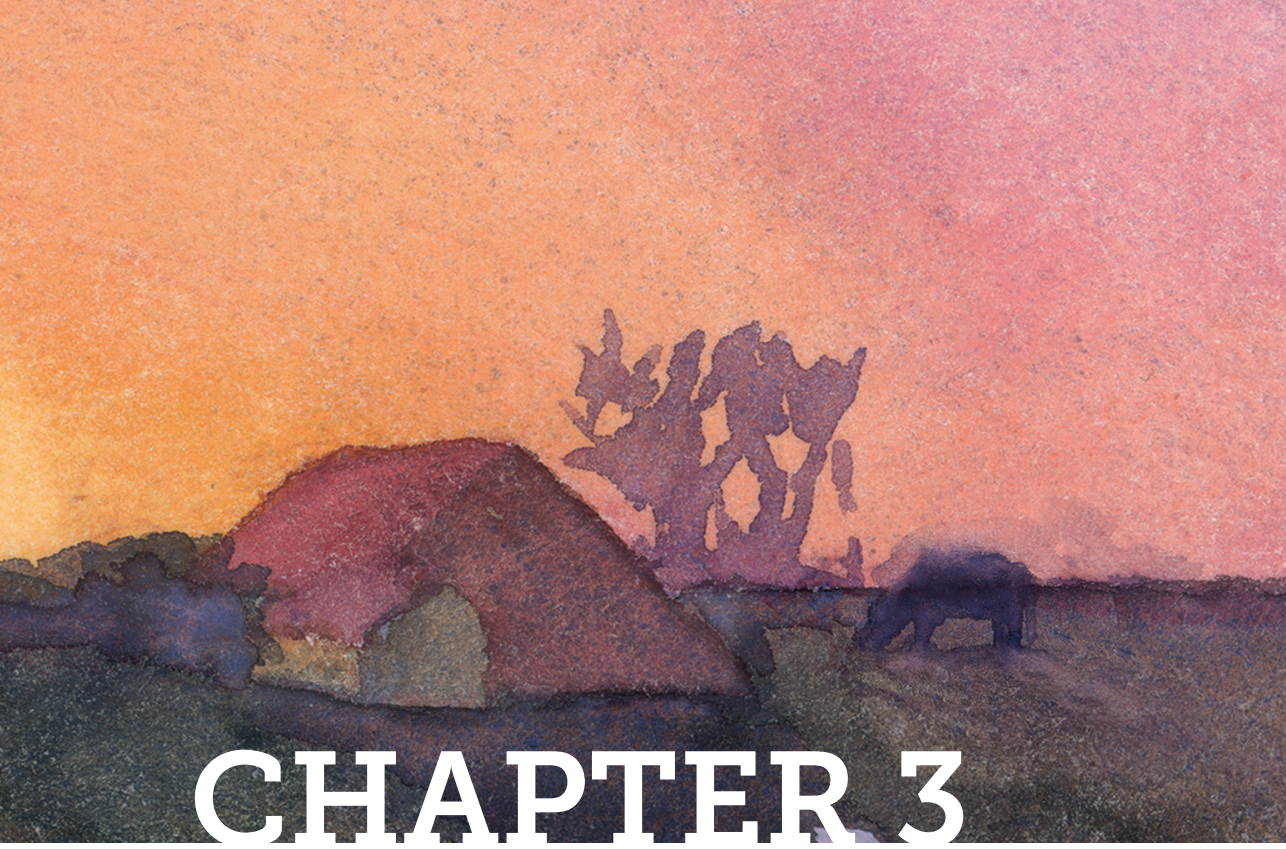
S2 Figure. Prevalence of ESBL genes and other genes associated with antimicrobial resistance in ESBL-E isolates cultured from retail chicken meat. Abbreviations: Sulfon, sulfonamide; Phen, phenicol; Li, lincosamide; Fo, fosfomycin; ESBL, extended-spectrum beta-lactamase; AQ, aminoglycoside and quinolone.



S3 Figure. Neighbour-joining tree based on the wgMLST analysis of 204 ESBL-producing *E. coli* isolates cultured from retail chicken meat in the Netherlands, using a "pairwise ignore missing values" approach. Legend, circles from inside out: conventional sequence type; shading in light or dark grey of the sequence type indicates clustering in whole genome multilocus sequence typing analyses; method of farming, light green is conventional and dark green is free range; supermarket chains: red SC4, light-green SC3, purple SC2, grey-cyan SC1; the outer most ring shows the detected ESBL genes in each isolate.



S4 Figure. Frequency of clonality with increasing time between the samples from which the isolates were cultured.



CHAPTER 3

Extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E) isolated from bean sprouts in the Netherlands

Pepijn Huizinga, Eefje Schrauwen, Silvia García-Cobos, Ina Willemsen, Carlo Verhulst, Alexander W. Friedrich, Paul H.M. Savelkoul, John W. Rossen, Jan Kluytmans

Adapted from: PLoS ONE 2019; 14(12): e0226828

ABSTRACT

Community-acquired carriage and infections due to extended-spectrum beta-lactamase producing *Enterobacteriaceae* (ESBL-E) are increasing worldwide, resulting in increased morbidity, mortality and healthcare costs. The origins of community-acquired ESBL-E carriage and infections remain unclear. Bean sprouts are a potential source of *Enterobacteriaceae* for the community, as illustrated by outbreaks of pathogenic *Enterobacteriaceae* in the past. The current study focuses on contamination of retail bean sprouts with ESBL-E in the Netherlands. Of 131 bean sprout samples purchased between 2013 and 2016, 25 (19%) were contaminated with ESBL-E. The detected isolates were almost exclusively *Klebsiella spp.* and co-resistance to other antibiotics was observed frequently. Over time there was substantial genetic diversity between isolates. On the other hand, isolates from samples closely matched in time were frequently clonally related, indicative of batch contamination. Remarkably, no *Escherichia coli* was found. In conclusion, bean sprouts frequently harbor ESBL-E, which is a potential source for consumers.

INTRODUCTION

Over the past 15 years human carriage and infections due to antimicrobial resistant *Enterobacteriaceae* have increased substantially, and concomitantly the impact on morbidity, mortality and healthcare costs are rising [1–4]. Infections caused by extended-spectrum beta-lactamase producing *Enterobacteriaceae* (ESBL-E) originally were a hospital related problem, however, this has shifted to a largely community-acquired problem [5]. The reservoirs and transmission routes of community acquired ESBL-E are poorly understood and seem to be a multifactorial problem.

Risk factors for ESBL-E carriage can be classified as intrinsic and extrinsic. Intrinsic risk factors decrease the natural barriers of the body, such as decreased gastric acid production caused by proton-pump inhibitors or decreased colonization resistance due to antibiotic use [6–8]. Extrinsic risk factors largely entail the frequency and intensity of contact with ESBL-E. Travel to high endemic areas and contact with ESBL-E positive family members and pets are typical examples [9–12]. Many food items have been shown to contain ESBL-E and as such are potential sources for acquisition of ESBL-E by humans. In recent years meat has gained much interest as a potential source, but a large variety of food items are contaminated with ESBL-E, including vegetables and drinking water [13–20]. One study from the Netherlands and one from South-Korea reported ESBL-E on bean sprouts, among other vegetables [19,21]. This is relevant as bean sprouts are often consumed raw, and as such have a higher risk of transmission than food items that are cooked before consumption [22]. It has been shown in the past that bean sprouts carry the potential to be the source of large-scale community outbreaks with pathogenic *Enterobacteriaceae*, as was the case with *E. coli* O104:H4 causing hemolytic-uremic syndrome in Germany in 2011 [23,24]. The aim of the current study is to investigate to what extend bean sprouts in the Netherlands are contaminated with ESBL-E.

MATERIALS AND METHODS

Study design

Bean sprout samples were purchased from supermarkets and grocery stores (including: ethnic markets and green grocers) in the Netherlands from December 2013 until January 2016. For the ESBL-E prevalence survey a maximum of one bean sprout sample per store per day was included. For all the samples, the following variables were noted: store of purchase, date of purchase and if available the expiration date. Besides the samples for the prevalence survey, extra samples per store per day were

obtained in the first sampling period (2013-2014) to determine the presence of batch contamination. Isolates from the additional samples were used only to determine the presence of batch contamination and were excluded from the other analyses.

Microbiological methods

Per sample, twelve grams of bean sprouts were enriched in 15 mL tryptic soy broth (TSB). After overnight incubation, 100 µL of the TSB was transferred to a selective TSB, containing cefotaxime (0.25 mg/L) and vancomycin (8 mg/L) (TSB-VC). After overnight incubation, 10 µL of the TSB-VC was subcultured on an ESBL screening agar, EbSA (AlphaOmega, 's-Gravenhage, the Netherlands), consisting of a split McConkey agar plate containing cloxacillin (400 mg/L), vancomycin (64 mg/L) and either cefotaxime or ceftazidime (1 mg/L). Species identification (VITEK-MS, bioMérieux, Marcy l'Etoile, France) and antibiotic susceptibility testing (VITEK2, bioMérieux, Marcy l'Etoile, France) were performed for all oxidase-negative Gram-negative isolates that grew on the EbSA. Minimal inhibitory concentrations (MIC) are given in mg/L. The production of ESBL was phenotypically confirmed with the combination disk diffusion method for cefotaxime (30 µg), ceftazidime (30 µg) and cefepime (30 µg). All with and without clavulanic acid (10 µg) (Rosco, Taastrup, Denmark). Test results were considered positive if the diameter of the inhibition zone was ≥5 mm larger for the disk with clavulanic acid as compared to the disk without clavulanic acid [25,26]. For interpretation of the phenotypic susceptibility testing EUCAST clinical breakpoints – bacteria (v 7.1) was used [27].

Whole Genome Sequencing (WGS), genome assembly and quality control (QC)

Phenotypically confirmed ESBL-E isolates were sequenced on a 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 [28]. As quality control parameters, the following criteria were used: coverage: ≥ 30; number of scaffolds: ≤1000; N50: ≥ 15,000 bases and maximum scaffold length: ≥ 50,000 bases.

Analyses of WGS data: species determination, resistance gene detection, Multi Locus Sequence Typing (MLST) and whole-genome MLST (wgMLST)

Assembled genomes were analyzed using an open access bioinformatics web tool (https://cge.cbs.dtu.dk/services/cge/, DTU, Copenhagen). This was done with ResFinder for analyses of resistance genes, PlasmidFinder for plasmid replicons and MLST 1.8 for MLST [29–31]. The services are combined in the bacterial analysis pipeline – batch upload mode [32]. This analysis pipeline also incorporates species determination with KmerFinder [33]. In case of conflicting results between the phenotypical MALDI-TOF and genetic KmerFinder 2.0, final species determination was based on the *rpoB* sequence [34].

wgMLST was performed using Ridom SeqSphere+, version 3.4.0 (Ridom, Münster, Germany). The species specific wgMLST typing schemes used in this study (*K. pneumoniae* and *K. oxytoca)* are described by Kluytmans – van den Bergh et al [28]. The pairwise genetic difference between isolates was calculated by dividing the 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. Species-specific thresholds for relatedness were used [28]. Using pairwise comparisons, a distance matrix was built. The relatedness of the isolates was inferred using the Neighbor-Joining method [35]. The Neighbor-Joining trees were constructed using MEGA6 [36].

Statistical analyses

Data were analyzed using Statistical Package for Social Science software (IBM SPSS Statistics 24.0, Armonk, NY). To test for differences in ESBL-E prevalence between supermarket chains and grocery stores and between the different supermarket chains, the Fischer exact test was used. As a measure of diversity between the isolates the Simpson Diversity Index (SID) was calculated based on MLST [37,38]. Confidence intervals of percentages were calculated with GraphPad QuickCalcs (GraphPad Software, La Jolla, California).

Accession number

Raw sequencing reads were submitted to the European Nucleotide Archive of the European Bioinformatics Institute and are available under the study accession number PRJEB25080.

RESULTS

ESBL-E prevalence in bean sprouts

A total of 131 bean sprout samples were tested for the presence of ESBL-E of which 25 (19.1%) tested positive (Table 1). The ESBL-E prevalence varied depending on the store of purchase. Between supermarket chains the largest difference in ESBL-E prevalence was between chains three and four, with a prevalence of 45.0% and 4.8%, respectively. In general, samples from supermarkets were more frequently contaminated with ESBL-E than samples from grocery stores (ESBL-E prevalence of 25.3% and 5.0% respectively, $p = 0.007$).

Table 1. ESBL-E prevalence in bean sprout samples in the Netherlands, 2013 – 2016.

	No. samples	ESBL-E positive (%)	95% CI	P
Prevalence survey samples	131	25 (19.1)	13.2 - 26.7	
Store of purchase (n=131)				0.013
Supermarket chain 1	23	5 (21.7)	9.2 - 42.3	
Supermarket chain 2	23	7 (30.4)	15.4 - 51.1	
Supermarket chain 3	20	9 (45.0)	25.8 - 65.8	
Supermarket chain 4	21	1 (4.8)	<0.01 - 24.4	
Supermarket chain 5	4	1 (25.0)	3.4 - 71.1	
Grocery store 1	10	0 (0)	0.00 - 32.1	
Grocery store 2	10	1 (10)	<0.01 - 42.6	
Grocery store 3	10	1 (10)	<0.01 - 42.6	
Grocery store 4	10	0 (0)	0.00 - 32.1	
Supermarket (n=131)				0.007
Yes	91	23 (25.3)	17.4 - 35.1	
No	40	2 (5)	0.5 - 17.4	

CI confidence interval, P p-value of the Fischer exact test.

K. pneumoniae was the predominant species (n = 21, 80.8%), followed by *K. oxytoca* (n = 3, 11.5%) and *K. variicola* (n = 1, 3.8%). One sample contained an ESBL-producing *E. cloacae* (3.8%) besides an ESBL-producing *K. pneumoniae*. ESBL-producing *E. coli* was not found.

Results of antimicrobial-susceptibility testing are shown in Tables 2 and 3. Besides the ESBL phenotype, high rates of resistance were found against ciprofloxacin (69.2%), trimethoprim-sulfamethoxazole (80.8%) and tobramycin (84.6%). Combined resistance against these three antibiotics was present in 50.0% of the isolates. Resistance against piperacillin-tazobactam was found in two of 26 isolates (7.7%). All isolates were susceptible to meropenem and colistin.

Genetic characteristics of ESBL-E isolated from bean sprouts

Quality control results and recoded file names to access files from ENA are displayed in Tables S1 and S2 respectively. All of the phenotypically confirmed ESBL-E isolates contained at least one ESBL gene (Table 2). The following ESBL genes were detected: in 9 (34.6%) isolates the bla_{SHV-2} gene, in 5 (19.2%) the $bla_{CTX-M-3}$ gene, in 4 (15.4%) both the bla_{SHV-27} gene and $bla_{CTX-M-3}$ gene, in 3 (11.5%) the $bla_{CTX-M-14}$ gene, in 3 (11.5%) the $bla_{CTX-M-27}$ gene and in 2 (7.7%) the $bla_{CTX-M-15}$ gene. In two isolates containing the $bla_{CTX-M-3}$ gene, the

bla$_{SHV-99}$ gene was also detected, with one mismatching nucleotide. The most frequently detected plasmid replicons as reported by PlasmidFinder 1.2 were IncFIA, IncFIB, IncFII, Col and IncR (Table 2). Most of the plasmid replicons called by PlasmidFinder were variants on the genes in the database [31]. MLST results of the isolates are shown in Table 2. Unknown MLST types were submitted to the corresponding databases. Three new MLST types were added for *K. pneumoniae* (ST2657, ST2658 and ST2659) and two were added for *K. oxytoca* (ST195 and ST196). The Simpson index of diversity (1-D) based on MLST was 0.96, 95% CI 0.92 – 1.00, demonstrating a high diversity between the isolates. Genes associated with resistance to aminoglycosides, quinolones, trimethoprim, sulphonamides, tetracyclines and macrolides were frequently present (Table 3).

wgMLST

The genetic relatedness of the 21 *K. pneumoniae* isolates from the prevalence survey is shown in Figure 1. Isolates were either clonally related or had large genetic diversity. The median genetic distance of *K. pneumoniae* isolate-to-isolate comparisons was 0.0002; range, 0.0000 – 0.0016 for clonally related isolates (n = 11), and 0.8524; range 0.1082 – 0.8721 for non-clonally related isolates (n = 199). Four clusters were identified; one cluster consisted of four isolates, one cluster contained three isolates and two clusters contained two isolates. Isolates within clusters came from samples that had expiration dates closely matched in time. The longest time between expiration dates within a cluster was 18 days. Clustering isolates were detected in samples purchased from different supermarkets. wgMLST analysis of the three *K.* oxytoca isolates from the prevalence study revealed no clonal relatedness. The smallest genetic distance was between isolates 29 and 33, which was 0.71. The genetic distance between isolates 29 and 32 and between 32 and 33 were both 0.99.

Batch contamination of bean sprout samples

In the period from December 2013 until March 2014 additional samples were purchased from different supermarkets, to investigate the occurrence of batch contamination with ESBL-E. Twenty-seven samples, coming from seven batches; three batches consisted of two samples, three batches consisted of five samples and one batch consisted of six samples. Analyzing the samples in a batch-by-batch manner shows three separate batch contamination events: two batches with *K.* pneumoniae (batch F and G) and one with *K. oxytoca* (batch F; Table 4). When comparing the 10 *K. pneumoniae* isolates from the batch contamination experiment with each other using wgMLST, contamination of five samples with one clone was found (Figure 2). This cluster occurred over a time period of thirteen days and was spread over two supermarket chains.

Table 2. Susceptibility profiles to different beta-lactams, detected extended-spectrum beta-lactamase (ESBL) genes, plasmid replicons and multi-locus sequence types (MLST) of ESBL-producing *Enterobacteriaceae* isolates from bean sprouts in the Netherlands.

Species ID	**AMC**	**TZP**	**CTX**	**CAZ**	**ESBL genes**
K. pneumoniae					
13	8	≤4	4	≤1	blaCTX-M-14
5	16	8	8	2	blaCTX-M-3
19	4	≤4	≤1	≤1	blaSHV-2
16	8	≤4	8	≤1	blaCTX-M-14
6	16	32	≥64	4	blaSHV-2
20	16	16	8	2	blaCTX-M-3, blaSHV-99[a]
21	16	8	8	2	blaCTX-M-3, blaSHV-99[a]
18	16	8	32	≤1	blaSHV-2
14	8	8	16	16	blaCTX-M-27
15	8	8	≥64	16	blaCTX-M-27
17	16	8	8	2	blaSHV-2
8	8	≤4	≤1	≤1	blaSHV-2
7,9	4	≤4	≤1	≤1	blaSHV-2
11	16	≤4	8	≤1	blaSHV-2
12	8	≤4	≥64	8	blaCTX-M-15
10	8	8	2	2	blaSHV-2
4	16	8	≥64	≤1	blaCTX-M-3, blaSHV-27
1,2,3	16	≤4	8	≤1	blaCTX-M-3, blaSHV-27
K. oxytoca					
29	8	≤4	8	≤1	blaCTX-M-14
33	8	≤4	4	≤1	blaCTX-M-3
32	8	≤4	≥64	4	blaCTX-M-27
K. variicola					
34	8	≤4	8	≤1	blaCTX-M-3
E. cloacae					
31	≥32	8	≥64	4	blaCTX-M-15

[a]ESBL genes called with a less than 100% identity and or length less than 100%. Minimum identity and length for call: 90.00% and 60% respectively. All plasmid replicons called by PlasmidFinder in default settings were reported. Shading within table indicates susceptibility interpretation according to EUCAST breakpoint table version 7.1, dark grey: resistant,

	Plasmid replicons	MLST
	IncFII,IncFIB(K),ColRNAI	ST-1296
	IncFII(K)	ST-1565
	IncFIA(HI1),IncFIB(K), IncHI1B,ColRNAI	ST-2176
	IncFII,IncFIB(K),ColRNAI	ST-2657
	IncFIA(HI1),IncR	ST-2658
	IncFIA(HI1),IncFIB(pKPHS1),IncFIB(K),IncFII(K),IncR,IncQ1,ColRNAI	ST-2659
	IncFIA(HI1),IncFIB(pKPHS1),IncFIB(K),IncFII(K),IncR,IncQ1,ColRNAI	ST-2659
	IncFIA(HI1),IncR	ST-280
	IncFIA(HI1),IncFIB(K)	ST-37
	IncFIA(HI1),IncFIB(K)	ST-37
	IncFIA(HI1),IncFII, IncFIB(K)	ST-39
	IncFiA(HI1),IncR,ColRNAI	ST-392
	IncFiA(HI1),IncR,ColRNAI	ST-392
	IncFIA(HI1),IncR,ColRNAI	ST-45
	IncFIB(K)	ST-45
	IncFIA(HI1),Col(BS512),IncR, Col(MG828),ColRNAI	ST-485
	IncFII(K),IncQ1	ST-661
	IncFII(K),IncQ1	ST-661
	IncFIA(HI1), IncFIB(pKPHS1),IncN	ST-195
	IncN,IncU	ST-196
	IncFIA(HI1),IncR	ST-2
	IncN2,IncFIB(K),ColRNAI	ST-1142
	IncFII(pECLA),IncFIB(pENTE01), IncFIB(pECLA),ColRNAI	ST-144

light grey: intermediate and white: susceptible. ID isolate identification number, AMC amoxicillin-clavulanic acid, TZP piperacillin-tazobactam, CTX cefotaxim, CAZ ceftazidime, ESBL extended-spectrum beta-lactamase, MLST multilocus sequencing typing, ST sequence type.

Table 3. Detected genes associated with resistance to different classes of antibiotics and phenotypic susceptibility profiles to most of these classes of antibiotics.

Species ID	**TOB**	**CIP**	**TMP**	**SXT**	**Aminoglycoside**[ǂ]
K. pneumoniae					
13	8	0.5	≥16	≥320	aac(3)-IId[a],strA,strB
5	8	≥4	≥16	≥320	aadA16[a],aac(6')Ib-cr
19	8	1	≤0.5	≤20	aac(3)-IId[a],strA,strB
16	8	0.5	≥16	≤20	aac(3)-IId[a]
6	8	≥4	≥16	≥320	aac(3)-IId[a],aadA16[a], strA[a],strB[a], aac(6')Ib-cr
20,21	8	≥4	≥16	≥320	aac(3)-IId[a],aadA16[a], aph(3')-Ia[a],strA,strB, aac(6')Ib-cr
18	8	0.5	8	40	aac(3)-IId[a],aadA16[a], strA[a],strB[a],aacA4[a], aac(6')Ib-cr[a]
14	2	≥4	≥16	≥320	aadA16[a],aac(6')Ib-cr
15	4	≥4	≥16	≥320	aadA16[a],aac(6')Ib-cr
17	8	0.5	≥16	≥320	aac(3)-IId[a], strA[a],strB[a]
7,8,9	8	≥4	≥16	≥320	aac(3)-IId[a],aadA16[a], aac(6')Ib-cr
11	8	≤0.25	≤0.5	≤20	aac(3)-IId[a], strA[a],strB[a]
12	≤1	1	≥16	≥320	strA,strB
10	8	≥4	≥16	≥320	aac(3)-IId[a],aadA16[a], aac(6')Ib-cr
4	8	2	≥16	≥320	aac(3)-IId[a],aadA16[a], aph(3')-Ia[a],strA,strB, aac(6')Ib-cr
1,2,3	8	2	≥16	≥320	aac(3)-IId[a],aadA16[a], aph(3')-Ia[a],strA,strB, aac(6')Ib-cr
K. oxytoca					
29	≤1	≤0.25	≤0.5	≤20	aph(3')-Ia[a]
33	8	0.5	≥16	≥320	strA[a],strB[a],aacA4, aac(6')Ib-cr[a]
32	8	≥4	≥16	≥320	aac(3)-IId[a],aadA16[a], aac(6')Ib-cr
K. variicola					
34	≤1	≤0.25	≤0.5	≤20	
E. cloacae					
31	8	0.5	≥16	≥320	aac(3)-IIa[a], aadA1[a],strA,strB, aac(6')Ib-cr

[a]Genes called with a less than 100% identity and or length less than 100%. Minimum identity and length for call: 90.00% and 60% respectively. ǂ aac(6')Ib-cr confers resistance to aminoglycosides and quinolones. Shading within table indicates susceptibility interpretation according to EUCAST breakpoint table version 7.1, dark grey: resistant,

Quinolone	TMP	SUL	TET	MAC
oqxA[a],oqxB[a], QnrS1	dfrA1	sul1, sul2	tet(A), tet(D)	
oqxA[a],oqxB[a], QnrB49[a],QnrS1	dfrA27	sul1	tet(A)[a]	mph(A)
oqxA[a],oqxB[a], QnrS1		sul2	tet(D)	
oqxA[a],oqxB[a], QnrS1	dfrA1	sul1	tet(A)	
oqxA[a],oqxB[a], QnrB6	dfrA27	sul1, sul2	tet(D)	
oqxA[a],oqxB[a], QnrB49[a],QnrS1	dfrA27	sul2	tet(A)[a]	mph(A)
oqxA[a],oqxB[a], QnrB6	dfrA27	sul1, sul2	tet(D)	
oqxA,oqxB	dfrA27	sul1	tet(D)	
oqxA,oqxB	dfrA27	sul1	tet(D)	
oqxA[a],oqxB[a], QnrS1	dfrA14[a]	sul2	tet(A)[a]	mph(A)
oqxA[a],oqxB[a]	dfrA27	sul1, sul2	tet(A)[a], tet(D)	
oqxA[a],oqxB[a], QnrB6		sul1, sul2	tet(D)	
oqxA[a],oqxB[a], QnrS1	dfrA14[a]	sul2		
oqxA[a],oqxB[a], QnrB49[a]	dfrA27	sul1, sul2	tet(A)[a], tet(D)	
oqxA[a],oqxB[a], QnrB49[a],QnrS1	dfrA27	sul1, sul2	tet(A)[a]	mph(A)
oqxA[a],oqxB[a], QnrB49[a],QnrS1	dfrA27	sul1, sul2	tet(A)[a]	mph(A)
QnrS1	dfrA14[a]	sul2		
QnrB52	dfrA27	sul1	tet(A)[a]	
oqxA[a],oqxB[a]				
QnrB1[a]	dfrA14[a]	sul2	tet(A)[a]	

light grey: intermediate and white: susceptible. ID isolate identification number, TOB tobramycin, CIP ciproflacin, TMP trimethoprim, SXT trimethoprim-sulfamethoxazole, SUL sulphonamide, TET tetracyclin, MAC macrolide

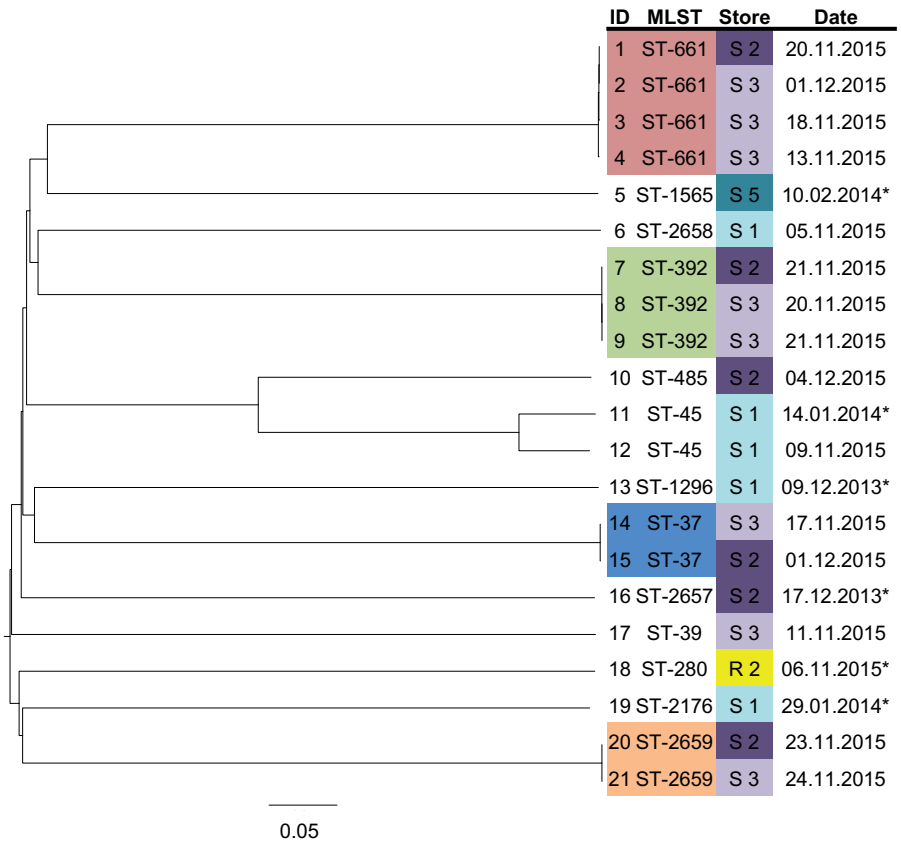


Figure 1. Neighbor-joining tree based on the wgMLST analysis of *K. pneumonia* isolates from the prevalence survey of bean sprout samples (2013 – 2016), using a pairwise ignore missing values approach. Scale shows genetic distance. wgMLST clusters are color coded (ID and MLST) as is the store of purchase. The date is the expiration date unless labelled with * in which case date of purchase of the sample is depicted. ID isolate identification number, MLST multilocus sequence typing, ST sequence type, S supermarket chain, R retailer

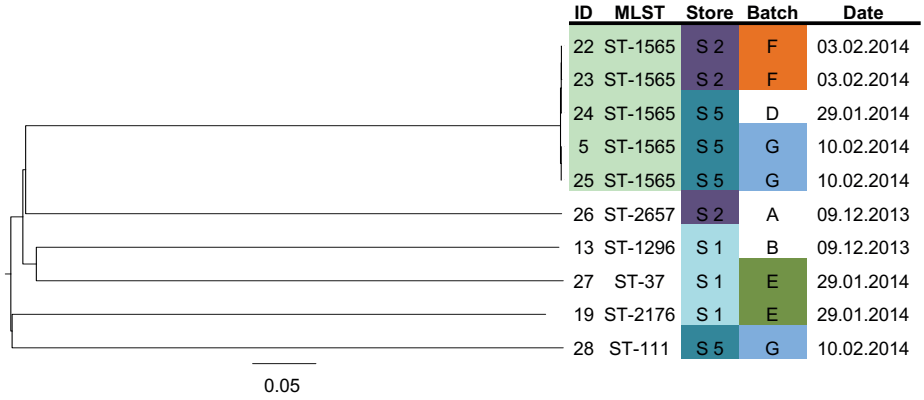


Figure 2. Neighbor-joining tree based on the wgMLST analysis of *K. pneumonia* isolates from multiple batches of bean sprouts, using a pairwise ignore missing values approach. Scale shows genetic distance. A batch was defined as bean sprout samples purchased on the same day from the same supermarket chain. wgMLST clusters are color coded (ID and MLST) as are store and batch of purchase. The date is the date of purchase of the sample. ID isolate identification number, MLST multilocus sequence typing, ST sequence type, S supermarket chain, R retailer

Table 4. Presence of ESBL-E in bean sprout samples purchased from supermarkets in batches.

Batch	No. samples in batch	No. samples ESBL-E positive (%) in batch	Clones present in >1 sample in batch
A	2	1 (50)	no
B	2	1 (50)	no
C	2	0 (0)	no
D	5	1 (20)	no
E	5	2 (40)	no
			no
F	5	4 (80)	yes
			yes
G	6	3 (50)	yes
			no

[a] Isolates with the same ST are also clonally related on basis of wgMLST analyses. ESBL-E, extended-spectrum beta-lactamase producing *Enterobacteriaceae*,

DISCUSSION

To our knowledge this is the first study focusing specifically on the ESBL-E prevalence in retail bean sprouts to date. An ESBL-E prevalence of 19% was found, being almost exclusively ESBL-producing *Klebsiella* spp. No ESBL-producing *E. coli* were found. The ESBL-E isolates found over time were either genetically highly diverse or clearly within the thresholds of clonal relatedness between epidemiologically related isolates as described by Kluytmans – van den Bergh et al [28] . The clonally related isolates always came from samples that were purchased within weeks of each other, which is suggestive for batch contamination. These findings indicate that there is a continuous influx of unrelated ESBL-E isolates and no prolonged persistence of specific clones.

A remarkable finding is that 96.2% of the isolates are of the genus *Klebsiella,* 80.8% being *K. pneumoniae.* The complete absence of ESBL-producing *E. coli* is noteworthy. We are unaware of factors favoring the growth of *K. pneumoniae* or suppressing the growth of other pathogens in the bean sprout production process. Other studies that present data on ESBL-E from bean sprouts reported similar high percentages of *K. pneumoniae*, namely, 80% and 84% in the studies by Reuland et al. (the Netherlands) and Kim et al. (South Korea) respectively [19,21].

No. samples with clone and species	MLST of clone	Date of purchase	Store	ID
1x K. pneumoniae	ST-2657	09/12/13	2	26
1x K. pneumoniae	ST-1296	09/12/13	1	13
-		17/12/13	5	
1x K. pneumoniae	ST-1565[a]	29/01/14	5	24
1x K. pneumoniae	ST-2176	29/01/14	1	19
1x K. pneumoniae	ST-37			27
2x K. oxytoca	ST-195[a]	03/02/14	2	29,30
2x K. pneumoniae	ST-1565[a]			22,23
2x K. pneumoniae	ST-1565[a]	10/02/14	5	5,25
1x K. pneumoniae	ST-111			28

MLST multilocus sequence typing, ST sequence type, isolate identification number

Besides the resistance to beta-lactams, we found high levels of co-resistance to important antimicrobial agents like ciprofloxacin, trimethoprim-sulfamethoxazole and tobramycin. Combined resistance to these three antibiotics and the ESBL phenotype was present in 50% of the isolates. This rate of co-resistance is higher than what is found in ESBL-E isolates from human carriage, namely 12% [39]. Combined resistance to the three antibiotics and the ESBL phenotype for *K. pneumoniae* from blood cultures in four peripheral hospitals in the South of the Netherlands was 18.3% (11 of 60 isolates, time period 1-1-2010 – 1-1-2018, unpublished data). None of the isolates from the current study showed resistance to carbapenems or colistin.

The ESBL-genes detected in bean sprouts have also been detected in the human population. For instance the $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-14}}$ genes, which were detected in bean sprouts in the current study, are among the most frequently detected ESBL genes in human carriage and bloodstream infections in the Netherlands [40–42]. Also $bla_{\text{CTX-M-3}}$ and $bla_{\text{CTX-M-27}}$ have been reported to be present in bloodstream infections in the Netherlands [42]. The most commonly detected ESBL gene in the current study, $bla_{\text{SHV-2}}$, has also been reported to be present in clinical samples [40]. However, the bla_{SHV} genes are not always typed to the specific variants within the group, making comparison of the exact SHV types difficult [41,43]. Although similar ESBL genes are found in bean sprouts and humans, it is difficult to judge the impact of the ESBL-gene

reservoir in bean sprouts on humans, as this study was not designed to make a direct comparison. However, bean sprouts have the potential to spread *Enterobacteriaceae* to humans, as has been shown by multiple outbreaks of pathogenic *Enterobacteriaceae* in humans in the past [23,24,44]. Therefore, our findings should be considered as a potential threat for humans but based on the current information we cannot quantify the size of the effect.

Our study has some strengths and limitations. Strengths of the study are the fact that samples were taken in different time periods, showing that ESBL-E contamination of bean sprouts is not an incidental finding. In addition, sensitive culture techniques were employed, using broth enrichment and a validated ESBL screening agar [45]. Furthermore, genetic confirmation of ESBL genes and precise typing and clustering methods using whole genome sequencing were used.

A limitation of the study is that we did not use a (semi-) quantitative culture method to quantify the load of the ESBL-E in bean sprouts. This information could have been important to estimate the possible impact for humans [22].

A further limitation of the study is the lack of information on the label of the bean sprouts. For instance, no information on place of production or production batches was given on the label and expiration dates were not always present. For the analyses, batches were defined as samples purchased from the same supermarket chain or store, with the same expiration date or date of purchase, depending on the availability of the information. No tracking codes or batch numbers from producers were present on the packages, which would have allowed for more precise analyses. Care was taken to achieve a representative sample of bean sprouts sold in the Netherlands and minimize the effect of batch contamination on the reported ESBL-E prevalence.

A final limitation of the study was the analysis of the plasmid content of the detected isolates. PlasmidFinder results are reported which enable comparisons to other studies, but further analyses into the epidemiology of the plasmids would have been a valuable addition. However, unravelling plasmid DNA from chromosomal DNA from the available short-read sequencing data is still a conundrum and beyond the scope of this study.

Despite these caveats several conclusions can be made on the ESBL-E prevalence of the different stores. First, there are differences in ESBL-E prevalence between supermarket chains. Supermarket chain one, two and three have an ESBL-E prevalence of more than 20%, whereas supermarket chain four has a prevalence of 4.8%. The bean

sprouts sold by supermarket chain two and three are likely to have an overlapping origin, as clonality in isolates from these supermarkets is common. Supermarket chain five was discontinued during the study period explaining the small number of samples. Second, a higher ESBL-E prevalence was found in samples from supermarkets compared to those from smaller retailers. The underlying causes of the differences in ESBL-E prevalence in the different stores are unknown. This may be caused by differences in the production process, network of transportation and differences in production scales with intrinsic possibilities of cross-contamination. Further studies are warranted to reveal the causes of contamination to decrease the overall ESBL-E prevalence in bean sprouts.

A final point on the ESBL-E contamination of bean sprout samples is the presence of batch contamination. High genetic variability of the ESBL-E isolates was seen in the bean sprout samples over time. In contrast, from samples purchased within three weeks of each other clonally related isolates were frequently cultured. Furthermore, in the experiment focusing on batch contamination, a single wgMLST clone was found in five different packages purchased from two different supermarket chains (Figs 1 and 2). These two observations support the hypothesis of batch contamination, however, not all samples from these batches were ESBL-E positive. This may indicate a varying load of ESBL-E or overgrowth with abundantly present non-fermenting bacteria. Also, the previously mentioned limitation of the definition of a batch may play a role.

In conclusion, 19.1% of bean sprout samples are contaminated with ESBL-E, with a remarkable high percentage of *Klebsiella* isolates in the absence of *E. coli.* The isolates are resistant to several other classes of antibiotics and are genetically highly diverse over time. Therefore, bean sprouts are a possible community source of ESBL-producing *Klebsiella spp.* Further investigations to the points of entry of ESBL-E into the production process and countermeasures against these entry points are warranted. Unfortunately, research looking into food as a vehicle for the spread of antimicrobial resistance in humans is greatly hampered by the lack of transparency in the food production process. In the current article the factory or even country of origin of the samples was not traceable for the researchers. We strongly suggest working towards a situation where basic information such as which country or countries the products were produced are clearly marked on the food items.

ACKNOWLEDGMENTS

We thank the team of curators of the Institut Pasteur MLST and whole genome MLST databases for curating the data and making them publicly available at http://bigsdb.pasteur.fr. This publication also made use of the PubMLST website (https://pubmlst.org/) developed by Keith Jolley (Jolley & Maiden 2010, BMC Bioinformatics, 11:595) and sited at the University of Oxford. The development of that website was funded by the Wellcome Trust.

REFERENCES

1. Woerther PL, Burdet C, Chachaty E, Andremont A. Trends in human fecal carriage of extended-spectrum beta-lactamases in the community: Toward the globalization of CTX-M. Clin Microbiol Rev. 2013;26: 744–758. doi:10.1128/CMR.00023-13
2. Karanika S, Karantanos T, Arvanitis M, Grigoras C, Mylonakis E. Fecal Colonization with Extended-spectrum Beta-lactamase-Producing Enterobacteriaceae and Risk Factors among Healthy Individuals: A Systematic Review and Metaanalysis. Clin Infect Dis. 2016; doi:10.1093/cid/ciw283
3. European Centre for Disease Prevention and Control (ECDC). Antimicrobial resistance surveillance in Europe 2014. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm: ECDC; 2015 [Internet]. 2015. doi:10.2900/23549
4. Tumbarello M, Spanu T, Di Bidino R, Marchetti M, Ruggeri M, Trecarichi EM, et al. Costs of bloodstream infections caused by Escherichia coli and influence of extended-spectrum-beta-lactamase production and inadequate initial antibiotic therapy. Antimicrob Agents Chemother. 2010;54: 4085–4091. doi:10.1128/AAC.00143-10
5. Livermore DM, Canton R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, et al. CTX-M: Changing the face of ESBLs in Europe [Internet]. Journal of Antimicrobial Chemotherapy. 2007. pp. 165–174. doi:10.1093/jac/dkl483
6. Huizinga P, van den Bergh MK, van Rijen M, Willemsen I, van 't Veer N, Kluytmans J. Proton Pump Inhibitor Use Is Associated With Extended-Spectrum β-Lactamase–Producing Enterobacteriaceae Rectal Carriage at Hospital Admission: A Cross-Sectional Study. Clin Infect Dis. 2016;702: ciw743. doi:10.1093/cid/ciw743
7. Reuland EA, Naiemi N, Kaiser AM, Heck M, Kluytmans JAJW, Savelkoul PHM, et al. Prevalence and risk factors for carriage of ESBL-producing Enterobacteriaceae in Amsterdam. J Antimicrob Chemother. 2016;71: 1–7. doi:10.1093/jac/dkv441
8. Shitrit P, Reisfeld S, Paitan Y, Gottesman BS, Katzir M, Paul M, et al. Extended-spectrum beta-lactamase-producing Enterobacteriaceae carriage upon hospital admission: Prevalence and risk factors. J Hosp Infect. Elsevier Ltd; 2013;85: 230–232. doi:10.1016/j.jhin.2013.07.014
9. Arcilla MS, Hattem JM van, Haverkate MR, Bootsma MCJ, Genderen PJJ van, Goorhuis A, et al. Import and spread of extended-spectrum β-lactamase-producing Enterobacteriaceae by international travellers (COMBAT study): a prospective, multicentre cohort study. Lancet Infect Dis. 2017;17: 78–85. doi:10.1016/S1473-3099(16)30319-X
10. Barreto Miranda IDM, Ignatius RPDM, Pfuller RDM, Friedrich-Janicke BDM, Steiner FDM, Paland MDM, et al. High carriage rate of ESBL-producing Enterobacteriaceae at presentation and follow-up among travellers with gastrointestinal complaints returning from India and Southeast Asia. J Travel Med. 2016;23: 1–7. doi:10.1093/jtm/tav024
11. Valverde A, Grill F, Coque TM, Pintado V, Baquero F, Cantón R, et al. High rate of intestinal colonization with extended-spectrum-β- lactamase-producing organisms in household contacts of infected community patients. J Clin Microbiol. 2008;46: 2796–2799. doi:10.1128/JCM.01008-08
12. Meyer E, Gastmeier P, Kola A, Schwab F. Pet animals and foreign travel are risk factors for colonisation with extended-spectrum beta-lactamase-producing Escherichia coli. Infection. 2012;40: 685–687. doi:10.1007/s15010-012-0324-8

13. Overdevest I, Willemsen I, Rijnsburger M, Eustace A, Xu L, Hawkey P, et al. Extended-spectrum β-lactamase genes of Escherichia coli in chicken meat and humans, the Netherlands. Emerg Infect Dis. 2011;17: 1216–1222. doi:10.3201/eid1707.110209
14. Kluytmans JAJW, Overdevest ITMA, Willemsen I, Kluytmans-Van Den Bergh MFQ, Van Der Zwaluw K, Heck M, et al. Extended-spectrum beta-lactamase-producing escherichia coli from retail chicken meat and humans: Comparison of strains, plasmids, resistance genes, and virulence factors. Clin Infect Dis. 2013;56: 478–487. doi:10.1093/cid/cis929
15. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Voets GM, van den Munckhof MP, van Essen-Zandbergen A, et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect. 2011;17: 873–880. doi:10.1111/j.1469-0691.2011.03497.x
16. de Boeck H, Miwanda B, Lunguya-Metila O, Muyembe-Tamfum JJ, Stobberingh E, Glupczynski Y, et al. ESBL-positive enterobacteria isolates in drinking water. Emerg Infect Dis. 2012;18: 1019–1020. doi:10.3201/eid1806.111214
17. Egervärn M, Englund S, Ljunge M, Wiberg C, Finn M, Lindblad M, et al. Unexpected common occurrence of transferable extended spectrum cephalosporinase-producing Escherichia coli in Swedish surface waters used for drinking water supply. Sci Total Environ. 2017;587–588: 466–472. doi:10.1016/j.scitotenv.2017.02.157
18. van Hoek AHAM, Veenman C, van Overbeek WM, Lynch G, de Roda Husman AM, Blaak H. Prevalence and characterization of ESBL- and AmpC-producing Enterobacteriaceae on retail vegetables. Int J Food Microbiol. 2015;204: 1–8. doi:10.1016/j.ijfoodmicro.2015.03.014
19. Reuland EA, al Naiemi N, Raadsen SA, Savelkoul PHM, Kluytmans JAJW, Vandenbroucke-Grauls CMJE. Prevalence of ESBL-producing Enterobacteriaceae in raw vegetables. Eur J Clin Microbiol Infect Dis. 2014;33: 1843–1846. doi:10.1007/s10096-014-2142-7
20. Blaak H, van Hoek AHAM, Veenman C, Docters van Leeuwen AE, Lynch G, van Overbeek WM, et al. Extended spectrum beta-lactamase- and constitutively AmpC-producing Enterobacteriaceae on fresh produce and in the agricultural environment. Int J Food Microbiol. 2014;168–169: 8–16. doi:10.1016/j.ijfoodmicro.2013.10.006
21. Kim H-S, Chon J-W, Kim Y-J, Kim D-H, Kim M, Seo K-H. Prevalence and characterization of extended-spectrum-β-lactamase-producing Escherichia coli and Klebsiella pneumoniae in ready-to-eat vegetables. Int J Food Microbiol. 2015;207: 83–6. doi:10.1016/j.ijfoodmicro.2015.04.049
22. Evers EG, Pielaat A, Smid JH, Van Duijkeren E, Vennemann FBC, Wijnands LM, et al. Comparative exposure assessment of esblproducing Escherichia coli through meat consumption. PLoS One. 2017;12. doi:10.1371/journal.pone.0169589
23. Bielaszewska M, Mellmann A, Zhang W, Köck R, Fruth A, Bauwens A, et al. Characterisation of the Escherichia coli strain associated with an outbreak of haemolytic uraemic syndrome in Germany, 2011: A microbiological study. Lancet Infect Dis. 2011;11: 671–676. doi:10.1016/S1473-3099(11)70165-7
24. Buchholz U, Bernard H, Werber D, Böhmer MM, Remschmidt C, Wilking H, et al. German Outbreak of Escherichia coli O104:H4 Associated with Sprouts. N Engl J Med. 2011;365: 1763–1770. doi:10.1056/NEJMoa1411087
25. Netherlands Society for Medical Microbiology. NVMM Guideline Laboratory detection of highly resistant microorganisms, version 2.0, 2012. Leeuwarden, Netherlands. 2012; Available: http://www.nvmm.nl/system/files/2012.11.15 richtlijn BRMO %2528version 2.0%2529 - RICHTLIJN.pdf

26. European Committee on Antimicrobial Susceptibility Testing. EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and / or epidemiological importance. European Society of Clinical Microbiology and Infectious Diseases, Basel, Switzerland. 2013; Available: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_v1.0_20131211.pdf
27. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 7.1 [Internet]. 2017. Available: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf
28. Kluytmans-van den Bergh MFQ, Rossen JWA, Bruijning-Verhagen PCJ, Bonten MJM, Friedrich AW, Vandenbroucke-Grauls CMJE, et al. Whole genome multilocus sequence typing of extended-spectrum beta-lactamase-producing Enterobacteriaceae. J Clin Microbiol. 2016;54: 2919–2927. doi:10.1128/JCM.01648-16
29. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012;67: 2640–2644. doi:10.1093/jac/dks261
30. Larsen M V., Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, et al. Multilocus sequence typing of total-genome-sequenced bacteria. J Clin Microbiol. 2012;50: 1355–1361. doi:10.1128/JCM.06094-11
31. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother. 2014;58: 3895–903. doi:10.1128/AAC.02412-14
32. Thomsen MCF, Ahrenfeldt J, Cisneros JLB, Jurtz V, Larsen MV, Hasman H, et al. A bacterial analysis platform: An integrated system for analysing bacterial whole genome sequencing data for clinical diagnostics and surveillance. PLoS One. 2016;11: 1–14. doi:10.1371/journal.pone.0157718
33. Larsen M V., Cosentino S, Lukjancenko O, Saputra D, Rasmussen S, Hasman H, et al. Benchmarking of methods for genomic taxonomy. J Clin Microbiol. 2014;52: 1529–1539. doi:10.1128/JCM.02981-13
34. Martínez-Romero E, Rodríguez-Medina N, Beltrán-Rojel M, Silva-Sánchez J, Barrios-Camacho H, Pérez-Rueda E, et al. Genome misclassification of Klebsiella variicola and Klebsiella quasipneumoniae isolated from plants, animals and humans. Salud Publica Mex. 2018;60: 56–62. doi:10.21149/8149
35. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4: 406–25. Available: http://www.ncbi.nlm.nih.gov/pubmed/3447015
36. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013;30: 2725–2729. doi:10.1093/molbev/mst197
37. Simpson EH. Measurement of Diversity. Nature. 1949;163: 688–688. doi:10.1038/163688a0
38. Grundmann H, Hori S, Tanner G. Determining Confidence Intervals When Measuring Genetic Diversity and the Discriminatory Abilities of Typing Methods for Microorganisms. J Clin Microbiol. 2001;39: 4190–4192. doi:10.1128/JCM.39.11.4190
39. Reuland EA, Overdevest ITMA, al Naiemi N, Kalpoe JS, Rijnsburger MC, Raadsen SA, et al. High prevalence of ESBL-producing Enterobacteriaceae carriage in Dutch community patients with gastrointestinal complaints. Clin Microbiol Infect. 2013;19: 542–549. doi:10.1111/j.1469-0691.2012.03947.x

40. Voets GM, Platteel TN, Fluit AC, Scharringa J, Schapendonk CM, Stuart JC, et al. Population distribution of Beta-lactamase conferring resistance to third-generation cephalosporins in human clinical Enterobacteriaceae in the Netherlands. PLoS One. 2012;7: e52102. doi:10.1371/journal.pone.0052102
41. Willemsen I, Oome S, Verhulst C, Pettersson A, Verduin K, Kluytmans J. Trends in Extended Spectrum Beta-Lactamase (ESBL) producing enterobacteriaceae and ESBL genes in a Dutch teaching hospital, measured in 5 yearly point prevalence surveys (2010-2014). PLoS One. 2015;10: 1–10. doi:10.1371/journal.pone.0141765
42. Van Der Bij AK, Peirano G, Goessens WHF, Van Der Vorm ER, Van Westreenen M, Pitout JDD. Clinical and molecular characteristics of extended-spectrum-beta-lactamase-producing Escherichia coli causing bacteremia in the Rotterdam Area, Netherlands. Antimicrob Agents Chemother. 2011;55: 3576–3578. doi:10.1128/AAC.00074-11
43. Stuart JC, Dierikx C, Naiemi N Al, Karczmarek A, Van Hoek AHAM, Vos P, et al. Rapid detection of TEM, SHV and CTX-M extended-spectrum beta-lactamases in Enterobacteriaceae using ligation-mediated amplification with microarray analysis. J Antimicrob Chemother. 2010;65: 1377–1381. doi:10.1093/jac/dkq146
44. German institut for risk Assesment (BfR). Bundesinstitut für Risikobewertung. EHEC outbreak: BfR confirms contamination of sprouts with O104:H4 [Internet]. 2011 [cited 30 Dec 2017]. Available: http://www.bfr.bund.de/de/presseinformation/2011/17/ehec_ausbruch__bfr_bestaetigt_kontamination_von_sprossen_mit_o104_h4-70934.html
45. Overdevest ITMA, Willemsen I, Elberts S, Verhulst C, Kluytmans JAJW. Laboratory detection of extended-spectrum-beta-lactamase-producing Enterobacteriaceae: evaluation of two screening agar plates and two confirmation techniques. J Clin Microbiol. 2011;49: 519–22. doi:10.1128/JCM.01953-10

SUPPORTING INFORMATION

S1 Table. Summary of the quality control parameters of the WGS assemblies used in the study.

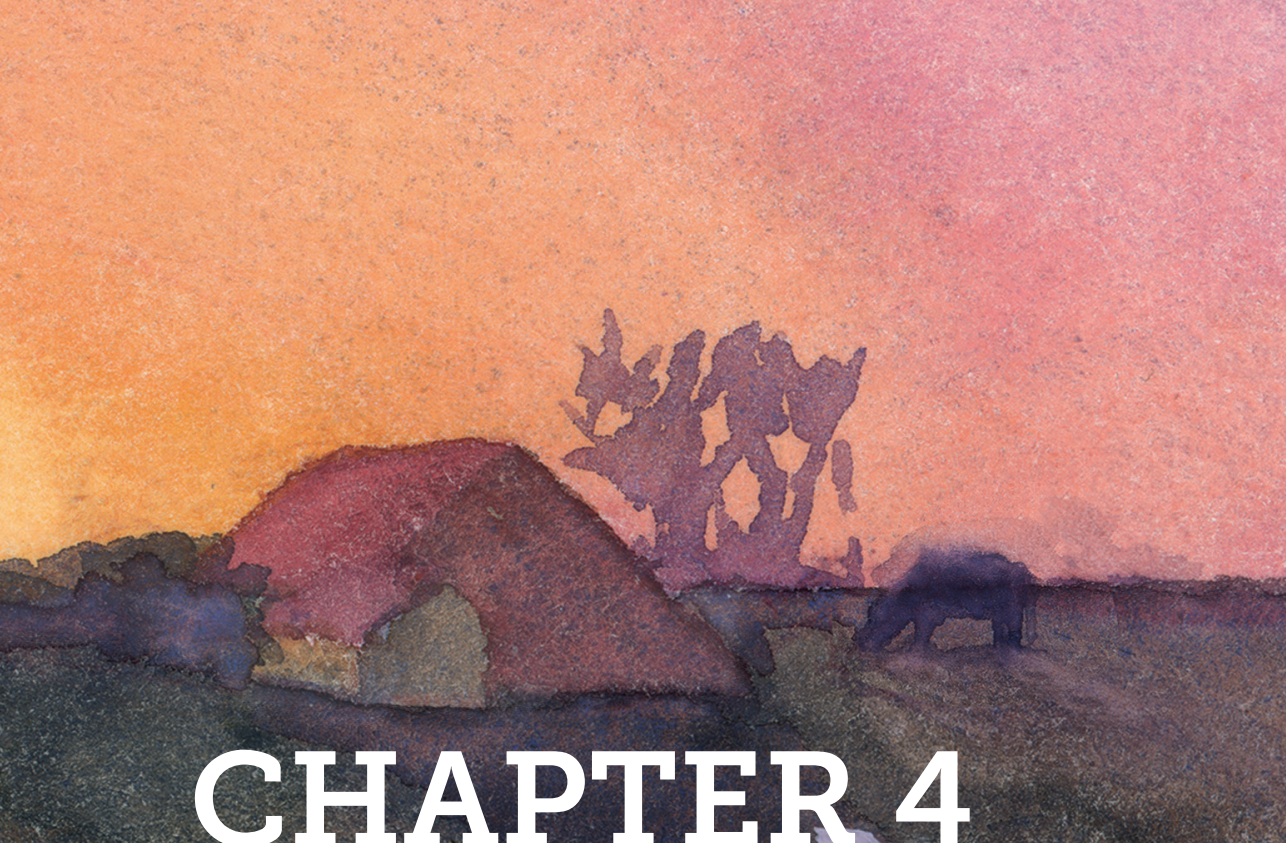
	Mean	Median	Minimum	Maximum
K. pneumoniae (n = 28)				
No. Scaffolds, *n*	58.4	53.0	35	99
N50, *n*	291716.1	295293.5	142946	426749
Max. scaffold length, *n*	659241.7	685594.5	416351	915597
Genome size, *n*	5389681.1	5364165.0	5222704	5572072
Coverage, *n*	69.2	72.3	31.5	98.2
Percentage of reads used, %	97.5	97.8	94.5	98.7
K. oxytoca (n = 4)				
No. Scaffolds, *n*	78.8	74.0	73	94
N50, *n*	181404.3	181331.0	138416	224539
Max. scaffold length, *n*	504542.5	493858.5	417814	612639
Genome size, *n*	6054428.0	6059100.5	5871338	6228173
Coverage, *n*	61.2	61.7	55.1	66.2
Percentage of reads used, %	97.9	98.2	96.9	98.5
K. variicola (n = 1)				
No. Scaffolds, *n*	77.0	77.0	77	77
N50, *n*	162401.0	162401.0	162401	162401
Max. scaffold length, *n*	423756.0	423756.0	423756	423756
Genome size, *n*	5673740.0	5673740.0	5673740	5673740
Coverage, *n*	61.6	61.6	61.6	61.6
Percentage of reads used, %	97.5	97.5	97.5	97.5
E. cloacae (n = 1)				
No. Scaffolds, *n*	53.0	53.0	53	53
N50, *n*	298624.0	298624.0	298624	298624
Max. scaffold length, *n*	625903.0	625903.0	625903	625903
Genome size, *n*	4710742.0	4710742.0	4710742	4710742
Coverage, *n*	105.0	105.0	105.0	105.0
Percentage of reads used, %	98.3	98.3	98.3	98.3

N50 is the shortest scaffold length such that 50% of the entire assembly is contained in scaffolds equal to or larger than this length, max. maximum

S2 Table. Recoding of isolate names for correspondence of fastq files on the ENA site.

Sample ID	Names of fastq files	Present in prevalence study	Batch number
1	15M105753-1	yes	
2	15M109131-1	yes	
3	15M104880-1	yes	
4	15M102484-1	yes	
5	14M013120-1	yes	G
6	15M100995-2	yes	
7	15M105754-1	yes	
8	15M104882-1	yes	
9	15M105761-1	yes	
10	15M110134-1	yes	
11	14M004312-1	yes	
12	15M101835-3	yes	
13	13M104391-1	yes	B
14	15M103835-2	yes	
15	15M109129-1	yes	
16	13M106860-4	yes	
17	15M101900-2	yes	
18	15M101907-2	yes	
19	14M009417-2	yes	E
20	15M105756-1	yes	
21	15M105764-1	yes	
29	14M010914-7	yes	F
31	15M101907-1	yes	
32	15M103834-1	yes	
33	15M112937-1	yes	
34	16M002405-1	yes	
22	14M010915-5		F
23	14M010919-3		F
24	14M009431-3		D
25	14M013121-1		G
26	13M104441-1		A
27	14M009420-2		E
28	14M013125-1		G
30	14M010921-6		F

Sample ID sample identification number,



CHAPTER 4

Proton-pump inhibitor (PPI) use is associated with extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E) rectal carriage at hospital admission: a cross-sectional study

Pepijn Huizinga, Marjolein Kluytmans - van den Bergh, Miranda van Rijen, Ina Willemsen, Nils van 't Veer, Jan Kluytmans

Adapted from: Clin Infect Dis 2017: 10/1093/cid/ciw743

ABSTRACT

In this cross-sectional study, 8.5% of patients using proton-pump inhibitors (PPI) were rectal carriers of extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E), compared to 2.9% of non-PPI users. In multivariable analysis PPI use was independently associated with ESBL-E rectal carriage at hospital admission (adjusted OR 3.89; 95% CI 1.65 – 9.19).

INTRODUCTION

Rectal carriage of extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E) in the general community has become endemic globally [1]. Several reservoirs for ESBL-E in the community setting have been described, including contaminated food. Whether oral exposure to ESBL-E results in ESBL-E rectal carriage will depend on several factors, such as the bacterial load of the exposure, functionality of the gastric barrier and antibiotic use.

The use of medication that neutralises or reduces the production of gastric acid has been shown to increase the risk of gastrointestinal infections [2]. Whether the use of such medication is associated with an increase in ESBL-E rectal carriage is less clear, and studies have reported conflicting results [3,4]. The objective of this cross-sectional study was, therefore, to assess the association between PPI use and ESBL-E rectal carriage at hospital admission.

MATERIALS AND METHODS

This cross-sectional study was performed in the Amphia Hospital, an 850-bed teaching hospital. Yearly, voluntary prevalence surveys for ESBL-E rectal carriage are performed as part of the routine infection control program. The current analysis is based on the prevalence surveys performed in November 2014 and November 2015, and includes all patients above 18 years, who were hospitalised for at most two days or received day care at the day of the survey. Patients who were hospitalised for more than two days were excluded to prevent the influence of hospital stay on ESBL-E rectal carriage.

ESBL-E rectal carriage was defined as the presence of genotypically confirmed ESBL-E in the rectal swab, and was detected by selective culturing of rectal swabs with subsequent phenotypic and genotypic confirmation of ESBL-production for all Enterobacteriaceae grown, as described previously (see supplementary data) [5].

Infection control practitioners retrieved demographic and clinical patient data from the electronic medical patient records. These included gender, date of birth, date of admission, date of culture, hospital admission within the 6 months prior to current admission, medical speciality by which the patient was admitted, antibiotic use on the day of the survey, and pre-admission use of PPIs and H_2-receptor antagonists. Data on pre-admission medication were collected from home-medication lists, which were verified on the day of admission by an assistant pharmacist. Infection control practitioners and assistant pharmacists were blinded for the culture results.

Table 1. Univariable and multivariable logistic regression analyses of PPI use and ESBL-E rectal carriage.

	ESBL-E-carriage	
	n = 31	
PPI use, *n (%)*	22	(71.0)
Type of PPI used, *n (%)*		
No PPI	9	(29.0)
Pantoprazole	9	(29.0)
Omeprazole	13	(41.9)
Esomeprazole	0	(0.0)
Lansoprazole	0	(0.0)
Antibiotic use at day of culture, *n (%)*	7	(22.6)
Prior hospital admission[A], *n (%)*	18	(58.1)
Age (years), *median (IQR)*	64	(51 – 72)

A. Hospital admission in the 6 months prior to the current hospital admission.

Data were analysed using Statistical Package for Social Science software (IBM SPSS Statistics 22.0, Armonk, NY). Univariable and multivariable logistic regression analyses were performed to examine whether PPI use was independently associated with ESBL-E rectal carriage. Based on the available literature on ESBL-E carriage and PPI use, age, antibiotic use on the day of culture, and hospital admission within the 6 months prior to the current hospital admission were included as potential confounding variables in the logistic regression analysis. The Chi-square test was used to test for differences in the distribution of ESBL genes between PPI users and non-PPI users.

According to the Dutch regulations for research with human subjects, ethical approval was not required, since conduct of the ESBL-E prevalence surveys is part of the local hospital infection control program.

RESULTS

Valid rectal cultures were obtained from 570 (81.2%) of 702 hospitalised or day care patients. Of these 570 patients, 259 (45.4%; 95% CI: 41.4% - 49.5%) used PPIs on admission. Antacids (n=5) and histamine H_2-receptor antagonists (n=9) were used infrequently,

No ESBL-E carriage		Univariable		Multivariable	
n = 539		OR	95% CI	OR	95% CI
237	(44.0)	3.12	1.41 – 6.89	3.89	1.65 – 9.19
302	(56.0)	ref			
111	(20.6)	2.72	1.05 – 7.03		
95	(17.6)	4.59	1.90 – 11.08		
28	(5.2)	NA	NA		
3	(0.6)	NA	NA		
103	(19.1)	1.24	0.52 – 2.94	1.16	0.48 – 2.85
277	(51.4)	1.31	0.63 – 2.73	1.03	0.48 – 2.22
65	(51 – 76)	0.988	0.968 – 1.01	0.976	0.955 – 0.998

which precluded analysis of a relation between these types of medication and ESBL-E rectal carriage. The distribution of gender, ward type, and medical speciality by which the patient was admitted were comparable for PPI users and non-PPI users. In PPI users, prior hospitalisation and antibiotic use were more frequent, and median age was higher (Table S1).

ESBL-E rectal carriage was detected in 31 (5.4%; 95% CI: 3.8% – 7.6%) of 570 patients. *Escherichia coli* (n=27; 87.1%) was the predominant species, followed by *Klebsiella pneumoniae* (n=3; 9.7%) and *Enterobacter cloacae* (n=1; 3.2%). No statistically significant differences in the distribution and diversity of ESBL genes were observed between PPI users and non-PPI users (Chi-square 5.84; p=0.322, data shown in Table S2).

In the univariable analysis, PPI use was found to be statistically significantly associated with ESBL-E rectal carriage (OR 3.12; 95% CI: 1.41 – 6.89) (Table 1). The effects of omeprazole and pantoprazole on ESBL-E rectal carriage were comparable. Hence, overall PPI use was analysed in the multivariable model. In the multivariable model, PPI use and age were independently associated with ESBL-E rectal carriage (Table 1). The prevalence of ESBL-E carriage at admission was higher in PPI users (adjusted OR 3.89; 95% CI: 1.65 – 9.19) but lower with increasing age (adjusted OR 0.976; 95% CI: 0.955 – 0.998).

DISCUSSION

In the present study PPI use was independently associated with ESBL-E rectal carriage at hospital admission. The relation between the use of medication that affects gastric acidity and ESBL-E rectal carriage has been investigated before. A recent Dutch study described a similar effect of PPIs on ESBL-E rectal carriage in primary care patients [3]. This indicates that the effect of PPIs is present in both the community and in patients with more frequent exposure to healthcare. In an Israeli study the use of histamine H_2-receptor antagonists, but not PPIs, was associated with ESBL-E rectal carriage [4]. The authors provided no clear explanation for the lack of effect of PPI use. Three other studies on risk factors for ESBL-E rectal carriage did not find an association with gastric-acid suppression therapy in general, but no data were provided on the specific effect of PPIs [6–8].

Antibiotic use on the day of culture was not associated with ESBL-E rectal carriage. This is due to the small time window between start of antibiotic use and time of culture in the study population. Unfortunately, no data were available on antibiotic use prior to hospital admission, which has previously been described as a risk factor for ESBL-E rectal carriage by others [9]. However, a recent Dutch study, found that adjustment for prior antibiotic use did not change the effect size of the association between PPI use and ESBL-E carriage [3].

Strengths of the present study include the high response rate (82.9%), the use of a sensitive culture method, including selective broth enrichment, and the blinded ascertainment of data on medication use and other patient characteristics.

In the present study, data on PPI use pertain to pre-admission use. This ensures the use of PPIs shortly before screening for ESBL-E carriage. Other studies have used time windows for PPI use of up to one year before culture, which may dilute the observed effect of PPI use on ESBL-E rectal carriage. Data on the duration and dosage of PPI use were not available in this study, which precluded analysis of a dose-effect relationship. Yet, taking into account the frequency and duration of PPI use is expected to increase the observed effect of PPI use on ESBL-E carriage. No data were available on the presence of co-morbidities, which may be a (residual) confounder of the observed association between PPI use and ESBL-E carriage.

Reports on the association between age and ESBL-E carriage have described no association, positive- and negative associations. These conflicting results may in part be due to the positive association between age and PPI use. This may mask the effect of age on ESBL-E rectal carriage when analyses are not adjusted for PPI use, as was shown in the current study.

The relevance of PPI use as a risk factor for ESBL-E carriage should be seen in the perspective of PPIs being one of the most frequently prescribed drugs in the world [10]. In the Netherlands, between 10 to 15% of the population uses a PPI. Above 65 years of age, the prevalence of PPI use increases to roughly 30% [11]. In the present study this was even higher, i.e. 45.4%. This is probably due to the fact that this hospital-derived patient population is older, and has more co-morbidities and medication use than the general population [12]. The extensive use of PPIs in the general population may facilitate the acquisition of ESBL-E in the community setting.

In conclusion, we found that PPI use is associated with ESBL-E rectal carriage at hospital admission. Prospective studies are warranted to further elucidate the role of PPI use in the acquisition of ESBL-E rectal carriage and may provide insight in the effect of different PPIs or dosage schedules.

FUNDING AND CONFLICTS OF INTEREST

The study was performed without financial support from third parties. None of the authors have associations that might pose a conflict of interest.

ACKNOWLEDGEMENTS

We would like to express our gratitude to the laboratory technicians responsible for the culture work, and the research nurses and infection control practitioners who collected the patient data.

REFERENCES

1. Woerther PL, Burdet C, Chachaty E, Andremont A. Trends in human fecal carriage of extended-spectrum beta-lactamases in the community: Toward the globalization of CTX-M. Clin. Microbiol. Rev. **2013**; 26:744–758. Available at: http://dx.doi.org/10.1128/CMR.00023-13.
2. Hassing R-J, Verbon A, de Visser H, Hofman A, Stricker BH. Proton pump inhibitors and gastroenteritis. Eur. J. Epidemiol. **2016**; Available at: http://link.springer.com/10.1007/s10654-016-0136-8.
3. Reuland EA, Naiemi N, Kaiser AM, et al. Prevalence and risk factors for carriage of ESBL-producing Enterobacteriaceae in Amsterdam. J. Antimicrob. Chemother. **2016**; 71:1–7. Available at: http://dx.doi.org/10.1093/jac/dkv441.
4. Ben-Ami R, Schwaber MJ, Navon-Venezia S, et al. Influx of Extended-Spectrum β-Lactamase-Producing Enterobacteriaceae into the Hospital. Clin. Infect. Dis. **2006**; 42:925–934. Available at: http://cid.oxfordjournals.org/content/42/7/925.abstract.
5. Kluytmans-van den Bergh MFQ, Verhulst C, Willemsen LE, Verkade E, Bonten MJM, Kluytmans JAJW. Rectal Carriage of Extended-Spectrum-Beta-Lactamase-Producing Enterobacteriaceae in Hospitalized Patients: Selective Preenrichment Increases Yield of Screening. J. Clin. Microbiol. **2015**; 53:2709–12. Available at: http://dx.doi.org/10.1128/JCM.01251-15. Accessed 1 April 2016.
6. Jans B, Schoevaerdts D, Huang TD, et al. Epidemiology of Multidrug-Resistant Microorganisms among Nursing Home Residents in Belgium. PLoS One **2013**; 8:e64908. Available at: http://dx.doi.org/10.1371/journal.pone.0064908.
7. Leung W, Malhi G, Willey BM, et al. Prevalence and predictors of MRSA, ESBL, and VRE colonization in the ambulatory IBD population. J. Crohn's Colitis **2012**; 6:743–749. Available at: http://dx.doi.org/10.1016/j.crohns.2011.12.005.
8. Rodriguez-Bano J, Lopez-Cerero L, Navarro MD, de Alba PD, Pascual A. Faecal carriage of extended-spectrum beta-lactamase-producing Escherichia coli: Prevalence, risk factors and molecular epidemiology. J. Clin. Microbiol. **2010**; 48:1726–1731. Available at: http://dx.doi.org/10.1128/JCM.02353-09.
9. Shitrit P, Reisfeld S, Paitan Y, et al. Extended-spectrum beta-lactamase-producing Enterobacteriaceae carriage upon hospital admission: Prevalence and risk factors. J. Hosp. Infect. **2013**; 85:230–232. Available at: http://dx.doi.org/10.1016/j.jhin.2013.07.014.
10. Forgacs I, Loganayagam A. Overprescribing proton pump inhibitors. BMJ **2008**; 336:2–3. Available at: http://dx.doi.org/10.1136/bmj.39406.449456.BE.
11. The Dutch Foundation for Pharmaceutical Statistics (SFK). Van Rennie tot PPI. 2014. Available at: https://www.sfk.nl/nieuws-publicaties/PW/2014/van-rennie-tot-ppi. Accessed 8 May 2016.
12. Pham CQD, Regal RE, Bostwick TR, Knauf KS. Acid suppressive therapy use on an inpatient internal medicine service. Ann. Pharmacother. **2006**; 40:1261–1266. Available at: http://dx.doi.org/10.1345/aph.1G703.

SUPPLEMENTARY MATERIALS

Detection of ESBL-E rectal carriage

Screening for ESBL-E rectal carriage was performed in accordance with national and international guidelines [1,2]. Nylon flocked swabs with liquid Amies medium (Eswab, Copan, Italy) were used for collection and transport of rectal samples [3]. Swabs were kept at 2 to 7°C and were processed within 24 h of sampling, at the local microbiology laboratory. A sheep blood agar plate was inoculated with 20 µl of the Eswab Amies medium, and served as growth control. The remaining Amies medium was selectively enriched in 5 ml of a tryptic soy broth containing 0.25 mg/L cefotaxime and 8 mg/L vancomycin (TSB-VC) [4]. After overnight incubation at 35 to 37°C, 10 µl of the TSB-VC was subcultured on an ESBL screening agar (EbSA, AlphaOmega, 's-Gravenhage, the Netherlands), consisting of two McConkey agars containing cloxacillin (400 mg/L), vancomycin (64 mg/L) and either cefotaxime or ceftazidime (1 mg/L). Species identification (VITEK-MS, bioMérieux, Marcy l'Etoile, France) and antibiotic susceptibility testing (VITEK2, panel N199, bioMérieux, Marcy l'Etoile, France) were performed for all oxidase-negative Gram-negative isolates that grew on the EbSA agar plate. The production of ESBL was phenotypically confirmed with the combination disk diffusion method for cefotaxime (30 µg), ceftazidime (30 µg) and cefepime (30 µg), with and without clavulanic acid (10 µg) (Rosco, Taastrup, Denmark). Test results were considered positive if the diameter of the inhibition zone was >=5 mm larger for the disk with clavulanic acid as compared to the disk without clavulanic acid [1,2]. Genotypic confirmation of the presence of ESBL genes was performed with the Check-MDR CT103 DNA microarray (Check-Points, Wageningen, the Netherlands) [5]. Cultures were rejected when no bacterial growth was observed on the blood agar plate, which was considered to be indicative of inappropriate sampling.

RESULTS

Table S1. Patient characteristics according to PPI use.

	PPI use		No PPI use	
	n = 259		n = 311	
Age (years), *median (interquartile range)*	70	(59 - 79)	62	(45 - 73)
Gender, *n (%)*				
Male	120	(46.3)	130	(41.8)
Female	139	(53.7)	181	(58.2)
Ward type, *n (%)*				
Intensive care unit (ICU)	4	(1.5)	3	(1.0)
Clinical	153	(59.1)	194	(62.4)
Day care	102	(39.4)	114	(36.7)
Medical speciality, *n (%)*				
Cardiology	22	(8.5)	23	(7.4)
Anaesthesiology (non-ICU)	15	(5.8)	15	(4.8)
Anaesthesiology (ICU)	4	(1.5)	3	(1.0)
Surgery	38	(14.7)	67	(21.5)
Internal medicine	89	(34.4)	48	(15.4)
Otorhinolaryngology	3	(1.2)	12	(3.9)
Pulmonology	18	(6.9)	9	(2.9)
Neurology	19	(7.3)	10	(3.2)
Obstetrics and gynaecology	7	(2.7)	27	(8.7)
Ophthalmology	8	(3.1)	27	(8.7)
Orthopaedics	18	(6.9)	29	(9.3)
Urology	6	(2.3)	19	(6.1)
Gastroenterology and hepatology	9	(3.5)	18	(5.8)
Other	3	(1.2)	4	(1.3)
Antibiotic use on day of culture, *n (%)*				
Yes	63	(24.3)	47	(15.1)
No	196	(75.7)	264	(84.9)
Type of antibiotic used[A], *n (%)*				
Penicillins or cephalosporins	37	(14.3)	32	(10.3)
Beta-lactam/beta-lactamase inhibitor	20	(7.7)	12	(3.9)
Carbapenems	1	(0.4)	0	(0.0)

Table S1. Continued.

	PPI use		No PPI use	
	n = 259		n = 311	
Fluoroquinolones	9	(3.5)	2	(0.6)
Aminoglycosides	7	(2.7)	5	(1.6)
Nitrofurantoin	3	(1.2)	0	(0.0)
Trimethoprim/sulfamethoxazole	3	(1.2)	0	(0.0)
Macrolides or lincosamides	4	(1.5)	2	(0.6)
Glycopeptides	0	(0.0)	1	(0.3)
Metronidazol	0	(0.0)	3	(1.0)
Tetracyclines	4	(1.5)	0	(0.0)
Prior hospital admission[B], *n (%)*				
Yes	170	(65.6)	125	(40.2)
No	89	(34.4)	186	(59.8)
ESBL-E rectal carriage, *n (%)*				
Yes	22	(8.5)	9	(2.9)
No	237	(91.5)	302	(97.1)

A. Twenty-five PPI users and 10 non-PPI users used multiple antibiotics on the day of culture.
B. Hospital admission in the 6 months prior to the current hospital admission.

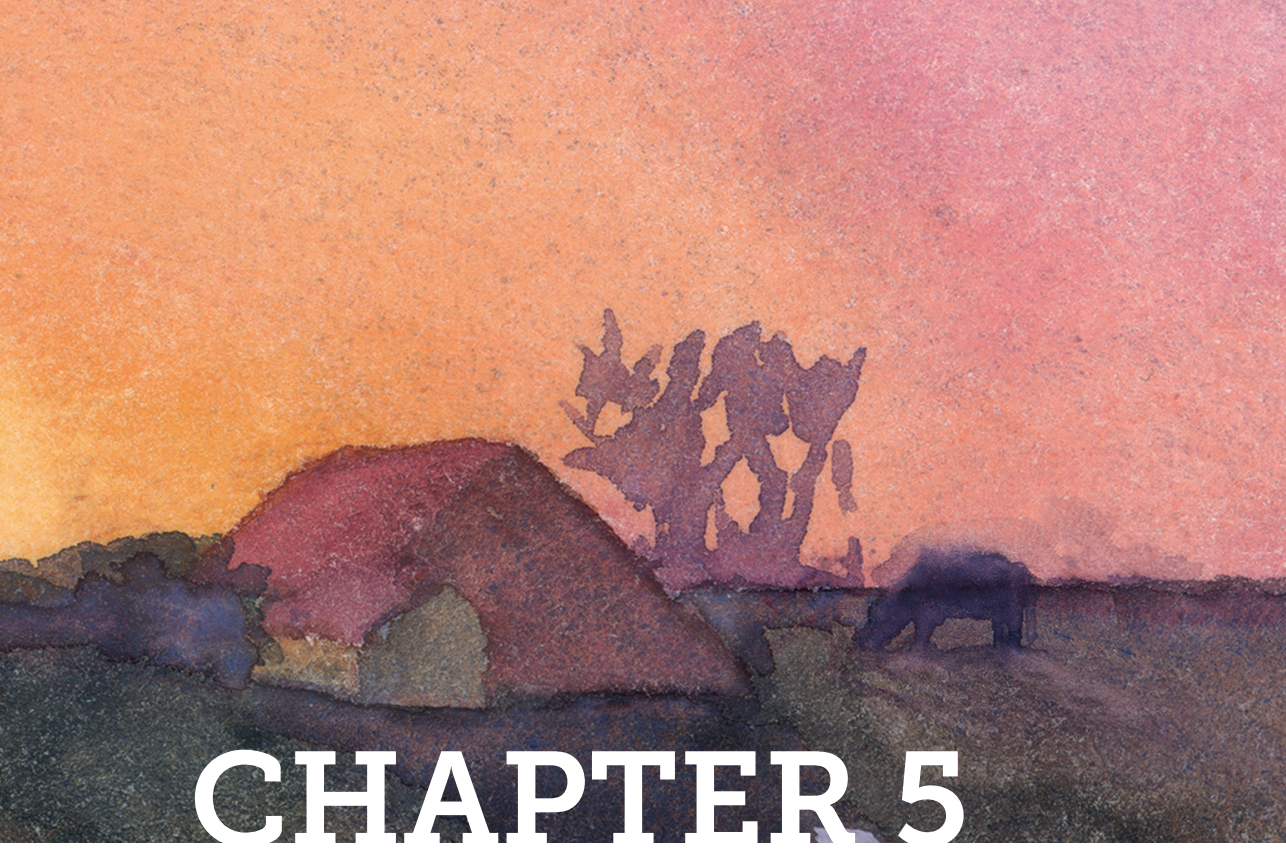
Table S2. Distribution of ESBL encoding genes in ESBL-E rectal carriers at hospital admission.

	Overall	PPI use[A]	no PPI use[B]
ESBL encoding gene	**n = 31**	**n = 22**	**n = 9**
$bla_{\text{CTX-M-1-like}}$	3	3	0
$bla_{\text{CTX-M-15-like}}$	19	14	5
$bla_{\text{CTX-M-9-group}}$	6	3	3
$bla_{\text{SHV 238S+240K}}$	1	1	0
$bla_{\text{CTX-M-9-group}}$ + $bla_{\text{SHV 238S+240K}}$	1	0	1
$bla_{\text{CTX-M-15-like}}$ + $bla_{\text{SHV 238S}}$	1	1	0

Chi-square$_{\text{PPI use vs. no PPI use}}$ = 5.84; p = 0.322.

REFERENCES

1. Netherlands Society for Medical Microbiology. NVMM Guideline Laboratory detection of highly resistant microorganisms, version 2.0, 2012. Leeuwarden, Netherlands. **2012**; Available at: http://www.nvmm.nl/system/files/2012.11.15 richtlijn BRMO %28version 2.0%29 - RICHTLIJN.pdf.
2. European Committee on Antimicrobial Susceptibility Testing. EUCAST guidelines for detection of resistance mechanisms and specific resistances of clinical and / or epidemiological importance. European Society of Clinical Microbiology and Infectious Diseases, Basel, Switzerland. **2013**; Available at: http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_v1.0_20131211.pdf.
3. Van Horn KG, Audette CD, Sebeck D, Tucker KA. Comparison of the Copan ESwab system with two Amies agar swab transport systems for maintenance of microorganism viability. J. Clin. Microbiol. **2008**; 46:1655–8. Available at: http://jcm.asm.org/content/46/5/1655.abstract?ijkey=a3513981af9b48bce88dffba2c7d748a5c849e49&keytype2=tf_ipsecsha. Accessed 4 April 2016.
4. Kluytmans-van den Bergh MFQ, Verhulst C, Willemsen LE, Verkade E, Bonten MJM, Kluytmans JAJW. Rectal Carriage of Extended-Spectrum-Beta-Lactamase-Producing Enterobacteriaceae in Hospitalized Patients: Selective Preenrichment Increases Yield of Screening. J. Clin. Microbiol. **2015**; 53:2709–12. Available at: http://dx.doi.org/10.1128/JCM.01251-15. Accessed 1 April 2016.
5. Cuzon G, Naas T, Bogaerts P, Glupczynski Y, Nordmann P. Evaluation of a DNA microarray for the rapid detection of extended-spectrum beta-lactamases (TEM, SHV and CTX-M), plasmid-mediated cephalosporinases (CMY-2-like, DHA, FOX, ACC-1, ACT/MIR and CMY-1-like/MOX) and carbapenemases (KPC, OXA-48, VIM, IMP and ND. J. Antimicrob. Chemother. **2012**; 67:1865–1869. Available at: http://dx.doi.org/10.1093/jac/dks156.
6. Simpson EH. Measurement of Diversity. Nature **1949**; 163:688–688. Available at: http://www.nature.com/nature/journal/v163/n4148/pdf/163688a0.pdf. Accessed 26 April 2016.
7. Grundmann H, Hori S, Tanner G. Determining Confidence Intervals When Measuring Genetic Diversity and the Discriminatory Abilities of Typing Methods for Microorganisms. J. Clin. Microbiol. **2001**; 39:4190–4192. Available at: http://dx.doi.org/10.1128/JCM.39.11.4190-4192.2001.



CHAPTER 5

Comparative genomics of extended-spectrum beta-lactamase-producing E. coli (ESBL-EC) isolates from human perianal and blood cultures and retail chicken meat cultures in the Netherlands between 2013 and 2015

Pepijn Huizinga, Marjolein Kluytmans-van den Bergh, Maarten van Smeden, Suzan D. Pas, Silvia García-Cobos, Ina Willemsen, Carlo Verhulst, Paul H. M. Savelkoul, John W. Rossen, Alexander W. Friedrich, Jan Kluytmans

Manuscript in preparation

ABSTRACT

Background: extended-spectrum beta-lactamase-producing *E. coli* (ESBL-EC) are regularly cultured from retail chicken meat. Whether and to what extent the presence of ESBL-EC in retail chicken meat leads to transmission to humans remains unclear. Studies using conventional typing methods have frequently identified overlap in Multi-Locus Sequence Type (MLST) and ESBL genes, but studies using whole-genome sequencing have questioned the conclusions. This study investigates the molecular characteristics of ESBL-EC isolates derived from human perianal cultures, blood cultures, and retail chicken meat cultures using whole-genome sequencing.

Methods: ESBL-EC isolates from blood cultures, perianal cultures and retail chicken meat cultures collected between 2013 and 2015 from the same geographical area in Netherlands were genetically compared using whole-genome sequencing. The presence or absence of genes usually located on mobile genetic elements was used as data for discriminant analyses of principal components. From this analysis, predicted group membership was compared to the observed origin of the culture to identify mispredicted isolates.

Findings: 90 blood culture isolates, 85 perianal culture isolates, and 231 isolates from retail chicken meat cultures were genetically compared. Three (4%) carriage-derived ESBL-EC isolates were found in wgMLST clades that were specific to chicken meat. These isolates were also mispredicted as chicken meat-derived isolates in the discriminant analysis of principal components based (DAPC) on mobile genetic elements. Based on these two types of analyses, these human-derived ESBL-EC isolates were likely to be of chicken meat origin. Based on mobile genetic elements alone, the current study found that 6.7% (95% confidence interval (CI): 2.8% – 14.1%) of ESBL-EC isolates from blood cultures and 8.2% (95% CI: 3.8% – 16.3%) of ESBL-EC isolates from perianal cultures were mispredicted as chicken meat-derived ESBL-EC isolates in the DAPC. One carriage-derived ESBL-EC isolate was clonally related to a chicken meat-derived ESBL-EC isolate, both of ST1304 and containing $bla_{\text{CTX-M-1}}$.

Interpretation: The current study shows that human-derived and chicken meat-derived ESBL-EC isolates can, to a large extent, be distinguished from one another based on mobile-genetic elements. Some ESBL-EC isolates cultured from humans have genetic characteristics usually found in chicken meat-derived ESBL-EC isolates suggesting chicken meat as a source.

INTRODUCTION

Infections with extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E) cannot be treated reliably with second-and third-generation cephalosporins, that are frequently used as empirical sepsis therapy in the Netherlands [1]. Infections with ESBL-E are associated with increased morbidity, mortality and healthcare costs [2–7], and are often preceded by carriage of ESBL-E [8]. Historically, the most commonly detected ESBL genes were bla_{SHV} and bla_{TEM} that were mainly a healthcare-associated problem. This changed to bla_{CTX-M} genes being the most common with acquisition in the general community [9,10].

ESBL-E have been found in a multitude of different locations, including food products [11]. Poultry meat is known to frequently be contaminated with ESBL-E [12–15]. Typing of the ESBL genes and other conventional typing techniques on ESBL-E isolates such as multilocus sequence typing and pulsed-field gel electrophoreses suggested overlap between ESBL-E derived from retail chicken meat and ESBL-E derived from human carriage and disease [13,15]. Based on the available data and the fact that in the Netherlands human antimicrobial use was low and veterinary antimicrobial use was high, programs were initiated to decrease the use of veterinary antimicrobials, which reduced veterinary antibiotic use between 2009 and 2017 by 63%; for broiler farms, this was 72% [16–19].

With advancements in whole-genome sequencing techniques, more in-depth analyses can be performed than with older typing techniques, increasing the resolution of the typing methods. The study's primary aim was to investigate the genetic similarity between ESBL-EC derived from human perianal cultures and blood cultures and ESBL-EC derived from retail chicken meat. As a secondary aim, we tried to quantify the number of ESBL-EC isolates cultured from humans that were genetically so similar to ESBL-EC derived from retail chicken meat that they were mispredicted as being chicken meat-derived ESBL-EC isolates based on their mobile genetic elements.

METHODS

Study Setting

ESBL-producing *E. coli* isolates (ESBL-EC) from blood cultures, perianal cultures, and cultures from retail chicken meat were examined. All isolates were collected in the Netherlands between 2013 and 2015. All human and the majority of the chicken meat isolates were from the south of the Netherlands. Chicken meat samples from Groningen, the north of the Netherlands, were added.

Blood culture isolates

All first available phenotypically confirmed ESBL-EC isolates per patient from blood cultures between January 1, 2013, until December 31, 2015, from the following hospitals in the south of the Netherlands were collected: Amphia hospital, Elisabeth TweeSteden hospital, and Bravis hospital. The first two are large teaching hospitals, with approximately 39k admitted patients per year (2018); the latter is a general hospital with 28k patients admitted per year.

A Maldi-TOF identification was performed on all isolates from positive blood cultures (VITEK-MS, bioMérieux, Marcy l'Etoile, France or Maldi-Biotyper, Bruker, Bremen, Germany). Susceptibility testing was performed using automated machines (VITEK2, bioMérieux, Marcy l'Etoile, France or Phoenix, Becton, Dickinson and Company, Franklin Lakes, New Jersey, United States of America) [20]. If the minimal inhibitory concentration for ceftazidime, ceftriaxone or cefotaxime was >= 1 mg/L, ESBL combination disk diffusion tests were performed (Rosco, Taastrup, Denmark) [21,22].

Human perianal cultures

Isolates from perianal cultures were taken from yearly point prevalence surveys in the Amphia hospital between 2013 and 2015. Cultures were performed as described before (Wilemsen *et al.*) [23]. Only ESBL-producing *E. coli* isolates were included in the study.

Chicken meat isolates

Chicken meat samples were collected between December 2013 and August 2015. For culture methods see Huizinga *et al.* [24]. Only one of clonally related isolates in periods of two weeks was included in the study to decrease effects of batch contamination and overrepresentation of specific clones caused by the sampling strategy while keeping all detected variability.

Whole-genome sequencing

All phenotypically confirmed ESBL-E isolates were sequenced using next generation sequencing techniques. DNA was prepared using the Nextera XT library preparation kit (Illumina, San Diego, United States). Sequencing was performed on a MiSeq (Illumina, San Diego, United States) aiming at 250- to 300-bp paired-end reads using the MiSeq reagent kit v2 or v3, respectively.

Assembly was performed using SPAdes genome assembler v3.13.0, default settings with the 'careful' option [25]. Quality control parameters were extracted using Quast v5.0.1 [26]. Average nucleotide coverage was calculated from the median kmer coverages given in contig headers, multiplied by the length of contig and divided

by the length of assembled genome for contigs larger than 1000bp. The following minimum values for quality parameters were maintained; otherwise, sequencing was repeated or the isolate excluded: average nucleotide coverage ≥ 20; number of scaffolds ≤1000; N50 ≥ 15,000 bases and maximum scaffold length ≥ 50,000 bases.

Draft genomes were screened for resistance genes, plasmid replicons (for those plasmid types of which no plasmidMLST schemes are available) and virulence genes using Abricate v0.8.10 to screen the ResFinder, PlasmidFinder and VirulenceFinder databases respectively [27–30]. Databases were downloaded on May 4, 2019. A minimum nucleotide coverage and identity of 60% and 90% was used, respectively. All beta-lactam genes with less than 100% coverage and identity were checked using the raw reads with KMA v1.2.10a and the ResFinder database downloaded from www.bitbucket.org/genomicepidemiology on August 9, 2019 [31].

The updated *E. coli* phylogroups were determined using the ClermonTyping tool v1.0.0 (https://github.com/A-BN/ClermonTyping) [32,33]. Discrepancies between the *in-silico* PCR method and the Mash genome-clustering tool were resolved using the phylogroup of the nearest neighbour in the whole-genome Multi Locus Sequence Typing (wgMLST) phylogentic tree built using nearest-neighbour joining method.

The conventional MLSTs (Achtman scheme) were determined using 'mlst' by T. Seeman v2.16.1 [34,35]. Isolates with unknown ST were submitted to EnteroBase to determine a new sequence type, https://enterobase.warwick.ac.uk/species/ecoli v1.1.2 [36].

Individual pMLST alleles for the schemes: IncAC, IncF, Inchi1, inchi2, inci1, incn and pbssb1-family were determined using pMLST downloaded from bitbucket.org/genomicepidemiology/pmlst on September 25, 2019. [29]

wgMLST was determined using Ridom SeqSphere + v5.1.0 as described by Kluytmans-van den Bergh *et al.*[37]. A wgMLST pairwise distance matrix was built, and a neighbour-joining tree was constructed. Visual representation and annotation of the neighbour-joining tree were performed on the website's interactive tree of life website, www.itol.embl.de [38]. For the wgMLST coverage-based rarefaction curves, clusters were determined using hierarchical clustering in R v3.6.0 using hclust (method = "average") and cutree from the *stats* package with a genetic distance cut-off value for clonality of 0.0095 [37]. A much less stringent cut-off value of 0.1 was arbitrarily used in the neighbour-joining tree to indicate cross domain isolates pairs located near one another.

Statistics

Discriminant analysis of principal components (DAPC) was performed using R with the adegenet (v2.1.1) package [39,40]. Predicted group memberships were compared to the origin of the culture. The number of principal components to retain was set so that the model explained 95% of the variance. As input a binary matrix of presence or absence of the genes from the following databases were used: ResFinder, pMLST, PlasmidFinder if no pMLST scheme was available and VirulenceFinder, an alternative is presented with the same data with exception of genes from the VirulenceFinder database. To gain insight into the amount of overfitting, 10-fold cross-validation was used. Coverage-based rarefaction and extrapolation sampling curves were constructed for ESBL genes, MLST and wgMLST clusters using the iNEXT package (v2.0.20) using q=0 [41,42]. If multiple ESBL genes were present in an isolate, the combination was interpreted as a new ESBL gene.

RESULTS

From blood cultures, 102 phenotypically confirmed ESBL-EC isolates were collected and sequenced and yielded 90 genetically confirmed ESBL-EC isolates. From 1625 perianal cultures, 87 phenotypically confirmed ESBL-EC isolates were collected; this resulted in 85 genotypically confirmed ESBL-EC isolates from 81 individual patients. From 465 chicken meat cultures, 333 ESBL-EC isolates were collected and sequenced; after applying the exclusion criteria specified in Figure 1, 231 genotypically confirmed ESBL-EC isolates remained for further analyses. The exact number of cultures per domain and number of selected sequenced isolates for analyses are shown in Figure 1.

The largest differences in phenotypically determined susceptibilities between human- and chicken meat-derived ESBL-EC are for amoxicillin-clavulanic acid and piperacillin-tazobactam where the human isolates are resistant in more than 50% and 20% of the isolates respectively compared to 7.8% and 0.0% for the isolates from retail chicken meat. For the aminoglycosides, resistance in human-derived ESBL-EC isolates is detected in between 20.0 – 50.0% of the isolates where this is lower than 4.0% in ESBL-EC isolates derived from retail chicken meat. Results of phenotypic susceptibility testing are shown in Supplementary Table 1.

The detected ESBL genes and the percentage of isolates the genes were detected in per source are shown in Table 1. In ESBL-EC isolates derived from humans, $bla_{\text{CTX-M-15}}$ was the most frequently detected gene, found in 52.2% of the ESBL positive blood cultures and 43.5% of ESBL positive perianal cultures. The four most commonly

detected ESBL genes in chicken meat were *bla*$_{CTX-M-1}$ (40.3%), *bla*$_{SHV-12}$ (38.5%), *bla*$_{TEM-52C}$ (10.8%) and *bla*$_{TEM-52B}$ (7.8%), corresponding to 97.4% of the detected ESBL genes in chicken meat. These four genes represented 15.6%, and 27.1% of the ESBL genes detected in blood cultures and carriage isolates, respectively.

Blood cultures	n
Unique patients with ESBL-EC bacteremia	102
Isolates selected for sequencing	102

Perianal cultures	n
Patients eligible	1907
Cultures not obtained	258
Cultures with negative growth control	24
Cultures available	1625
Cultures negative for ESBL-EC	1541
Cultures positive for ESBL-EC	84
Cultures with 1 ESBL-EC isolate	81
Cultures with 2 ESBL-EC isolates	3
Isolates selected for sequencing	87

Retail chicken meat cultures	n
Cultures available	465
Cultures negative for ESBL-EC	196
Cultures positive for ESBL-EC	269
Cultures with 1 ESBL-EC isolate	210
Cultures with 2 ESBL-EC isolates	54
Cultures with 3 ESBL-EC isolates	5
Isolates selected for sequencing	333

Exclusion criteria	Blood cultures	Perianal cultures	Chicken meat
Isolates not available for sequencing	6	0	2
Sequence quality insufficient	0	1	20
cgMLST < 90%	0	0	1
No ESBL gene detected	6	1	4
Escherichia fergusonii	0	0	1
Assumed batch clonality	0	0	74
Total isolates included for analyses	90	85	231

Figure 1. The number of included human perianal cultures, human blood cultures, and retail chicken meat cultures collected between 2013 and 2015 in the Netherlands with the phenotypically selected isolates from those cultures in the top row. Exclusion criteria of these selected ESBL-EC isolates per domain in the bottom table.

There were seven isolates that had two ESBL genes; one from a blood culture with bla$_{CTX-M-3}$ and *bla*$_{CTX-M-14b}$, three from human carriage with *bla*$_{CTX-M-32}$ and *bla*$_{CTX-M-27}$, *bla*$_{SHV-12}$ and *bla*$_{CTX-M-15}$, *bla*$_{TEM-52C}$ and *bla*$_{CTX-M-14}$, and three from chicken meat two with the combination *bla*$_{SHV-12}$ and *bla*$_{CTX-M-1}$ and one with *bla*$_{CTX-M-2}$ and *bla*$_{CTX-M-1}$.

In human-derived ESBL-EC isolates, the dominant phylogroup was B2 with 46.7% of blood culture isolates, and 32.9% of the carriage isolates coming from this group. In chicken meat-derived ESBL-EC isolates the most common phylogroups were A (32.5%), B1 (23.4%) and F (19.9%); together encompassing 75.8% of the chicken meat isolates. From blood cultures 23.3% and from perianal cultures 32.3% of the ESBL-EC isolates fell in these three phylogroups, see Table 1.

Table 1. ESBL genes and phylogroups detected in the ESBL-producing *E. coli* from blood cultures, perianal cultures and cultures from retail chicken meat. The percentage is based on the number of isolates the ESBL gene was detected in; some isolates contained more than one ESBL gene.

	Blood		Perianal		Chicken	
	n	%	n	%	n	%
ESBL genes						
blaCTX-M-1	12	13.3%	17	20.0%	93	40.3%
blaSHV-12	1	1.1%	5	5.9%	89	38.5%
blaCTX-M-15	47	52.2%	37	43.5%	4	1.7%
blaTEM-52C	1	1.1%	1	1.2%	25	10.8%
blaCTX-M-14	11	12.2%	10	11.8%	0	0.0%
blaTEM-52B	0	0.0%	0	0.0%	18	7.8%
blaCTX-M-27	6	6.7%	8	9.4%	0	0.0%
blaCTX-M-14b	5	5.6%	4	4.7%	0	0.0%
blaCTX-M-32	1	1.1%	3	3.5%	1	0.4%
blaCTX-M-3	2	2.2%	1	1.2%	0	0.0%
blaCTX-M-2	1	1.1%	0	0.0%	2	0.9%
other	4	4.4%	2	2.4%	2	0.9%
Total number of detected genes	91		88		234	
Phylogroup						
A	9	10.0%	13	15.3%	75	32.5%
B1	7	7.8%	10	11.8%	54	23.4%
B2	42	46.7%	28	32.9%	2	0.9%
C	8	8.9%	6	7.1%	14	6.1%
Clade I	0	0.0%	1	1.2%	2	0.9%
D	19	21.1%	19	22.4%	18	7.8%
E	0	0.0%	1	1.2%	20	8.7%
F	5	5.6%	7	8.2%	46	19.9%
Number of isolates	90		85		231	

Eighteen multi locus sequence types (ST) were found in both human and retail chicken meat cultures. Most commonly detected ST in ESBL-EC isolates derived from retail chicken meat was ST117 (14.7%), which was also found in 2.4% of the ESBL-EC isolates derived from human perianal cultures. ST38 was found in 8.9%, 10.6% and 2.2% of ESBL-EC isolates from blood cultures, perianal cultures and cultures from retail chicken meat respectively, see Supplementary Table 2.

Discriminant analyses of principal components (DAPC)

DAPC was performed to test if isolates could be classified into the domain of origin based on genes usually located on mobile genetic elements: antimicrobial resistance genes, pMLST alleles or plasmid replicons and virulence genes. DAPC maximises between-group variance and minimises within-group variance [39]. The DAPC plot showed a clear separation between human- and chicken-derived ESBL-E isolates (x-axis), whilst the human blood and carriage isolates remain more admixed (y-axis), Figure 2. Using group membership probabilities per isolate obtained from the model, the predicted domains were compared to the actual origin, see Table 2. Isolates derived from chicken meat were almost always (99.6%) predicted as chicken meat isolates. Among the human-derived isolates, 6.7% of blood culture and 8.2% of carriage isolates were predicted as having chicken meat origin. The blood and carriage ESBL-EC isolates were more admixed between themselves, resulting in more frequent mispredictions.

During cross-validation, the mispredictions slightly increased. However, the conclusions did not change: low percentage of chicken-derived isolates mispredicted as human, higher percentage of human-derived isolates predicted as having a chicken origin and mixed human carriage and blood culture isolates, Table 2. The analysis was also repeated, excluding the VirulenceFinder database's genes leading to identical conclusions, see Supplementary Table 3.

Table 2. Predicted origin of the isolates by discriminant analysis of principal components against the actual origin of the isolates. Resistance genes, pMLST alleles, plasmid replicons and virulence genes are used for the primary model. Ten-fold cross-validation was performed, averages (95% confidence intervals) of mispredictions are shown.

Model	Predicted origin	Actual origin		
		Blood (n=90)	Carriage (n=85)	Chicken (n=231)
Primary model	Blood	78.9% (71)	21.2% (18)	0.0% (0)
	Carriage	14.4% (13)	70.6% (60)	0.4% (1)
	Chicken	6.7% (6)	8.2% (7)	99.6% (230)
		Average % (95% CI) of mispredictions in ten-fold cross-validation		
Cross-validation	Blood	52.2% (37.7 - 66.8)	43.5% (35.7 - 51.3)	0.9% (0.0 - 2.2)
	Carriage	36.7% (28.2 - 45.1)	39.3% (24.6 - 54.1)	1.3% (0.0 - 2.8)
	Chicken	11.1% (3.6 - 18.6)	17.2% (7.1 - 27.3)	97.8% (96.2 - 99.5)

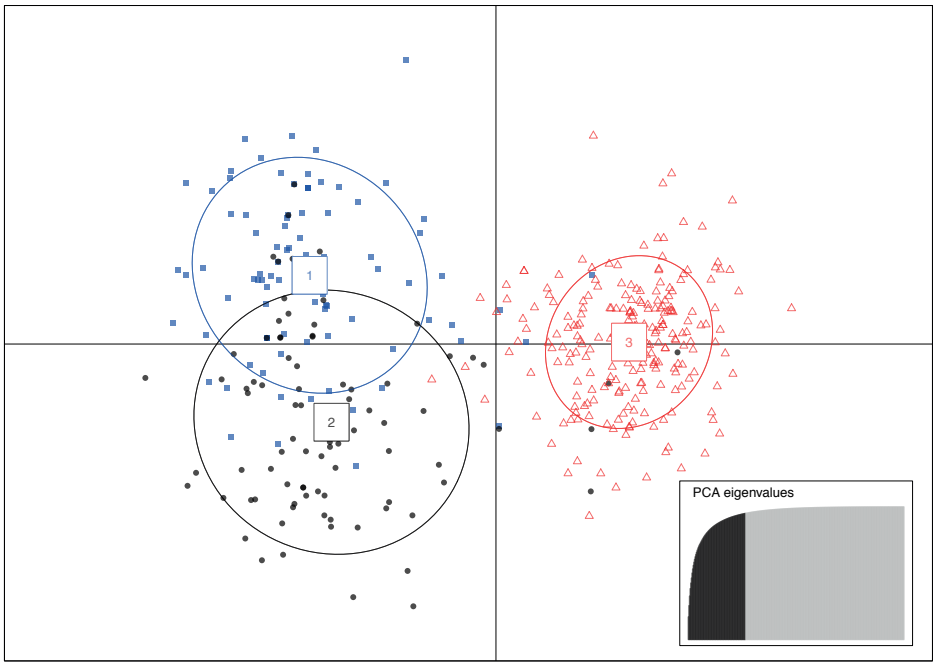


Figure 2. Scatterplot of the discriminant analyses of principal components based on antimicrobial resistance genes, pMLST alleles, plasmid replicons, and virulence genes. The axes are the two discriminant functions with the highest discriminatory power. Legend: blue squares are blood culture-derived isolates, black circles are carriage-derived isolates, and red triangles are chicken meat-derived isolates. Principal Component Analyses (PCA) eigenvalues, in black the retained PCA eigenvalues in the dimension-reduction step.

Whole-genome MLST

The neighbour-joining tree of the wgMLST analysis annotated with the domain of origin, phylogroup, ESBL gene and MLST of the ESBL-EC isolates is shown in Figure 3. Isolates that belonged to ST131 and ST8347 in phylogroup B2 showed little genomic variation and all isolates were cultured from humans. Isolates from ST117 in phylogroup F and isolates in phylogroup E were almost all cultured from chicken meat. Phylogroups A, B1, C and D were mixed for the origin of the isolates. Clusters that contained human- and chicken meat-derived isolates with a genetic distance ≤0.1 are shown in Figure 3. In phylogroup B1 and C isolates from humans and chicken meat were mixed throughout the phylogenetic clade. Whilst, in phylogroup A and D the isolates from the same source groups were more clustered together.

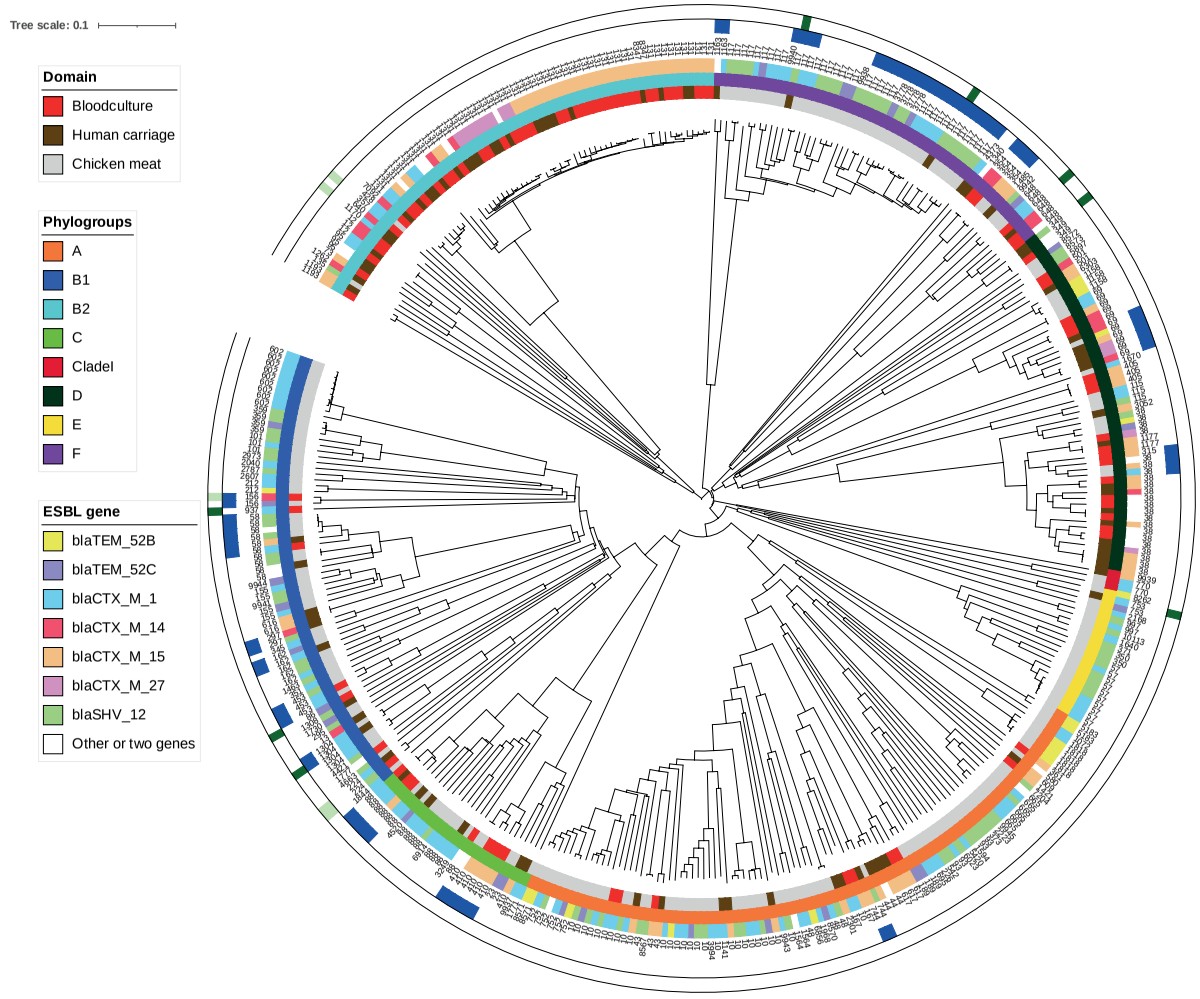


Figure 3. Neighbour-joining tree based on the wgMLST analysis of 406 ESBL-producing *E. coli* isolates derived from human blood cultures, human perianal cultures or retail chicken meat cultures, using a pairwise ignore missing values approach. Legend coloured rings from the inside out: domain of origin; phylogroup; ESBL genes; MLST; clusters from hierarchical clustering with a cut-off of 0.1 that contain both human- and chicken meat-derived isolates; human-derived isolates classified as chicken meat isolates in the discriminant analysis of principal components: dark green P > 75%, light green P < 75%.

The most closely related cross-domain isolate pairs had genetic distances of: 0.0066 (ST1304, carriage), 0.0183 (ST156, blood culture), 0.0220 (ST744, carriage), 0.0261 (ST453, blood culture), 0.0303 (ST1163, carriage), between 0.040 – 0.043 (ST410, two blood culture isolates and two chicken meat isolates) and between 0.049 – 0.055 (ST117, one carriage isolate related to six chicken meat isolates).

In the ST117 clade that was mainly populated with chicken meat-derived isolates, two carriage-derived isolates were located. Similarly, in phylogroup E that was also associated with chicken meat-derived isolates, one carriage-derived isolate was located. In phylogroup E, the genetic variation was larger compared to the ST117 clade.

The presence of human-derived isolates in phylogenetic clades specific to isolates with chicken meat origin could imply transmission from chicken meat to the human domain, based on the chromosomal gene content. These same three isolates were also predicted to have a chicken origin in the DAPC, which was based on genes not present in the wgMLST scheme.

Sample completeness for ESBL genes, MLST and wgMLST clusters
There is more diversity within wgMLST clusters than there are MLST than there are ESBL genes. To obtain an indication of how complete the sampling was for these entities, coverage-based rarefaction and extrapolation sampling curves were constructed, see Supplementary Figure 1. For ESBL genes and MLST the currently used sample sizes gave a reasonable indication of circulating variability; in chicken meat the sample coverage for ESBL genes was 98% (95% CI: 96 – 99%) and 76% (95% CI: 72 – 80%) for MLST. The variability in wgMLST was so large that the sample coverage for the three sources were all below 28%, see Supplementary Table 4. The sample coverage was higher for blood culture isolates due to more clonally related ST131 isolates.

DISCUSSION

The current study shows that some human-derived ESBL-EC isolates are genetically so similar to chicken meat-derived ESBL-EC isolates based on genes likely present on mobile-genetic elements, that they cannot be distinguished. This suggests a chicken meat-related origin for these isolates. Chicken meat-derived ESBL-EC isolates were almost never mispredicted as human-derived ESBL-EC isolates based on mobile genetic elements. Three human-derived ESBL-EC isolates (4%) were found in wgMLST clades specific to chicken meat-derived ESBL-EC isolates. These isolates were also mispredicted as chicken meat-derived isolates based on their mobile genetic elements.

Transmission of antimicrobial resistance can occur through horizontal transfer of mobile genetic elements and clonal transfer of bacteria [43,44]. For horizontal gene transfer, based on whole-genome sequencing analyses, several papers have found that mobile genetic elements are, to some extent, likely to spread from retail chicken meat to humans. A study from the United Kingdom described that 5% of human isolates potentially share antimicrobial resistance-associated mobile genetic elements with those found in livestock [45]. Another study from the Netherlands showed that plasmids found in humans were highly similar to plasmids found in poultry [15,46]. The current study confirms 6.7% (95% CI: 2.8% – 14.1%) of ESBL-EC isolates from blood cultures and 8.2% (95% CI: 3.8% – 16.3%) of ESBL-EC isolates from perianal cultures were predicted to have a chicken meat origin, based on mobile genetic elements. This indicates transfer of mobile genetic elements from retail chicken meat to humans. In contrast, chicken meat-derived isolates can almost always, 0.4% (95% CI: <0.01- 2.7%), be distinguished from human-derived isolates based on their mobile genetic elements.

When, like in the current study, clonal transmission is sought after in a larger geographical area without a direct epidemiological link between source and acceptor, the number of circulating variants and their abundancies are important. For ESBL genes and to a lesser extent for MLST, the maximum variability is quickly achieved in the currently studied dataset. For wgMLST the sample completeness curves showed that the variability was so large that even within the domains themselves the chance of finding clonal isolates was small unless they were of an abundant clade; ST131 in humans and ST117 in chicken meat.

In studies with similar designs, also using whole-genome sequencing, varying frequencies of cross-domain clonally-related isolate pairs were found. Liu *et al.* found 0.5% of *E. coli* in urinary tract infections in Flagstaff Arizona to be caused by ST131-*H22*+ColV, which most likely originated from retail meat [53]. In rural Ghana, around 10% of ESBL *E. coli* isolates in children were closely related to isolates found in locally produced poultry [54]. Ludden et al. showed clonally related isolates cultured from different farms sampled within the same month, which can be expected due to tight production pyramids [45,55]. They also showed highly related, <15 SNPs, non-ESBL-EC isolate pairs derived from animals and humans cultured 6 to 8 years apart. It is unknown whether cross-domain transmission led to prolonged colonisation in humans or whether more recent transmission events occurred. De Been *et al.* look at isolates previously identified as a potential clonal transfer from poultry meat to humans in the Netherlands as determined with MLST, plasmid typing and antibiotic resistance gene sequencing and found that there were large genetic differences between the five isolate pairs that were previously indistinguishable [15,46]. Although only one case of

cross-domain clonally-related isolates was found in the current study, the sample size is far from sufficient to exclude other cross-domain clonal-transmission events from occurring, as is also described by Hanage [60].

Compared to a study five years before the current study in identical setting, the ESBL gene distribution changed considerably. The proportion of $bla_{CTX\text{-}M\text{-}1}$ in carriage isolates was 58% in 2008 – 2009 and decreased to 20% in the current study. No evident change in ESBL-E carriage prevalence was seen between the two time periods [23]. For ESBL-EC isolates from blood cultures a similar decrease in the proportion of $bla_{CTX\text{-}M\text{-}1}$ occurred: from 33% in 2008 – 2009 to 13% in the current study. Van Hout *et al.* confirmed the lower prevalence of $bla_{CTX\text{-}M\text{-}1}$ in blood cultures; 9% in 2014 – 2016 [61]. The decrease of $bla_{CTX\text{-}M\text{-}1}$ in human-derived ESBL-EC isolates coincides with the initiation of a large decrease in veterinary antimicrobial use [24,62]. Over the same period the percentage of $bla_{CTX\text{-}M\text{-}15}$ ESBL-EC isolates from perianal cultures (8%) and from blood cultures (13%) increased from 2008 – 2009 to 53% and 44% in the current study, respectively. These changes are in percentages of all detected ESBL-EC isolates and should be confirmed by prevalence numbers in future.

The strengths of our study are that the human and chicken meat samples were collected during the same period and employing similar selective culture conditions. The retail chicken meat was purchased from the largest supermarket chains in the Netherland with a combined market share of 70%, and chicken breast fillet is the most sold cut of chicken meat in the Netherlands [63,64]. Advantages of culturing retail meat instead of livestock is that imported meat is also included, giving the most accurate representation of what the population at risk of acquisition is exposed to.

The study also has some limitations. The chicken meat and human blood cultures were collected in a more continues fashion than the point prevalence surveys of the human perianal samples [23,24]. Chicken meat isolates were excluded if one or more isolates from clonal pairs were cultured within two weeks of each other to decrease the effect of batch contamination further exaggerated by the sampling strategy. Occasionally, multiple samples were purchased at a single time point to find all present genetic variability, unfortunately at the cost of finding isolates more than once. This was only done for isolates from retail chicken meat cultures and not for isolates from the blood cultures and perianal cultures where the variability was similarly large.

The dilemma of short-read sequencing and plasmid reconstruction remains. Although different methods to extract plasmids from short-read sequence data have been published and are being developed it remains error-prone [65–68]. Sequencing

complete plasmids using long-read sequencing techniques and comparing them between the domains would have been an important addition to the study but unfortunately was beyond this project's scope.

Concluding, the current study finds evidence for cross-domain transmission of mobile genetic elements from retail chicken meat to humans. In the current dataset based on mobile genetic elements alone 6.7% (95% CI: 2.8% – 14.1%) of ESBL-EC isolates from blood cultures and 8.2% (95% CI: 3.8% – 16.3%) of ESBL-EC isolates from perianal cultures were mispredicted as chicken meat-derived ESBL-EC isolates. There are also a limited number (4%) of human-derived ESBL-EC isolates which suggest transmission of complete ESBL-producing *E. coli* clones from retail chicken meat to humans. Comparing ESBL-EC isolates from a similar study from 2008 - 2009 with the current study, $bla_{CTX\text{-}M\text{-}1}$ has become less frequent and the presence of $bla_{CTX\text{-}M\text{-}15}$ has increased [56]. During the same period, there was a considerable reduction in the use of antimicrobial agents in the veterinary sector as potential explanation for the decline in $bla_{CTX\text{-}M\text{-}1}$. The current study shows that there are clearly different genetic populations of ESBL-EC found in chicken meat and humans, but there are also remarkable similarities and overlap. We conclude that in general, sources of antimicrobial-resistant clones will remain a risk for introduction of a next high-risk clone to the human population [69]. Therefore, decreasing antimicrobial resistance by striving towards low antibiotic use across all sectors remains a logical preventative measure.

FUNDING

Part of the sequence data was generated and reused from the Dutch Food & Nutrition Delta Programme 2013 project PathoDetect.

This study was also partially funded by the Amphia Hospital and the medical specialist company Amphia (MSB-A), Breda, the Netherlands.

This study was also partly supported by the Interreg IVa-funded projects "EurSafety Health-Net - EURegional network for patient safety and protection against infections" and "SafeGuard", as part of a Dutch–German cross-border network supported by the European Commission, German Ministry of Economic Affairs, Energy and Industry of the State of North Rhine- Westphalia, and German Ministry for Economic Affairs, Labour, Transport and Digitalisation of the State of Lower Saxony, and the Dutch Provinces of Overijssel, Gelderland, and Limburg.

REFERENCES

1. de Greef S, Mouton J, Schoffelen A, Verduin C. NethMap 2019: Consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands / MARAN 2019: Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands in 2018. 2019.
2. Melzer M, Petersen I. Mortality following bacteraemic infection caused by extended spectrum beta-lactamase (ESBL) producing E. coli compared to non-ESBL producing E. coli. J Infect. 2007;55: 254–259. doi:10.1016/j.jinf.2007.04.007
3. Rottier WC, Ammerlaan HSM, Bonten MJM. Effects of confounders and intermediates on the association of bacteraemia caused by extended-spectrum b -lactamase-producing Enterobacteriaceae and patient outcome : a meta-analysis. 2012; 1311–1320. doi:10.1093/jac/dks065
4. Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect Dis. 2018;3099: 1–11. doi:10.1016/S1473-3099(18)30605-4
5. Tumbarello M, Spanu T, Di Bidino R, Marchetti M, Ruggeri M, Trecarichi EM, et al. Costs of bloodstream infections caused by Escherichia coli and influence of extended-spectrum-beta-lactamase production and inadequate initial antibiotic therapy. Antimicrob Agents Chemother. 2010;54: 4085–4091. doi:10.1128/AAC.00143-10
6. de Kraker MEA, Davey PG, Grundmann H. Mortality and hospital stay associated with resistant Staphylococcus aureus and Escherichia coli bacteremia: estimating the burden of antibiotic resistance in Europe. PLoS Med. 2011;8: e1001104. doi:10.1371/journal.pmed.1001104
7. Mauldin PD, Salgado CD, Hansen IS, Durup DT, Bosso JA. Attributable hospital cost and length of stay associated with health care-associated infections caused by antibiotic-resistant gram-negative bacteria. Antimicrob Agents Chemother. 2010;54: 109–115. doi:10.1128/AAC.01041-09
8. Rottier WC, Deelen JWT, Werkhoven CH Van, Bonten MJM. 'Development of diagnostic prediction tools for bacteraemia caused by 3rd generation cephalosporin-resistant Enterobacteriaceae in suspected bacterial infections' e Author' s reply. Clin Microbiol Infect. European Society of Clinical Microbiology and Infectious Diseases; 2018;24: 1349–1350. doi:10.1016/j.cmi.2018.09.003
9. Livermore DM, Canton R, Gniadkowski M, Nordmann P, Rossolini GM, Arlet G, et al. CTX-M: Changing the face of ESBLs in Europe [Internet]. Journal of Antimicrobial Chemotherapy. 2007. pp. 165–174. doi:10.1093/jac/dkl483
10. Bunt G Van Den, Fluit AC, Spaninks MP, Timmerman AJ, Geurts Y, Kant A, et al. Faecal carriage , risk factors , acquisition and persistence of ESBL- producing Enterobacteriaceae in dogs and cats and co-carriage with humans belonging to the same household. 2019; 1–9. doi:10.1093/jac/dkz462
11. Woolhouse M, Ward M, van Bunnik B, Farrar J. Antimicrobial resistance in humans, livestock and the wider environment. Philos Trans R Soc Lond B Biol Sci. 2015;370: 20140083. doi:10.1098/rstb.2014.0083

12. Overdevest I, Willemsen I, Rijnsburger M, Eustace A, Xu L, Hawkey P, et al. Extended-spectrum β-lactamase genes of Escherichia coli in chicken meat and humans, the Netherlands. Emerg Infect Dis. 2011;17: 1216–1222. doi:10.3201/eid1707.110209
13. Kluytmans JAJW, Overdevest ITMA, Willemsen I, Bergh MFQK Den, Zwaluw K Van Der, Heck M, et al. Extended-Spectrum β -Lactamase – Producing Escherichia coli From Retail Chicken Meat and Humans : Comparison of Strains , Plasmids , Resistance Genes , and Virulence Factors. 2013;56: 478–487. doi:10.1093/cid/cis929
14. Cohen Stuart J, van den Munckhof T, Voets G, Scharringa J, Fluit A, Hall ML Van. Comparison of ESBL contamination in organic and conventional retail chicken meat. Int J Food Microbiol. Elsevier B.V.; 2012;154: 212–214. doi:10.1016/j.ijfoodmicro.2011.12.034
15. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Voets GM, van den Munckhof MP, van Essen-Zandbergen A, et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect. 2011;17: 873–880. doi:10.1111/j.1469-0691.2011.03497.x
16. Veldman K, Mevius D. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2017 (MARAN 2018) [Internet]. 2018. Available: https://www.wur.nl/en/show/Maran-rapport-2018.htm
17. Bos MEH, Taverne FJ, van Geijlswijk IM, Mouton JW, Mevius DJ, Heederik DJJ. Consumption of Antimicrobials in Pigs, Veal Calves, and Broilers in The Netherlands: Quantitative Results of Nationwide Collection of Data in 2011. PLoS One. 2013;8. doi:10.1371/journal.pone.0077525
18. Bos MEH, Mevius DJ, Wagenaar J a., van Geijlswijk IM, Mouton JW, Heederik DJJ. Antimicrobial prescription patterns of veterinarians: introduction of a benchmarking approach. J Antimicrob Chemother. 2015; 1–3. doi:10.1093/jac/dkv104
19. Avined. Antibioticumgebruik pluimveesector in 2017 [Internet]. 2018. Available: https://www.avined.nl/sites/www.avined.nl/files/antibioticagebruik_-_sectorrapportage_2017.pdf
20. The European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters, version 7.0, 2017. http://www.eucast.org. 2017.
21. European Committee on Antimicrobial Susceptibility Testing. Guidelines for detection of resistance mechanisms and specific resistance of clinical and/or epidemiological importance. Version 2.0 [Internet]. 2017. Available: www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Resistance_mechanisms/EUCAST_detection_of_resistance_mechanisms_170711.pdf
22. Netherlands Society for Medical Microbiology. NVMM Guideline Laboratory detection of highly resistant microorganisms, version 2.0, 2012. Leeuwarden, Netherlands. 2012; Available: http://www.nvmm.nl/system/files/2012.11.15 richtlijn BRMO %2528version 2.0%2529 - RICHTLIJN.pdf
23. Willemsen I, Oome S, Verhulst C, Pettersson A, Verduin K, Kluytmans J. Trends in Extended Spectrum Beta-Lactamase (ESBL) producing enterobacteriaceae and ESBL genes in a Dutch teaching hospital, measured in 5 yearly point prevalence surveys (2010-2014). PLoS One. 2015;10: 1–10. doi:10.1371/journal.pone.0141765
24. Huizinga P, Van Den Bergh MK, Rossen JW, Willemsen I, Verhulst C, Savelkoul PHM, et al. Decreasing prevalence of contamination with extended-spectrum beta-lactamaseproducing Enterobacteriaceae (ESBL-E) in retail chicken meat in the Netherlands. PLoS One. 2019;14: 1–17. doi:10.1371/journal.pone.0226828
25. Bankevich A, Nurk S, Antipov D, Gurevich A a., Dvorkin M, Kulikov AS, et al. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. J Comput Biol. 2012;19: 455–477. doi:10.1089/cmb.2012.0021

26. Gurevich A, Saveliev V, Vyahhi N, Tesler G. QUAST: Quality assessment tool for genome assemblies. Bioinformatics. 2013; doi:10.1093/bioinformatics/btt086
27. Seemann T. Abricate [Internet]. Github; Available: https://github.com/tseemann/abricate
28. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012;67: 2640–2644. doi:10.1093/jac/dks261
29. Carattoli A, Zankari E, Garci??-Fern??ndez A, Larsen MV, Lund O, Villa L, et al. In Silico detection and typing of plasmids using plasmidfinder and plasmid multi-locus sequence typing. Antimicrob Agents Chemother. 2014; doi:10.1128/AAC.02412-14
30. Chen L, Zheng D, Liu B, Yang J, Jin Q. VFDB 2016: Hierarchical and refined dataset for big data analysis - 10 years on. Nucleic Acids Res. 2016;44: D694–D697. doi:10.1093/nar/gkv1239
31. Clausen PTLC, Aarestrup FM, Lund O. Rapid and precise alignment of raw reads against redundant databases with KMA. BMC Bioinformatics. BMC Bioinformatics; 2018;19: 1–8. doi:10.1186/s12859-018-2336-6
32. Beghain J, Bridier-Nahmias A, Le Nagard H, Denamur E, Clermont O. ClermonTyping: an easy-to-use and accurate in silico method for Escherichia genus strain phylotyping. Microb genomics. 2018;4. doi:10.1099/mgen.0.000192
33. Clermont O, Phane Bonacorsi S, Bingen E. Rapid and Simple Determination of the Escherichia coli Phylogenetic Group. Appl Environ Microbiol. 2000;66: 4555–4558.
34. Wirth T, Falush D, Lan R, Colles F, Mensa P, Wieler LH, et al. Sex and virulence in Escherichia coli: An evolutionary perspective. Mol Microbiol. 2006;60: 1136–1151. doi:10.1111/j.1365-2958.2006.05172.x
35. Seemann T. mlst. In: https://github.com/tseemann/mlst.
36. Alikhan NF, Zhou Z, Sergeant MJ, Achtman M. A genomic overview of the population structure of Salmonella. PLoS Genet. 2018;14: 1–13. doi:10.1371/journal.pgen.1007261
37. Kluytmans-van den Bergh MFQ, Rossen JWA, Bruijning-Verhagen PCJ, Bonten MJM, Friedrich AW, Vandenbroucke-Grauls CMJE, et al. Whole genome multi-locus sequence typing of extended-spectrum beta-lactamase-producing Enterobacteriaceae. J Clin Microbiol. 2016;54: 2919–2927. doi:10.1128/JCM.01648-16
38. Letunic I, Bork P. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. Oxford University Press; 2019;47: W256–W259. doi:10.1093/nar/gkz239
39. Jombart T, Devillard S, Balloux F. Discriminant analysis of principal components: A new method for the analysis of genetically structured populations. BMC Genet. 2010;11. doi:10.1186/1471-2156-11-94
40. Jombart T, Collins C. A tutorial for Discriminant Analysis of Principal Components (DAPC) using adegenet. Rvignette. 2015; 1–37. doi:10.1038/72708
41. Chao A, Gotelli NJ, Hsieh TC, Sander EL, Ma KH, Colwell RK, et al. Rarefaction and extrapolation with Hill numbers: a framework for sampling and estimation in species diversity studies. Ecol Monogr. 2014;84: 45–67. doi:10.1890/13-0133.1
42. Hsieh TC, Ma KH, Chao A. iNEXT: an R package for rarefaction and extrapolation of species diversity (Hill numbers). McInerny G, editor. Methods Ecol Evol. 2016;7: 1451–1456. doi:10.1111/2041-210X.12613
43. Muloi D, Ward MJ, Pedersen AB, Fèvre EM, Woolhouse MEJ, van Bunnik BAD. Are Food Animals Responsible for Transfer of Antimicrobial-Resistant *Escherichia coli* or Their Resistance Determinants to Human Populations? A Systematic Review. Foodborne Pathog Dis. 2018; doi:10.1089/fpd.2017.2411

44. Lipsitch M, Singer RS, Levin BR. Antibiotics in agriculture: When is it time to close the barn door? Proc Natl Acad Sci U S A. 2002;99: 5752–5754. doi:10.1073/pnas.092142499
45. Ludden C, Raven KE, Jamrozy D, Gouliouris T, Blane B, Coll F, et al. One Health Genomic Surveillance of Escherichia coli Demonstrates Distinct Lineages and Mobile Genetic Elements in Isolates from Humans versus Livestock. Sansonetti PJ, editor. MBio. 2019;10: 1–12. doi:10.1128/mBio.02693-18
46. de Been M, Lanza VF, de Toro M, Scharringa J, Dohmen W, Du Y, et al. Dissemination of Cephalosporin Resistance Genes between Escherichia coli Strains from Farm Animals and Humans by Specific Plasmid Lineages. PLoS Genet. 2014;10. doi:10.1371/journal.pgen.1004776
47. Kluytmans-van den Bergh MFQ, Bruijning-Verhagen PCJ, Vandenbroucke-Grauls CMJE, de Brauwer EIGB, Buiting AGM, Diederen BM, et al. Contact precautions in single-bed or multiple-bed rooms for patients with extended-spectrum β-lactamase-producing Enterobacteriaceae in Dutch hospitals: a cluster-randomised, crossover, non-inferiority study. Lancet Infect Dis. 2019;3099. doi:10.1016/S1473-3099(19)30262-2
48. Nguyen VT, Jamrozy D, Matamoros S, Carrique-Mas JJ, Ho HM, Thai QH, et al. Limited contribution of non-intensive chicken farming to ESBL-producing *Escherichia coli* colonisation in humans in Vietnam: an epidemiological and genomic analysis. J Antimicrob Chemother. 2019; doi:10.1093/jac/dky506
49. Fischer J, Hille K, Ruddat I, Mellmann A, Köck R, Kreienbrock L. Simultaneous occurrence of MRSA and ESBL-producing Enterobacteriaceae on pig farms and in nasal and stool samples from farmers. Vet Microbiol. Elsevier B.V.; 2017;200: 107–113. doi:10.1016/j.vetmic.2016.05.021
50. Dierikx C, van der Goot J, Fabri T, van Essen-Zandbergen A, Smith H, Mevius D. Extended-spectrum-β-lactamase- and AmpC-β-lactamase-producing Escherichia coli in Dutch broilers and broiler farmers. J Antimicrob Chemother. 2013;68: 60–67. doi:10.1093/jac/dks349
51. Dahms C, Hubner N-O, Kossow A, Mellmann A, Dittmann K, Kramer A. Occurrence of ESBL-Producing Escherichia coli in Livestock and Farm Workers in Mecklenburg-Western Pomerania, Germany. PLoS One. United States; 2015;10: e0143326. doi:10.1371/journal.pone.0143326
52. Dohmen W, Bonten MJM, Bos MEH, van Marm S, Scharringa J, Wagenaar JA, et al. Carriage of extended-spectrum β-lactamases in pig farmers is associated with occurrence in pigs. Clin Microbiol Infect. Elsevier Ltd; 2015;21: 917–923. doi:10.1016/j.cmi.2015.05.032
53. Liu CM, Stegger M, Aziz M, Johnson TJ, Waits K, Nordstrom L, et al. Escherichia coli ST131-H22 as a Foodborne Uropathogen. MBio. 2018;9: 1–11. doi:10.1128/mBio.00470-18
54. Falgenhauer L, Imirzalioglu C, Oppong K, Akenten CW, Hogan B, Krumkamp R, et al. Detection and Characterisation of ESBL-Producing Escherichia coli From Humans and Poultry in Ghana. Front Microbiol. 2019;9: 1–8. doi:10.3389/fmicb.2018.03358
55. Dierikx CM, van der Goot JA, Smith HE, Kant A, Mevius DJ. Presence of ESBL/AmpC-producing Escherichia coli in the broiler production pyramid: a descriptive study. PLoS One. United States; 2013;8: e79005. doi:10.1371/journal.pone.0079005
56. Kluytmans JAJW, Overdevest ITMA, Willemsen I, Kluytmans-Van Den Bergh MFQ, Van Der Zwaluw K, Heck M, et al. Extended-spectrum beta-lactamase-producing escherichia coli from retail chicken meat and humans: Comparison of strains, plasmids, resistance genes, and virulence factors. Clin Infect Dis. 2013;56: 478–487. doi:10.1093/cid/cis929

57. Mughini-Gras L, Dorado-García A, van Duijkeren E, van den Bunt G, Dierikx CM, Bonten MJM, et al. Attributable sources of community-acquired carriage of Escherichia coli containing β-lactam antibiotic resistance genes: a population-based modelling study. Lancet Planet Heal. 2019;3: e357–e369. doi:10.1016/S2542-5196(19)30130-5
58. Dorado-García A, Smid JH, van Pelt W, Bonten MJM, Fluit AC, van den Bunt G, et al. Molecular relatedness of ESBL/AmpC-producing Escherichia coli from humans, animals, food and the environment: a pooled analysis. J Antimicrob Chemother. 2018;73: 339–347. doi:10.1093/jac/dkx397
59. Day MJ, Rodríguez I, van Essen-Zandbergen A, Dierikx C, Kadlec K, Schink AK, et al. Diversity of STs, plasmids and ESBL genes among Escherichia coli from humans, animals and food in Germany, the Netherlands and the UK. J Antimicrob Chemother. 2016;71: 1178–1182. doi:10.1093/jac/dkv485
60. Hanage WP. Two health or not two health? That is the question. MBio. 2019;10: 1–4. doi:10.1128/mBio.00550-19
61. Hout D Van, Verschuuren TD, Bruijning-verhagen PCJ, Bosch T. Extended-spectrum beta-lactamase (ESBL) -producing and non-ESBL- producing Escherichia coli isolates causing bacteremia in the Netherlands (2014 – 2016) differ in ST distribution , antimicrobial resistance gene and virulence gene content. 2019;
62. Veldman K, Mevius D. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2015 (MARAN 2016) [Internet]. 2016. doi:10.21945/RIVM-2017-0056
63. Terluin I, Verhoog D, Dagevos H, van Horne P, Hoste R. Vleesconsumptie per hoofd van de bevolking in Nederland, 2005-2016 [Internet]. Wageningen; 2017. doi:10.18174/424550
64. Nielsen. Markets and Finances. In: Markets and Finances [Internet]. 2017 [cited 14 Oct 2019]. Available: https://www.nielsen.com/nl/nl/insights/article/2017/nielsen-release-market-shares/
65. Rozov R, Kav AB, Bogumil D, Shterzer N, Halperin E, Mizrahi I, et al. Recycler: An algorithm for detecting plasmids from de novo assembly graphs. Bioinformatics. 2017;33: 475–482. doi:10.1093/bioinformatics/btw651
66. Stohr JJJM, Kluytmans-Van Den Bergh MFQ, Wedema R, Friedrich AW, Kluytmans JAJW, Rossen JWA. Detection of extended-spectrum beta-lactamase (ESBL) genes and plasmid replicons in enterobacteriaceae using plasmidspades assembly of short-read sequence data. Microb Genomics. 2020;6: 6–12. doi:10.1099/mgen.0.000400
67. Arredondo-Alonso S, Rogers MRC, Braat JC, Verschuuren TD, Top J, Corander J, et al. Mlplasmids: a User-Friendly Tool To Predict Plasmid- and Chromosome-Derived Sequences for Single Species. Microb genomics. 2018;4: 1–15. doi:10.1099/mgen.0.000224
68. Arredondo-Alonso S, Van Schaik W, Willems RJ, Schürch AC. On the (im)possibility to reconstruct plasmids from whole genome short-read sequencing data. doi:10.1101/086744
69. Smith DL, Harris AD, Johnson JA, Silbergeld EK, Morris JG. Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. Proc Natl Acad Sci. 2002; doi:10.1073/pnas.082188899

SUPPLEMENTARY MATERIALS

Supplementary Table 1. Percentage of isolates phenotypically resistant to the antimicrobial. Abbreviations: AMC, amoxicillin-clavulanic acid; TZP, piperacillin-tazobactam; MEM, meropenem; CIP, ciprofloxacin; TMP trimethoprim; SXT, trimethoprim-sulfamethoxazole; GEN, gentamicin; TOB, tobramycin; NIT, nitrofurantoin; CST, colistin. *Results not available for two isolates.

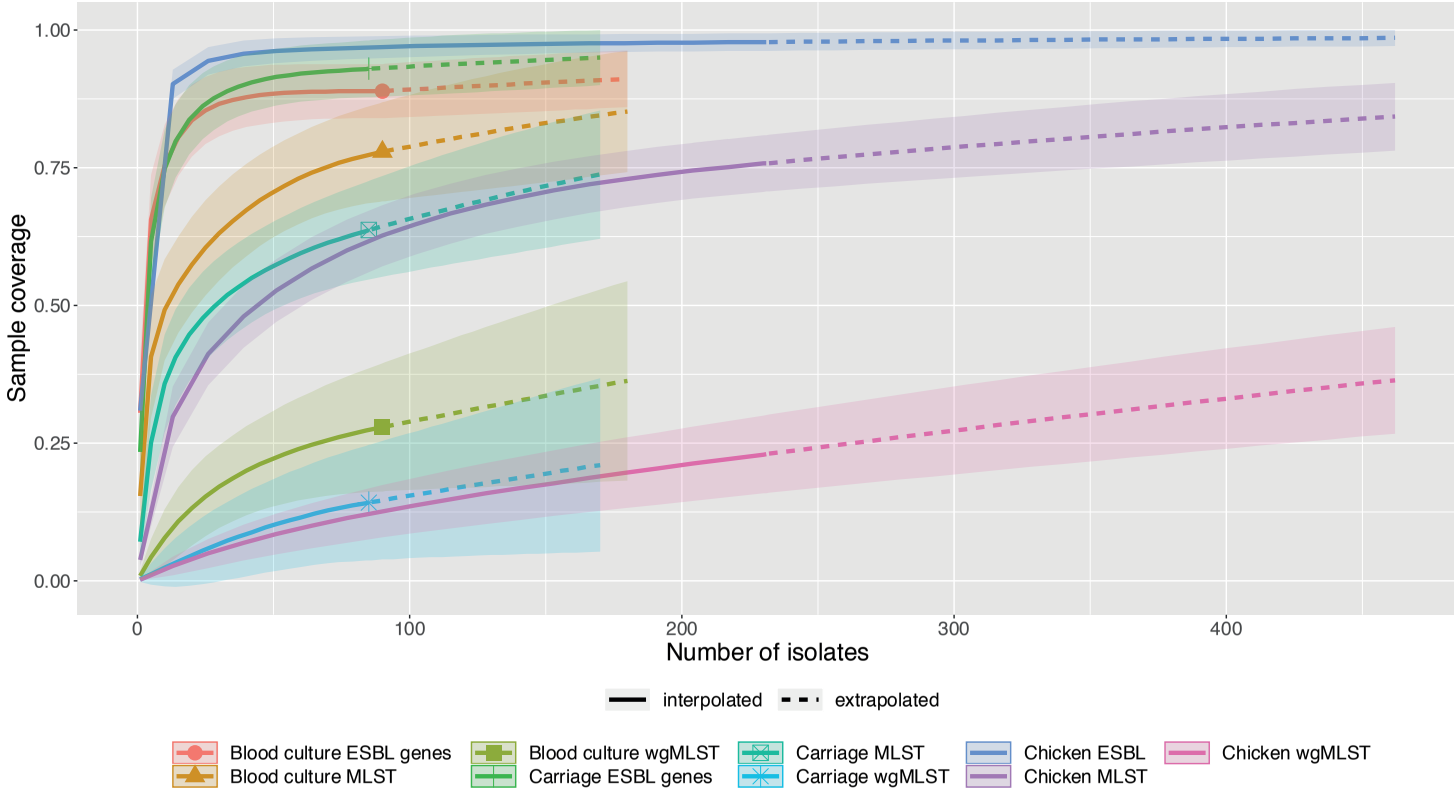
	Blood culture (n=90)		Carriage (n=85)		Chicken meat (n=231)	
	n	% resistant	n	% resistant	n	% resistant
AMC	47	52.2%	43	50.6%	18	7.8%
TZP	18	20.0%	19	22.4%	0	0.0%
MEM	0	0.0%	0	0.0%	0	0.0%
CIP	66	73.3%	48	56.5%	74	32.0%
TMP	52	57.8%	54	63.5%	110	47.6%
SXT	57	63.3%	56	65.9%	110	47.6%
GEN	32	35.6%	18	21.2%	9	3.9%
TOB	45	50.0%	23	27.1%	7	3.0%
NIT	1*	1.1%	1	1.2%	4	1.7%
CST	0	0.0%	0	0.0%	2	0.9%

Supplementary Table 2. The number of isolates per domain with a sequence type found in both a human and chicken meat domain.

ST	Blood		Carriage		Chicken meat	
	n	%	n	%	n	%
117	0	0.0%	2	2.4%	34	14.7%
10	3	3.3%	3	3.5%	22	9.5%
38	8	8.9%	9	10.6%	5	2.2%
88	4	4.4%	4	4.7%	6	2.6%
69	3	3.3%	5	5.9%	3	1.3%
58	1	1.1%	2	2.4%	7	3.0%
410	4	4.4%	1	1.2%	2	0.9%
648	3	3.3%	1	1.2%	2	0.9%
162	0	0.0%	1	1.2%	4	1.7%
1304	0	0.0%	1	1.2%	3	1.3%
354	2	2.2%	0	0.0%	2	0.9%
43	1	1.1%	1	1.2%	1	0.4%
453	1	1.1%	0	0.0%	2	0.9%
297	0	0.0%	1	1.2%	1	0.4%
744	0	0.0%	1	1.2%	1	0.4%
753	0	0.0%	1	1.2%	1	0.4%
1163	0	0.0%	1	1.2%	1	0.4%
156	1	1.1%	0	0.0%	1	0.4%
Non-cross-domain ST	59	65.6%	51	60.0%	133	57.6%
Total	90		85		231	

Supplementary Table 3. Predicted origin of the isolates by discriminant analysis of principal components against the actual origin of the isolates. The model used resistance genes, pMLST alleles and plasmid replicons as input. The virulence genes were excluded compared with the primary model.

Predicted origin	Actual origin		
	Blood (n=90)	Carriage (n=85)	Chicken (n=231)
Blood	76.7% (69)	23.5% (20)	0.0% (0)
Carriage	15.6% (14)	64.7% (55)	0.4% (1)
Chicken	7.8% (7)	11.8 (10)	99.6% (230)

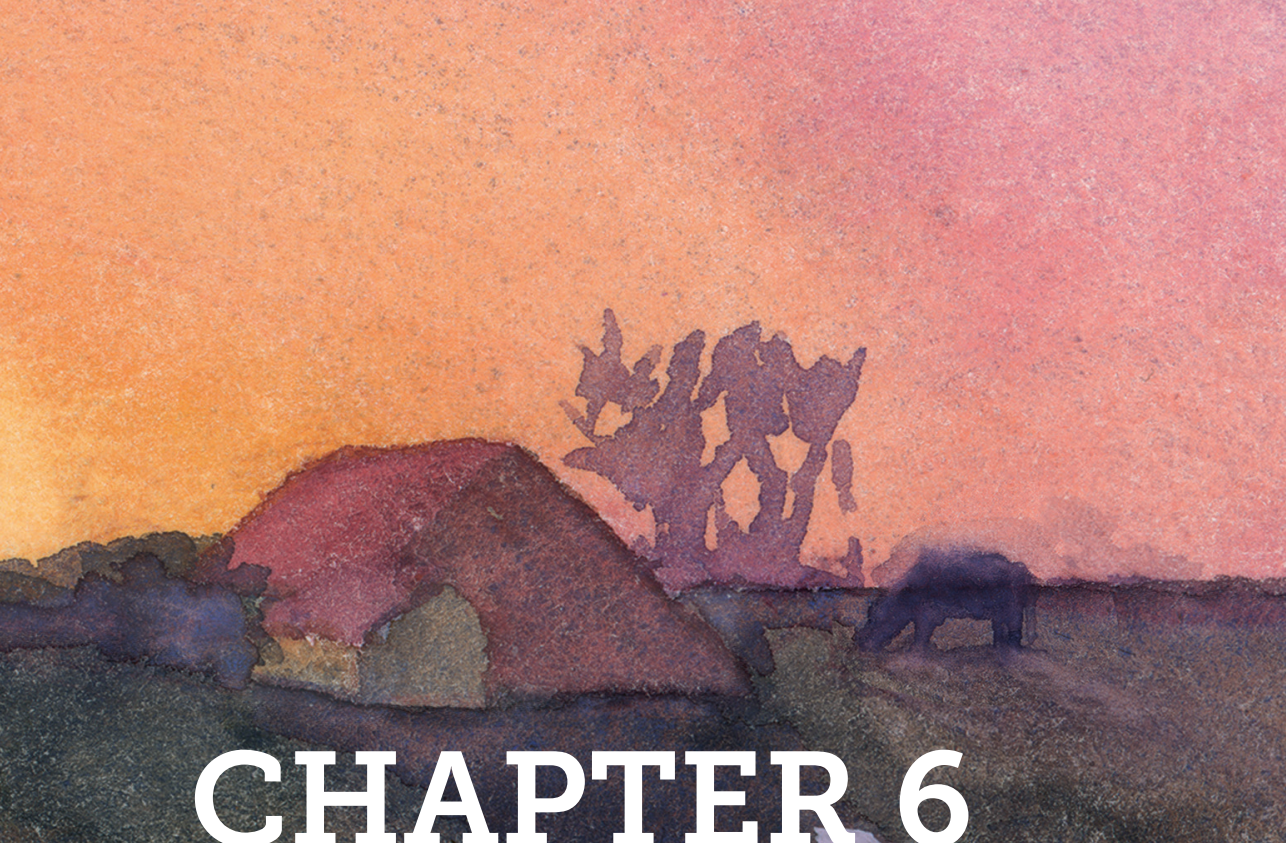


Supplementary Figure 1. Coverage-based rarefaction and extrapolation curves. Sample coverage is the proportion of the total number of different individuals that belong to the entity detected in the sample, in this case, ESBL genes, multi-locus sequence types or whole-genome multi-locus sequence-types.

Supplementary Table 4. Sample coverage, species richness and estimate of the richness asymptote for ESBL genes, MLST and wgMLST in the three domains.

	n	Observed sample coverage	Lower 95% CI	Upper 95%CI
ESBL genes in blood cultures	90	0,89	0,83	0,94
ESBL genes in carriage	85	0,93	0,88	0,98
ESBL genes in chicken meat	231	0,98	0,96	0,99
MLST in blood cultures	90	0,78	0,69	0,87
MLST in carriage	85	0,64	0,54	0,73
MLST in chicken meat	231	0,76	0,72	0,80
wgMLST in blood cultures	90	0,28	0,16	0,40
wgMLST in carriage	85	0,14	0,06	0,23
wgMLST in chicken meat	231	0,23	0,16	0,30

Observed species richness	Estimate of richness asymptote	Estimated bootstrap s.e.	Lower 95% CI	Upper 95% CI
15	59,5	29,8	28,5	161,5
13	30,8	23,3	15,5	139,5
11	23,4	17,1	12,7	104,4
32	81,4	33,9	46,6	198,9
42	137,0	55,3	74,9	316,0
90	220,1	52,4	150,8	368,3
73	595,3	292,4	260,7	1526,3
78	955,7	547,6	363,3	2778,4
199	1126,8	266,3	733,6	1809,5



CHAPTER 6

Presence of *mcr*-1-positive Enterobacteriaceae in retail chicken meat but not in humans in the Netherlands since 2009

Marjolein Kluytmans-van den Bergh, Pepijn Huizinga, Marc Bonten, Martine Bos, Katrien De Bruyne, Alexander Friedrich, John Rossen, Paul Savelkoul, Jan Kluytmans

Adapted from: Euro Surveill 2016;21(9):pii=30149.

ABSTRACT

Background

Recently, the plasmid-mediated colistin resistance gene *mcr-1* was found in Enterobacteriaceae from humans, pigs and retail meat in China. Several reports have documented the global presence of the *mcr-1* gene in Enterobacteriaceae from humans, livestock and food since.

Objective

This study aimed to screen several well-characterised collections of Enterobacteriaceae isolates, obtained from retail chicken meat and hospitalised patients in the Netherlands since 2009, for the presence of colistin resistance and the *mcr-1* gene.

Methods

Available antimicrobial susceptibility data and whole-genome sequence (WGS) data were screened for the presence of colistin resistance and the *mcr-1* gene, respectively.

Results

A total of 2,471 Enterobacteriaceae isolates, from surveys in retail chicken meat (196 isolates), prevalence surveys in hospitalised patients (1,247 isolates), clinical cultures (813 isolates) and outbreaks in healthcare settings (215 isolates), were analysed. The *mcr-1* gene was identified in three (2%) of 196 extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* isolates from retail chicken meat samples in 2009 and 2014. Two isolates were obtained from the same batch of meat samples, most likely representing contamination from a common source. No *mcr-1*-positive isolates were identified among 2,275 human isolates tested. All *mcr-1*-positive isolates were colistin resistant (minimum inhibitory concentration (MIC) >2 mg/L).

Conclusions

The findings of this study indicate that *mcr-1*-based colistin resistance currently poses no threat to healthcare in the Netherlands. However, continued monitoring of colistin resistance and its underlying mechanisms in humans, livestock, and food is needed.

INTRODUCTION

The worldwide emergence of extended-spectrum beta-lactamases (ESBL) and carbapenemases has severely limited the available treatment options for infections with Gram-negative bacteria.[1] Colistin is an antibiotic of last resort for the treatment of infections with carbapenem-resistant bacteria, and its use in humans is increasing [1].

In November 2015, the presence of a plasmid-mediated colistin resistance gene, *mcr-1*, in Enterobacteriaceae from food, livestock, and humans in China was reported [2]. The *mcr-1* gene was detected in 21% (166/804) of *E. coli* isolates cultured from pigs at slaughter and in 15% (78/523) of *E. coli* isolates cultured from retail meat between 2011 and 2014. In addition, the *mcr-1* gene was present in 1% (13/902) of *E. coli* isolates and 1% (3/420) of *Klebsiella pneumoniae* isolates from clinical cultures from patients in two Chinese hospitals in 2014. Directly following this publication, the *mcr-1* gene was reported to be present in <1% (1/417) of ESBL- and AmpC-producing *E. coli* isolates from human bloodstream infections, and in 2% (5/255) of ESBL- and AmpC-producing *E. coli* isolates cultured from imported chicken meat in Denmark since 2012 [3]. Hereafter, several reports have documented the global presence of the *mcr-1* gene in Enterobacteriaceae cultured from humans, livestock, and food [4–13].

Traditionally, colistin resistance was thought to be mediated by chromosomal mutations only, and to spread exclusively via clonal transmission of resistant isolates [14]. The emergence of plasmid-mediated colistin resistance enables the much more efficient horizontal transfer of colistin-resistance genes to other bacteria, making *mcr-1* a potential threat to public health. The aim of this study was to screen several well-documented collections of Enterobacteriaceae isolates, obtained from retail chicken meat and hospitalised patients in the Netherlands since 2009, for the presence of colistin resistance and the *mcr-1* gene.

METHODS

Enterobacteriaceae isolates

A total number of 2,471 Enterobacteriaceae isolates were analysed for the presence of colistin resistance and the *mcr-1* gene. Isolates originated from prevalence surveys in retail chicken meat (196 isolates), prevalence surveys in hospitalised patients (1,247 isolates), clinical cultures (813 isolates) and several outbreaks in healthcare settings (215 isolates), all collected in the Netherlands between 2009 and 2015.

Retail chicken meat

Two ESBL-producing Enterobacteriaceae (ESBL-E) prevalence surveys in Dutch retail chicken meat were performed in 2009 and in 2014 [15,16]. A total number of 196 ESBL-E isolates were obtained, 74 isolates from 71 ESBL-E-positive meat samples in 2009 (89 samples cultured), and 122 isolates from 86 ESBL-E-positive meat samples in 2014 (101 meat samples cultured).

Hospitalised patients, rectal samples

The retail chicken meat surveys in 2009 and 2014 were accompanied by hospital-wide prevalence surveys in patients who were admitted to four hospitals in the region where the chicken meat was bought [15,16]. In 2009, ESBL-E rectal carriage was detected in 45 (5%) of 876 patients, who carried 50 ESBL-E isolates. Two repeated prevalence surveys in one of the four hospitals in 2013 and 2014 yielded 63 ESBL-E isolates obtained from 63 ESBL- E carriers (6%) among 1,065 patients cultured [17].

A multi-centre cluster-randomised study comparing isolation strategies for known ESBL-E-positive patients was performed in 14 Dutch hospitals between 2011 and 2014 (SoM study) [18]. All consecutive adult patients with a routine clinical culture with ESBL-E were nursed under contact precautions and enrolled in the study (index patient). Ward-based ESBL-E prevalence surveys were performed one week after enrolment of the index patient. Perianal swabs were obtained from 10,691 patients and identified 992 (9%) ESBL-E carriers, from whom 1,134 ESBL-E isolates were cultured.

Hospitalised patients, clinical cultures

In 2009, 2013 and 2014, all consecutive ESBL-E isolates from blood cultures were prospectively collected in the four hospitals that participated in the ESBL-E rectal carriage prevalence surveys [15,16]. A total number of 102 ESBL-E isolates from blood cultures were obtained, 25 isolates from 23 patients with an ESBL-E-positive blood culture in 2009, and 77 isolates from 76 patients in 2013 and 2014. Three isolates that were collected in 2014 were not available for whole-genome sequencing (WGS). In the SoM study, a total number of 711 clinical ESBL-E isolates were obtained from 654 ESBL-E-positive patients.

Outbreaks in healthcare settings

Since 2009, several outbreaks with antimicrobial-resistant bacteria in Dutch hospitals and nursing homes have been documented. Six outbreaks, comprising 215 isolates, for which WGS data were available, were included in this analysis: (i) an outbreak of CTX-M-15-producing *K. pneumoniae* in several wards of a hospital and an associated rehabilitation centre between 2012 and 2015 (29 isolates) [19]; (ii)

an outbreak of *Enterobacter cloacae* in a surgical ward in 2014 (14 isolates); (iii) an outbreak of colistin-resistant *E. cloacae* in an intensive care unit between 2009 and 2014 (86 isolates); (iv) an outbreak of colistin-resistant KPC-producing *K. pneumoniae* in a nursing home in 2012 (10 isolates) [20]; (v) an outbreak of colistin-resistant *K. pneumoniae* in patients after endoscopic retrograde cholangiopancreaticography (ERCP) procedures in 2014 and 2015 (50 isolates); and (vi) an outbreak of (intrinsic) colistin-resistant *Serratia marcescens* in a neonatal intensive care unit in 2014 and 2015 (26 isolates).

WGS and analysis of sequence data

WGS was performed, on either a MiSeq, HiSeq 2500 or NextSeq sequencer (Illumina, San Diego, CA, USA). *De novo* assembly was performed using CLC genomics Workbench 7.0.4 (Qiagen, Hilden, Germany) or the open source SPAdes 3.5.0 software [21]. Sequence data were screened for the presence of the *mrc-1* gene by running the assembled sequences against a task template containing the *mcr-1* gene sequence in Ridom SeqSphere+ v3.0.1 (Ridom, Münster, Germany) or by uploading the assembled sequences to the open access bioinformatics webtool ResFinder (updated version 2.1, including the *mcr-1* sequence) of the Center for Genomic Epidemiology (Technical University Denmark, Lingby, Denmark) [22]. For isolates from two outbreaks (colistin-resistant *E. cloacae* and ERCP-related colistin-resistant *K. pneumonia*), the thresholds for sequence identity and coverage length were set to 98% and 60%, respectively, while for all other isolates both thresholds were set to 80%. The sequence data of the *mcr-1*-positive isolates were further analysed by using a genotyping plugin that allowed serotyping of the isolates and detection of acquired antibiotic resistance genes and plasmids with an 80% threshold for both sequence identity and percent alignment (BioNumerics v7.6 beta software, Applied Maths, Sint-Martens-Latem, Belgium). Reference data for acquired antimicrobial resistance genes and plasmid replicons were retrieved from the ResFinder and PlasmidFinder databases (version November 9th, 2015) of the Center for Genomic Epidemiology (Technical University Denmark, Lingby, Denmark) [22,23]. Whole-genome multilocus sequence typing (wgMLST) analysis was performed using a pan-genome MLST scheme comprising 9,347 genes, based on 19 well-annotated reference genomes of *E. coli* and *Shigella* spp. (BioNumerics v7.6 beta, Applied Maths, Sint-Martens-Latem, Belgium). Additionally, single-nucleotide polymorphism (SNP) calling was performed by mapping the paired-end reads of isolate 14M009387 and isolate 213 to the *de novo* assembled genome of isolate 14M009386, using Bowtie 2.5.5 and SAMtools [24,25]. Resulting Binary Alignment Maps (BAM) files were used to perform whole-genome SNP (wgSNP) analysis (BioNumerics v7.6 beta, Applied Maths, Sint-Martens-Latem, Belgium).

Table 1. Colistin resistance and presence of the *mcr-1* gene in 2,471 Enterobacteriaceae isolates from retail chicken meat, rectal samples, clinical cultures and outbreaks by origin, year of culture and type of isolate.

Origin	Year of culture
Retail chicken meat	
Prevalence survey (n=74)	2009
Prevalence survey (n=122)	2014
Hospitalised patients, rectal samples	
Prevalence survey, 4 hospitals (n=50)	2009
Prevalence surveys, 1 hospital (n=63)	2013–2014
Prevalence surveys, 14 hospitals (n=1,134)	2011–2014
Hospitalised patients, clinical cultures	
Blood cultures, 4 hospitals (n=25)	2009
Blood cultures, 4 hospitals (n=77)	2013–2014
Clinical cultures, 14 hospitals (n=711)	2011–2014

Type of isolate	Number of isolates		
	Total	Colistin resistant[a]	*mcr-1* positive
ESBL-producing *Escherichia coli*	68	NA[b]	1
ESBL-producing *Klebsiella pneumoniae*	6	NA	0
ESBL-producing *E. coli*	119	2	2
ESBL-producing *K. pneumoniae*	3	0	0
ESBL-producing *E. coli*	39	NA	0
ESBL-producing *K. pneumoniae*	11	NA	0
ESBL-producing *E. coli*	54	0	0
ESBL-producing *K. pneumoniae*	8	0	0
ESBL-producing *Klebsiella oxytoca*	1	0	0
ESBL-producing *E. coli*	821	2	0
ESBL-producing *K. pneumoniae*	172	3	0
ESBL-producing *K. oxytoca*	13	0	0
ESBL-producing *Enterobacter cloacae*	77	2	0
ESBL-producing *Citrobacter* spp.	38	1	0
ESBL-producing *Morganella morganii*	6	6[c]	0
Other ESBL-producing Enterobacteriaceae	7	0	0
ESBL-producing *E. coli*	16	NA	0
ESBL-producing *K. pneumoniae*	7	NA	0
ESBL-producing *K. oxytoca*	2	NA	0
ESBL-producing *E. coli*	67[d]	0	0
ESBL-producing *K. pneumoniae*	8[d]	0	0
ESBL-producing *K. oxytoca*	2	0	0
ESBL-producing *E. coli*	546	4	0
ESBL-producing *K. pneumoniae*	101	2	0
ESBL-producing *K. oxytoca*	5	0	0
ESBL-producing *E. cloacae*	46	3	0
ESBL-producing *Citrobacter* spp.	4	0	0
ESBL-producing *M. morganii*	3	3[c]	0
ESBL-producing *Proteus mirabilis*	2	2[c]	0
ESBL-producing *Proteus vulgaris* group	1	1[c]	0
Other ESBL-producing Enterobacteriaceae	3	0	0

Table 1. Continued.

Origin	Year of culture
Outbreaks in healthcare settings	
Several wards, including rehabilitation centre (n=29)[e]	2012–2015
Surgical ward (n=14)	2014
Intensive care unit (n=86)	2009–2014
Nursing home (n=10)	2012
ERCP-related procedures (n=50)	2014–2015
Neonatal intensive care unit (n=26)[e]	2014–2015

Data are n. ERCP=endoscopic retrograde cholangiopancreaticography; ESBL=extended-spectrum beta-lactamase; NA=not available.
a. Colistin resistance was defined as a colistin minimum inhibitory concentration >2 mg/L, according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints.[26]

Antimicrobial susceptibility testing

Isolates for which antimicrobial susceptibility data were available were screened for the presence of colistin resistance. Susceptibility testing of the three *mcr-1*-positive *E. coli* isolates was performed using the automated Vitek2 system (bioMérieux, Marcy l'Etoile, France) and Etest (bioMérieux, Marcy l'Etoile, France). The breakpoint tables of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used for the interpretation of minimum inhibitory concentrations (MICs) [26]. Isolates with a colistin MIC >2 mg/L were considered colistin resistant.

RESULTS

An overview of the 2,471 Enterobacteriaceae isolates from retail chicken meat, rectal samples, clinical cultures and outbreaks is presented in Table 1. Colistin resistance was found in two (2%) of 122 chicken meat-derived ESBL-E isolates, in 14 (1%) of 1,247 isolates from ESBL-E rectal carriers, and in 15 (2%) of 813 ESBL-E isolates from clinical cultures. The *mcr-1* gene was detected in three (2%) of 196 chicken meat-derived ESBL-producing *E. coli* isolates, one cultured in 2009 and two in 2014. For all three isolates, the *mcr-1* sequence showed 100% similarity to the gene reported in China [2]. None of the 2,275 human isolates harboured the *mcr-1* gene.

Type of isolate	Number of isolates		
	Total	Colistin resistant[a]	*mcr-1* positive
CTX-M-15 producing *K. pneumoniae*	29	0	0
E. cloacae	14	0	0
Colistin-resistant *E. cloacae*	86	86	0
Colistin-resistant KPC-producing *K. pneumoniae*	10	10	0
Colistin-resistant *K. pneumoniae*	50	43	0
Colistin-resistant *S. marcescens*	26	26[c]	0

b. The *mcr-1*-positive isolate was tested colistin resistant with Etest.
c. Intrinsic resistance.
d. Two *E. coli* isolates and one *K. pneumoniae* isolate were not available for whole-genome sequencing.
e. Outbreak and subsequent surveillance.

Table 2 shows the general and molecular characteristics of the three *mcr-1*-positive *E. coli* isolates. The isolate that was cultured in 2009 had sequence type ST2079, was CTX-M-1-producing and harboured 17 acquired resistance genes. Both isolates from 2014 had sequence type ST117, were SHV-12-positive and harboured five acquired resistance genes. Although these two isolates were cultured from different meat samples of non-Dutch origin, the meat samples had the same lot number and were bought in the same supermarket on the same day. Plasmid replicons were identified in all three isolates, eight in the isolate from 2009 and two in both isolates from 2014. However, none of the plasmid replicons could be linked to the *mcr-1* gene.

Antimicrobial susceptibilities for the three *mcr-1*-positive *E. coli* isolates are shown in Table 3. All three isolates were colistin resistant (MIC >2 mg/L). The isolate from 2009 tested colistin susceptible by Vitek2 (MIC = 2 mg/L), but resistant by Etest (MIC = 3 mg/L). wgMLST analysis showed that the two isolates from 2014 differed by only 3 of 4,243 shared loci, whereas the isolate from 2009 differed by 3,606 of 3,791 shared loci (Table 4). The two isolates from 2014 differed by only eight SNPs in wgSNP analysis.

Table 2. Characteristics of the *mcr*-1-positive *Escherichia coli* isolates from retail chicken meat.

Isolate	Origin	Date of purchase	Supermarket	MLST
213	Chicken meat	October 14th, 2009	A	ST2079
14M009386[a]	Chicken meat	January 29th, 2014	B	ST117
14M009387[a]	Chicken meat	January 29th, 2014	B	ST117

MLST=multilocus sequence typing; ST=sequence type.

Table 3. Antimicrobial susceptibility of *mcr*-1-positive *Escherichia coli* isolates from retail chicken meat.

Antimicrobial agent	Isolate					
	213		14M009386		14M009387	
	MIC	S/I/R[a]	MIC	S/I/R[a]	MIC	S/I/R[a]
Polymyxins						
Colistin	3[b]	R	≥16	R	≥16	R
Penicillins						
Ampicillin	≥32	R	≥32	R	≥32	R
Amoxicillin/clavulanic acid	8	S	≤2	S	4	S
Piperacillin/tazobactam	≤4	S	≤4	S	≤4	S
Cephalosporins						
Cefuroxime	≥64	R	16	R	16	R
Cefotaxime	8	R	4	R	4	R
Ceftazidime	≤1	S	16	R	16	R
Cefepime	2	I	≤1	S	≤1	S
Cefoxitin	≤4	S[c]	≤4	S[c]	≤4	S[c]
Carbapenems						
Meropenem	≤0.25	S	≤0.25	S	≤0.25	S
Imipenem	≤0.25	S	≤0.25	S	≤0.25	S
Aminoglycosides						
Gentamicin	≤1.0	S	≤1	S	≤1	S
Tobramycin	≤1.0	S	≤1	S	≤1	S

Serotype	Acquired resistance genes	Plasmid replicons
O8:H19	*aadA1, aadA2, aadA3, aph(3')-Ia, aph(3'')-Ib, aph(3')-Ic, aph(6)-Id, bla*$_{CTX-M-1}$*, bla*$_{TEM-1B}$*, tet*(A), *mcr-1, lnu*(F), *cmlA1, catA1, sul2, sul3, dfrA5*	FIB, FII, HI2, HI2A, I1, I2, P, p0111
O159:H4	*aadA1, bla*$_{SHV-12}$*, mcr-1, sul1, sul3*	FIB, FII
O159:H4	*aadA1, bla*$_{SHV-12}$*, mcr-1, sul1, sul3*	FIB, FII

a. Isolate 14M009386 and 14M009387 were cultured from different meat samples with the same lot number.

Table 3. Continued.

Antimicrobial agent	Isolate					
	213		14M009386		14M009387	
	MIC	S/I/R[a]	MIC	S/I/R[a]	MIC	S/I/R[a]
Fluoroquinolones						
Ciprofloxacin	0.5	S	≤0.25	S	≤0.25	S
Norfloxacin	2	R	≤0.5	S	≤0.5	S
Folate pathway inhibitors						
Trimethoprim/ sulfamethoxazole	≥16/304	R	≤1/19	S	≤1/19	S

Data are mg/L. I=intermediate; MIC=minimum inhibitory concentration; R=resistant; S=susceptible.
a. According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoints.[26]
b. Etest: MIC = 3 mg/L; Vitek2: MIC = 2 mg/L.
c. No clinical breakpoint available; S refers to the screening breakpoint for AmpC-producing Enterobacteriaceae.

Table 4. wgMLST and wgSNP analyses of *mcr*-1-positive *Escherichia coli* isolates from retail chicken meat.

	wgMLST[a]			wgSNP[a]
Isolate	Loci shared	Allele differences		SNP positions
14M009387	4,243	3	(0.001)	8
213	3,791	3,606	(0.951)	100,215

Data are n or n (proportion). SNP=single-nucleotide polymorphism; wgMLST=whole-genome multilocus sequence typing; wgSNP=whole-genome single-nucleotide polymorphism.
a. Isolate 14M0009386 was used as a reference.

DISCUSSION

The recently described plasmid-mediated colistin resistance gene, *mcr-1*, was identified in three ESBL-producing *E. coli* isolates from retail chicken meat samples obtained from Dutch supermarkets in 2009 and 2014. All three *mcr-1*-positive isolates were colistin resistant, and two of them were genetically closely related. No *mcr-1*-positive isolates were detected in a large collection of Enterobacteriaceae isolates of human origin that were collected during the same period and included isolates of four outbreaks with colistin-resistant Enterobacteriaceae.

In addition to recent reports on the global occurrence of the *mcr-1* gene in Enterobacteriaceae cultured from humans, livestock, and food [2–4,6–13], the findings of this study confirm the presence of the *mcr-1* gene in the European setting already since 2009.

The observed 2% prevalence of *mcr-1*-positive isolates is comparable to the reported 2% (5/255) prevalence in ESBL- and AmpC-producing *E. coli* isolates from imported chicken meat in Denmark, and is lower than the 15% (78/523) prevalence in *E. coli* from retail meat in China [2,3]. This lower prevalence may be related to the relatively low rates of polymyxin use in livestock in Europe. In 2014, polymyxins constituted <1% (0.34 defined daily dose animal (DDDA)/animal-year) of all antibiotics used in broilers in the Netherlands, with a decreasing trend over the last few years [27].

It is noteworthy that the observed 2% prevalence of *mcr-1*-positive isolates in ESBL-E isolates from retail chicken meat in this study is similar to the 2% phenotypic colistin resistance that was found in *E. coli* isolates cultured from Dutch retail chicken meat in 2014 [27]. Unfortunately, no data are currently available on the resistance mechanisms involved in this phenotypic colistin resistance.

The genetic identity of the two *mcr-1*-positive isolates that were obtained from the same batch of meat samples most likely represents batch contamination from a common source.

The *mcr-1*-positive isolates in this study belong to other sequence types than those found to be related to the *mcr-1* gene in the Chinese and Danish study[2,3]. *E. coli* ST2097 is uncommon in humans, but has been reported once before in a study on ESBL-producing bacteria in flies from broiler farms in the Netherlands [28]. *E. coli* ST117, on the other hand, is common in both poultry and humans [15,29]. The detection of the *mcr-1* gene in isolates that belong to different sequence types illustrates the potential for horizontal transfer of this resistance gene.

Although all chicken meat samples were bought in Dutch supermarkets, the labelling of the samples did not provide any clue with respect to the country where animals were raised. Available data on the origin of the chicken meat were limited to the producing country for the samples from 2014 (non-Dutch, European); for the 2009 isolate this information was not available. A non-European origin of the *mcr-1*-positive meat samples can, therefore, neither be confirmed, nor excluded.

The absence of the *mcr-1* gene in human isolates of various origins is in accordance with observations in previous studies that the presence of the *mcr-1* gene in clinical isolates is still rare. In China, 1% (13/902) of clinical *E. coli* isolates and 1% (3/420) of clinical *K. pneumoniae* isolates were *mcr-1* positive, and in Denmark <1% (1/417) of ESBL- and AmpC-producing *E. coli* isolates from bloodstream infections [2,3]. This absence of the *mcr-1* gene in current Dutch collections of human Enterobacteriaceae may in part be due to the low use of colistin and its analogues, the polymyxins, in humans in the Netherlands. In 2014, polymyxins constituted <1% (0.01 defined daily dose (DDD)/1,000 inhabitant-days) of all systemic antimicrobials used in primary care and <1% (0.2 DDD/100 patient-days) of systemic antimicrobials used in the hospital setting [30].

Short-read sequence data are not optimal for the assembly of plasmid sequences, which are known to contain multiple repetitive elements. This may explain why the analysis of our sequence data did not reveal a link between the *mcr-1* gene and the plasmid replicons identified.

Although the prevalence of *mcr-1*-positive isolates in meat samples was low, the presence of this colistin resistance gene in food represents a potential public health threat, as it is located on mobile genetic elements that have the potential to spread horizontally to other bacteria. With the increase in carbapenem resistance, the use of colistin is increasing and, herewith, the selective pressure for the spread of *mcr-1* gene-containing plasmids. As colistin has become one of the last-resort antibiotic options to treat severe infections with Gram-negative bacteria, the continued monitoring of colistin resistance and its underlying resistance mechanisms is important, not only in humans, but also in livestock and food. The emergence of plasmid-mediated colistin resistance underpins the recent proposal of veterinary experts to reconsider the use of colistin and its analogues in livestock [31].

In conclusion, the plasmid-mediated colistin resistance gene *mcr-1* was detected in three ESBL-producing *E. coli* isolates that had been cultured from retail chicken meat from Dutch supermarkets in 2009 and 2014. Two isolates were obtained from

the same batch of meat samples, which most likely represents contamination from a common source. The *mcr-1* gene was not present in a large collection of human Enterobacteriaceae isolates collected between 2009 and 2015 in the Netherlands. These findings indicate that *mcr-1*-based colistin resistance currently poses no threat to healthcare in the Netherlands, but requires continued monitoring of colistin resistance and its underlying mechanisms in humans, livestock, and food.

ACKNOWLEDGMENTS

We are grateful to the members of the SoM study group for their contribution to this study.

FINANCIAL SUPPORT

This study was supported by The Netherlands Organisation for Health Research and Development (ZonMw, project number 205100010) and the Food & Nutrition Delta Program 2013 (Pathodetect).

CONFLICTS OF INTEREST

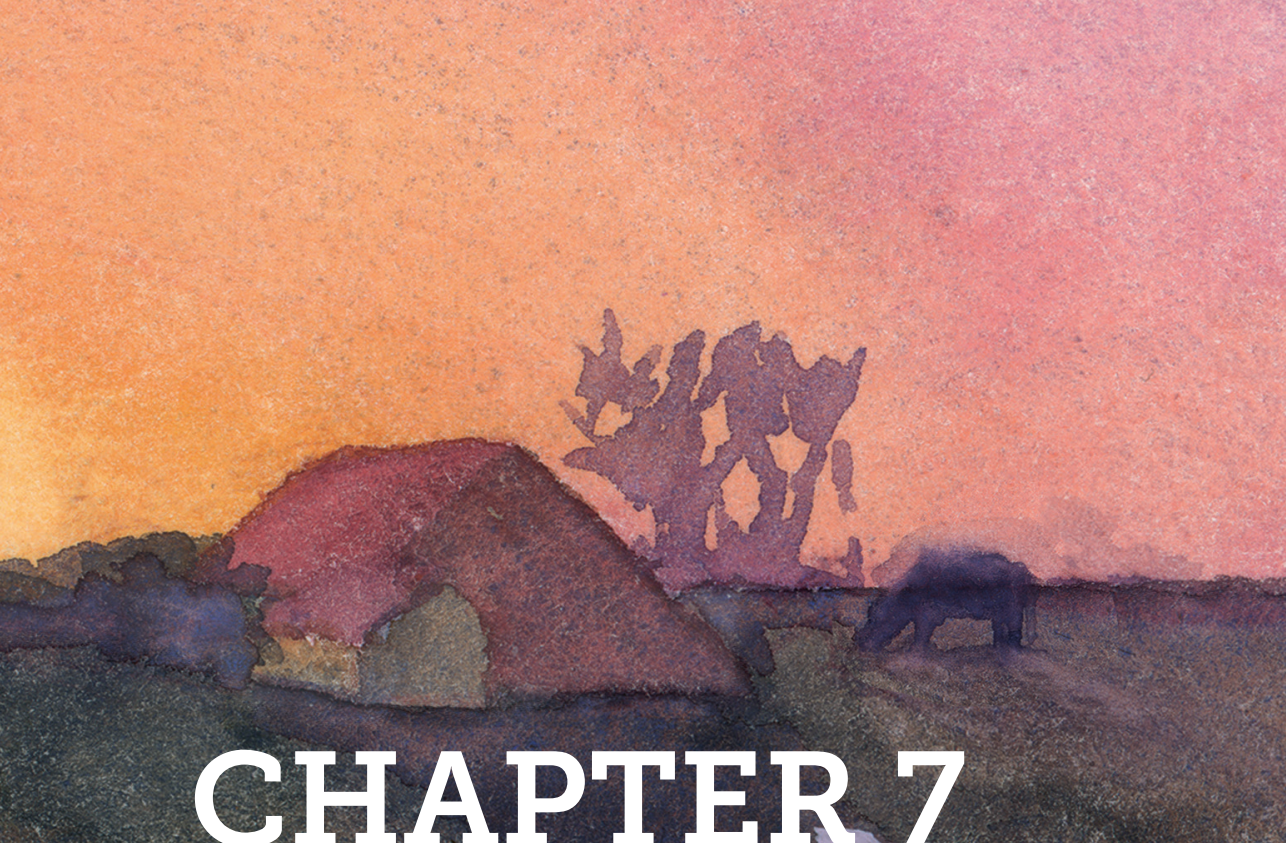
Katrien De Bruyne is an employee of Applied Maths (Sint-Martens-Latem), a company that develops and sells software for microbiological typing methods. All other authors report no conflicts of interest.

REFERENCES

1. Doi Y, Paterson DL. Carbapenemase-producing Enterobacteriaceae. Semin Respir Crit Care Med. 2015;36: 74–84. doi:10.1055/s-0035-1544208
2. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. Lancet Infect Dis. Elsevier Ltd; 2016;16: 161–168. doi:10.1016/S1473-3099(15)00424-7
3. Hasman H, Hammerum AM, Hansen F, Hendriksen RS, Olesen B, Agersø Y, et al. Detection of mcr-1 encoding plasmid-mediated colistin-resistant Escherichia coli isolates from human bloodstream infection and imported chicken meat, Denmark 2015. Euro Surveill. 2015;20: 1–5. doi:10.2807/1560-7917.ES.2015.20.49.30085
4. Arcilla MS, van Hattem JM, Matamoros S, Melles DC, Penders J, de Jong MD, et al. Dissemination of the mcr-1 colistin resistance gene. Lancet Infect Dis. Elsevier Ltd; 2016;16: 147–9. doi:10.1016/S1473-3099(15)00541-1
5. Olaitan AO, Chabou S, Okdah L, Morand S, Rolain J-M. Dissemination of the mcr-1 colistin resistance gene. Lancet Infect Dis. Elsevier Ltd; 2016;16: 147. doi:10.1016/S1473-3099(15)00540-X
6. Webb HE, Granier SA, Marault M, Millemann Y, den Bakker HC, Nightingale KK, et al. Dissemination of the mcr-1 colistin resistance gene. Lancet Infect Dis. Elsevier Ltd; 2016;16: 144–145. doi:10.1016/S1473-3099(15)00538-1
7. Public Health England (PHE). First detection of plasmid-mediated colistin resistance (mcr-1 gene) in food and human isolates in England and Wales (Serial number 2015/090). 2015.
8. Falgenhauer L, Waezsada SE, Yao Y, Imirzalioglu C, Käsbohrer A, Roesler U, et al. Colistin resistance gene mcr-1 in extended-spectrum β-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany. Lancet Infect Dis. 2016;16: 282–283. doi:10.1016/S1473-3099(16)00009-8
9. Malhotra-Kumar S, Xavier BB, Das AJ, Lammens C, Butaye P, Goossens H. Colistin resistance gene mcr-1 harboured on a multidrug resistant plasmid. Lancet Infect Dis. Elsevier Ltd; 2016;16: 283–4. doi:10.1016/S1473-3099(16)00012-8
10. Malhotra-Kumar S, Xavier BB, Das AJ, Lammens C, Hoang HTT, Pham NT, et al. Colistin-resistant Escherichia coli harbouring mcr-1 isolated from food animals in Hanoi, Vietnam. Lancet Infect Dis. Elsevier Ltd; 2016;16: 286–287. doi:10.1016/S1473-3099(16)00014-1
11. Stoesser N, Sheppard AE, Pankhurst L, de Maio N, Moore CE, Sebra R, et al. Evolutionary history of the global emergence of the Escherichia coli epidemic clone ST131. MBio. 2016;7: 1–15. doi:10.1128/mBio.02162-15
12. Suzuki S, Ohnishi M, Kawanishi M, Akiba M, Kuroda M. Investigation of a plasmid genome database for colistin-resistance gene mcr-1. Lancet Infect Dis. Elsevier Ltd; 2016;16: 284–285. doi:10.1016/S1473-3099(16)00008-6
13. Haenni M, Poirel L, Kieffer N, Châtre P, Saras E, Métayer V, et al. Co-occurrence of extended spectrum β lactamase and MCR-1 encoding genes on plasmids. Lancet Infect Dis. 2016;16: 281–282. doi:10.1016/S1473-3099(16)00007-4

14. Giani T, Arena F, Vaggelli G, Conte V, Chiarelli A, Henrici De Angelis L, et al. Large Nosocomial Outbreak of Colistin-Resistant, Carbapenemase-Producing Klebsiella pneumoniae Traced to Clonal Expansion of an mgrB Deletion Mutant. J Clin Microbiol. 2015;53: 3341–4. doi:10.1128/JCM.01017-15
15. Kluytmans JAJW, Overdevest ITMA, Willemsen I, Bergh MFQK Den, Zwaluw K Van Der, Heck M, et al. Extended-Spectrum β -Lactamase – Producing Escherichia coli From Retail Chicken Meat and Humans : Comparison of Strains , Plasmids , Resistance Genes , and Virulence Factors. 2013;56: 478–487. doi:10.1093/cid/cis929
16. Overdevest I, Willemsen I, Rijnsburger M, Eustace A, Xu L, Hawkey P, et al. Extended-spectrum β-lactamase genes of Escherichia coli in chicken meat and humans, the Netherlands. Emerg Infect Dis. 2011;17: 1216–1222. doi:10.3201/eid1707.110209
17. Willemsen I, Oome S, Verhulst C, Pettersson A, Verduin K, Kluytmans J. Trends in Extended Spectrum Beta-Lactamase (ESBL) producing enterobacteriaceae and ESBL genes in a Dutch teaching hospital, measured in 5 yearly point prevalence surveys (2010-2014). PLoS One. 2015;10: 1–10. doi:10.1371/journal.pone.0141765
18. Nederlands Trial Register. Trial ID NTR2799. In: http://www.trialregister.nl/trialreg/admin/rctview.asp?TC=2799.
19. Zhou K, Lokate M, Deurenberg RH, Arends J, Foe JL Ten, Grundmann H, et al. Characterization of a CTX-M-15 producing Klebsiella pneumoniae outbreak strain assigned to a novel sequence type (1427). Front Microbiol. 2015;6: 1–10. doi:10.3389/fmicb.2015.01250
20. Weterings V, Zhou K, Rossen JW, van Stenis D, Thewessen E, Kluytmans J, et al. An outbreak of colistin-resistant Klebsiella pneumoniae carbapenemase-producing Klebsiella pneumoniae in the Netherlands (July to December 2013), with inter-institutional spread. Eur J Clin Microbiol Infect Dis. 2015;34: 1647–1655. doi:10.1007/s10096-015-2401-2
21. Bankevich A, Nurk S, Antipov D, Gurevich A a., Dvorkin M, Kulikov AS, et al. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. J Comput Biol. 2012;19: 455–477. doi:10.1089/cmb.2012.0021
22. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012;67: 2640–2644. doi:10.1093/jac/dks261
23. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother. 2014;58: 3895–903. doi:10.1128/AAC.02412-14
24. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9: 357–359. doi:10.1038/nmeth.1923
25. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. Bioinformatics. 2009;25: 2078–2079. doi:10.1093/bioinformatics/btp352
26. European Committee on Antimicrobial Susceptibility Testing. Breakpoint tables for interpretation of MICs and zone diameters. Version 5.0. 2015.
27. Veldman K, Dierikx C, Mevius D. Monitoring of antimicrobial resistance and antibiotic asage in animals in the Netherlands in 2014 (MARAN 2015) [Internet]. 2015. Available: https://www.wur.nl/upload_mm/1/3/3/53df1fe1-ca3d-4050-a4ad-a1fde4ed158b_NethmapMaran2015.pdf

28. Blaak H, Hamidjaja RA, Van Hoek AHAM, De Heer L, De Roda Husman AM, Schets FM. Detection of extended-spectrum beta-lactamase (ESBL)-producing escherichia coli on flies at poultry farms. Appl Environ Microbiol. 2014;80: 239–246. doi:10.1128/AEM.02616-13
29. Wu G, Day MJ, Mafura MT, Nunez-Garcia J, Fenner JJ, Sharma M, et al. Comparative Analysis of ESBL-Positive Escherichia coli Isolates from Animals and Humans from the UK, The Netherlands and Germany. PLoS One. 2013;8: 1–10. doi:10.1371/journal.pone.0075392
30. de Greef S, Mouton J. NethMap 2015, consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands in 2014 [Internet]. 2015. Available: http://www.swab.nl/swab/cms3.nsf/uploads/4F5A0D8E6F0DD139C1257E6E0051833A/$FILE/NethmapMaran2015 _webversie.pdf
31. Catry B, Cavaleri M, Baptiste K, Grave K, Grein K, Holm A, et al. Use of colistin-containing products within the European Union and European Economic Area (EU/EEA): development of resistance in animals and possible impact on human and animal health. Int J Antimicrob Agents. 2015;46: 297–306. doi:10.1016/j.ijantimicag.2015.06.005



CHAPTER 7

High prevalence of the *mcr*-1 gene in retail chicken meat in the Netherlands in 2015

Eefje JA Schrauwen, Pepijn Huizinga, Nick van Spreuwel, Carlo Verhulst, Marjolein FQ Kluytmans–van den Bergh, Jan AJW Kluytmans

Adapted from: Antimicrob Resist Infect Control 2017; 6:83

ABSTRACT

Recently, plasmid-mediated colistin resistance was reported in humans, animals and food. We studied the presence of *mcr-1* and *mcr-2* in Dutch retail chicken meat. The prevalence of *mcr-1* was 24,8% (53/214), whereas *mcr-2* was not found. The presence of *mcr-1*-positive Enterobacteriaceae was confirmed by culture in 34/53 samples (64,2%). The prevalence depended on the supermarket chain and was lower in free-range chicken samples. The unexpected high prevalence of *mcr-1* in food is cause for concern.

INTRODUCTION

Recently, a plasmid-mediated colistin resistance gene, called *mcr-1*, was reported from China which was soon followed by several reports on *mcr-1* positive Enterobacteriaceae from food, animals and the environment across the world [1–3]. This is of particular concern as colistin is currently considered as a last resort agent for treatment of infections with isolates that contain other resistance traits, like extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae or carbapenem-resistant bacteria [4–6].

Recent investigations using metagenomics, indicated a substantial larger environmental reservoir regarding the *mcr-1* gene in the Chinese population [7]. This indicates that other approaches are needed to reveal the true reservoir of *mcr-1*.

In the Netherlands, *mcr-1* was detected at low prevalence in *E. coli* isolates from livestock and meat (< 2%) and at very low frequencies in the human population [6,8,9]. The aim of this study was to determine the prevalence of *mcr-1* and *mcr-2* in a collection of poultry samples from Dutch supermarkets using a PCR-based method.

METHODS

Chicken meat samples (n=214) were bought from four supermarket chains throughout the Netherlands in 2015. The number of samples was balanced across supermarkets and one sample per production batch was included. Meat samples were enriched overnight in non-selective tryptic soy broth (TSB) and subsequently stored at -80 °C until further testing. DNA was isolated from 50µl of the defrosted TSB using NucliSens EasyMAG (Biomérieux). Detection of *mcr-1* and *mcr-2* gene was performed by real-time multiplex PCR (ABI 7500 system) using the following primers and probes: mcr1-2_forward AAATGCCMTRCARACCGACCAAG, *mcr-1*-2_reverse TCTCACCGACGACGAACACCAC, *mcr-1*_probe YY-BHQ1 TTTGATGCGCCGATTGGGCTTGATC, *mcr-2* probe FAM-BHQ1 TGCAGACCACCAAGCCGAGCGAG. Control isolates that contained either *mcr-1* or *mcr-2* were used. Concurrently, 100 µl of TSB was inoculated in fresh TSB and incubated at 35-37 °C overnight. Subsequently, 10 µl of this overnight grown TSB was streaked onto a CLED-colistin-agar with 1.5 µg/ml colistin (Duchefa) and 10 µg/ml Daptomycin (Novartis). All colistin resistant isolates that could grow on the selective CLED-colistin-agar were confirmed by Vitek MS (Biomérieux) and non-intrinsic colistin resistant isolates found, were further tested for the presence of mcr genes by PCR. The isolates were tested by broth-micro-dilution (BMD), in cation-adjusted Mueller Hinton broth, for colistin susceptibility and Vitek2 (AST N344) (Biomérieux) to determine the susceptibility for various other antibiotics [10].

RESULTS

Prevalence of *mcr-1* in retail chicken samples

The prevalence of *mcr-1* on retail chicken meat using PCR on TSB was 24.8% (53/214 samples positive) and no *mcr-2* was detected. Using a selective culture method, the presence of *mcr-1* was confirmed in 34 of these 53 (64.2%) samples with a positive result by PCR. Using this culture method, intrinsically resistant isolates had the ability to grow as well. No mcr-positive Enterobacteriaceae were found in all other samples. *E. coli* was identified in 32 samples, and *K. pneumoniae* in two samples. The median CT-value of the culture positive samples was significantly lower: culture-positive, 25.6 and culture-negative, 30.1, ($p<0.001$, Mann-Whitney U-test).

The prevalence of *mcr-1* according to the method of farming husbandry (free range: yes/no) and supermarket chain is shown in table 1. Using multivariable regression analysis it was shown that both variables were statistically significant and independently related to the presence of *mcr-1*. We also investigated the country of origin (COO), as indicated on the label (Table 1). This variable showed co-linearity with the supermarket chain and was not included in the multivariate analysis (Figure 1).

Table 1. Determinants of the presence of *mcr-1* in Dutch retail chicken meat samples, 2015

Determinant	Samples n=214	*mcr*-1 PCR positive n (%)	OR (95% CI)	Adjusted OR (95%CI)
Labelling as free-range				
Yes	70	10 (14.3)	reference	reference
No	144	43 (29.8)	2.6 (1.2-5.5)	3.0 (1.3-6.6)
Supermarket chain				
A	53	1 (1.9)	reference	reference
B	53	10 (18.9)	12.1 (1.5-98.3)	12.5 (1.5-101.8)
C	54	21 (38.9)	33.1 (4.2-257.8)	34.6 (4.4-272.0)
D	54	21 (38.9)	33.1 (4.2-257.8)	37.5 (4.8-295.3)
Country of origin[a]				
NL	67	21 (30.3)	2.9 (1.3-6.5)	
GER	44	19 (43.2)	4.8 (2.0-11.4)	
DEN	9	1 (11.1)	0.8 (0.1-6.9)	
NL/GER	80	11 (13.8)	reference	
NL/GER/BE	12	0 (0.0)	not applicable	
Unknown	2	1 (50.0)	6.3 (0.4-107.8)	

[a]NL = The Netherlands, GER = Germany, DEN = Denmark and BE = Belgium

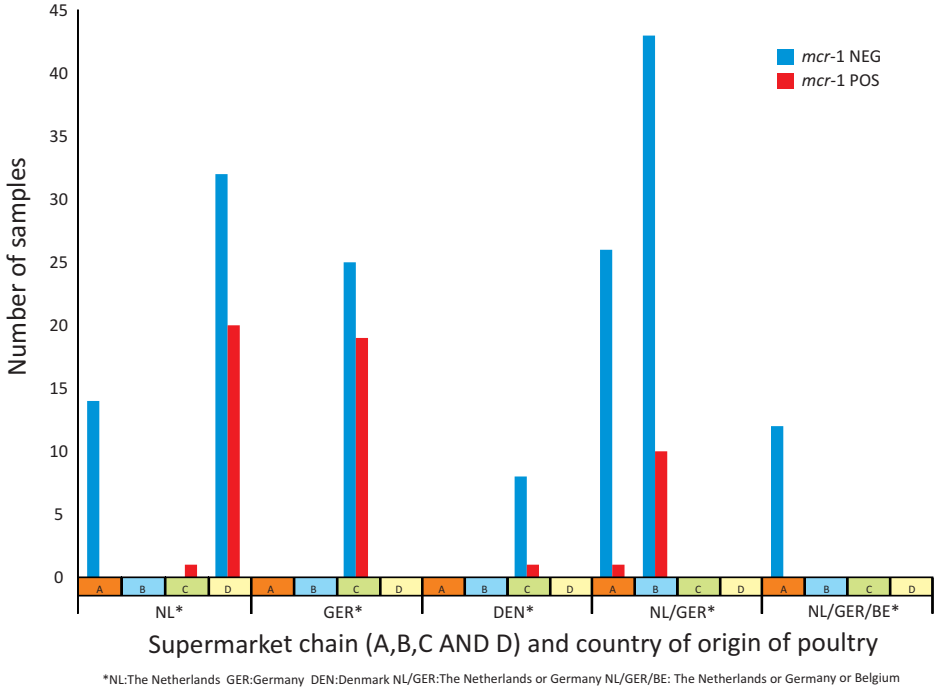


Figure 1. Distribution of the *mcr-1* positive and negative chicken meat samples across supermarket chains and country of origin (n=214)

The *in vitro* antimicrobial susceptibility for the 35 *mcr-1* positive isolates, which were found by culture, is shown in table 2. One sample (nr. 11) harboured two isolates which were *mcr-1* positive but with different susceptibility patterns. There were high levels of resistance against ampicillin (100%), amoxicillin-clavulanic acid (89%), trimethoprim/sulfamethoxazol (69%) and ciprofloxacin (57%). Only one *mcr-1* positive ESBL-producer was found (sample 34) and all isolates were susceptible to meropenem.

Table 2. Antimicrobial susceptibility of *mcr*-1 positive Enterobacteriaceae isolated from Dutch retail chicken meat, 2015

Sample	Spp	SMC	Colistin[a]	AMP[b]	AMC[b]	TZP[b]	CXM[b]
1	*K. pneu*	C	16	>= 32	<= 2	<= 4	2
2	*K. pneu*	C	16	>= 32	8	8	2
3	*E. coli*	D	4	>= 32	8	<= 4	4
4	*E. coli*	D	4	>= 32	>= 32	<= 4	4
5	*E. coli*	B	8	>= 32	16	<= 4	4
6	*E. coli*	C	4	>= 32	>= 32	<= 4	4
7	*E. coli*	A	4	>= 32	>= 32	<= 4	4
8	*E. coli*	B	8	>= 32	16	<= 4	4
9	*E. coli*	C	8	>= 32	16	<= 4	4
10	*E. coli*	D	4	>= 32	>= 32	<= 4	4
11A	*E. coli*	D	4	>= 32	>= 32	<= 4	4
12	*E. coli*	D	4	>= 32	>= 32	<= 4	4
13	*E. coli*	D	4	>= 32	>= 32	<= 4	4
14	*E. coli*	D	4	>= 32	>= 32	<= 4	2
15	*E. coli*	C	4	>= 32	16	<= 4	4
16	*E. coli*	C	8	>= 32	>= 32	<= 4	4
17	*E. coli*	C	4	>= 32	>= 32	<= 4	2
18	*E. coli*	C	4	>= 32	8	<= 4	2
19	*E. coli*	B	4	>= 32	16	<= 4	<= 1
20	*E. coli*	B	4	>= 32	>= 32	<= 4	4
11B	*E. coli*	D	4	>= 32	>= 32	<= 4	4
21	*E. coli*	D	4	>= 32	16	<= 4	4
22	*E. coli*	B	4	>= 32	16	<= 4	4
23	*E. coli*	C	4	>= 32	>= 32	<= 4	8
24	*E. coli*	B	4	>= 32	>= 32	16	4
25	*E. coli*	D	4	>= 32	>= 32	<= 4	16
26	*E. coli*	C	4	>= 32	16	<= 4	4
27	*E. coli*	C	8	>= 32	16	<= 4	4
28	*E. coli*	D	4	>= 32	>= 32	<= 4	4
29	*E. coli*	B	4	>= 32	>= 32	<= 4	4
30	*E. coli*	D	4	>= 32	>= 32	32	4
31	*E. coli*	D	4	>= 32	>= 32	32	4
32	*E. coli*	B	4	>= 32	>= 32	64	4
33	*E. coli*	D	4	>= 32	>= 32	>= 128	4
34	*E. coli*[c]	B	4	>= 32	16	<= 4	32

[a]BMD=broth micro-dilution

[b]Vitek2

[c]Phenotypic confirmed extended-spectrum beta-lactamase- producing *E. coli*

	CTX[b]	CAZ[b]	MEM[b]	GEN[b]	TOB[b]	CIP[b]	SXT[b]
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	<= 0.25	<= 20
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	<= 0.25	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	1	<= 20
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	<= 0.25	<= 20
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	<= 0.25	<= 20
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	<= 0.25	<= 20
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	<= 0.25	<= 20
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	<= 0.25	<= 20
	<= 0.25	0.25	<= 0.25	<= 1	<= 1	1	<= 20
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	>= 4	<= 20
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	0.5	>= 320
	<= 0.25	0.25	<= 0.25	<= 1	<= 1	<= 0.25	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	<= 0.25	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	<= 0.25	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	<= 0.25	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	>= 4	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	>= 4	>= 320
	<= 0.25	0.25	<= 0.25	<= 1	<= 1	>= 4	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	>= 4	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	>= 4	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	>= 4	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	1	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	1	>= 320
	<= 0.25	0.25	<= 0.25	<= 1	<= 1	2	>= 320
	<= 0.25	<= 0.12	<= 0.25	<= 1	<= 1	>= 4	>= 320
	<= 0.25	<= 0.12	<= 0.25	>= 16	8	>= 4	>= 320
	<= 0.25	<= 0.12	<= 0.25	>= 16	8	>= 4	>= 320
	<= 0.25	0.25	<= 0.25	>= 16	8	>= 4	>= 320
	<= 0.25	<= 0.12	<= 0.25	>= 16	2	>= 4	>= 320
	<= 0.25	<= 0.12	<= 0.25	>= 16	4	>= 4	>= 320
	<= 0.25	<= 0.12	<= 0.25	>= 16	4	>= 4	>= 320
	<= 0.25	<= 0.12	<= 0.25	>= 16	4	>= 4	>= 320
	32	4	<= 0.25	<= 1	<= 1	<= 0.25	<= 20

Abbreviations: AMP ampicillin, TZP Piperacillin tazobactam, CXM Cefuroxime, CTX cefotaxime, CAZ ceftazidime, MEM meropenem, GEN gentamicin, TOB tobramycin, CIP ciprofloxacin, SXT trimethoprim-sulfamethoxazole.

DISCUSSION

In this study, a PCR-based detection method identified an unexpected high prevalence (24.8%) of *mcr-1* in retail chicken meat samples and no *mcr-2* was found. The majority of the PCR positive samples were confirmed by selective culture. The 19 PCR positive samples that could not be confirmed by culture were all overgrown by intrinsically colistin-resistant bacterial species (e.g. *Serratia spp.* and *Proteus spp.*), which decreases the sensitivity of the culture for *mcr-1* harbouring bacteria. In addition, the higher CT-values in the culture-negative samples are indicative of a lower bacterial load, which may further explain the negative findings. Moreover, the freeze-thaw step of the samples might have played a role in the viability of the colistin-resistant bacteria.

A link between *mcr-1* in humans and food has been proposed in the first report from China, in which 28% of poultry samples harboured *mcr-1* [1]. In a study from south America, chicken meat was also identified as a reservoir for *mcr-1*-harboring *E. coli* isolates (19.5%) based on a selective culture approach. It should be realized that Brazil is the third-largest chicken meat producer and the largest exporter of this product [11]. Subsequent studies confirmed the presence of *mcr-1* in isolates from poultry and other meat products from Europe, but at much lower rates [2]. A recent study form Germany, including 580 *E. coli* isolates from chicken meat, found a decreasing prevalence of *mcr-1*, from 8.1% in 2011 to 0.5% in 2014, however, this was based on isolate screening [12].

At present, *mcr-1* is only sporadically found in humans in the Netherlands [6,8,9]. This is in a situation where colistin and other polymyxins are used at very low levels. In 2014, polymyxins constituted less than 0.1% (0.01 defined daily dose (DDD)/1,000 inhabitant-days) of all systemic antimicrobials used in primary care and 0.3% (0.2 DDD/100 patient-days) in the hospital setting [13]. Therefore, the selective pressure is currently low. Also, it should be taken into account that more selective approaches are necessary to reveal the true presence of *mcr-1* in humans. Both the current study and the study by Wang *et al* show that direct molecular techniques, molecular techniques after enrichment steps and selective culture techniques result in much higher prevalence compared to studies using non-targeted methods [7]. Considering the low selective pressure in humans and the lack of data on the resistome in humans it is not evident what the implications of these findings are for public health on the short or long term.

The culture approach showed that the majority of the *mcr-1* positive isolates were susceptible to cephalosporins, carbapenems and aminoglycosides. Apparently, the *mcr-1* gene is frequently present in isolates that are susceptible to most classes of

antibiotics. This might explain the relative low prevalence of *mcr-1* in studies that have primarily focused on isolates with other resistance traits [6,14–17].

The differences in *mcr-1* prevalence between supermarket chains are remarkable, with the two chains with the highest prevalence (C and D) having an odds ratio that is approximately 35 times higher compared to the supermarket chain with the lowest prevalence, after adjusting for free-range rearing of the animals. We attempted to extend the multivariable regression analysis to study the reservoir of the *mcr-1* gene to COO. However, in most cases, multiple countries are named on one sample without further specification. In addition, there was co-linearity with the supermarket chain (figure 1), prohibiting to include these variables in the regression model. Further details on the production process could not be studied as the label on the package does not provide further information. The conclusion is that there are large differences in the prevalence of *mcr*-1 between supermarkets which we cannot explain with the available information. As shown in Table 2, there were variable susceptibility patterns to other antibiotics, which showed a tendency to cluster within supermarket chains. We cannot draw conclusions based on our data. This would require additional research. It would be important to extend the investigations into the different chains of production of chicken meat to identify the determinants of the presence of *mcr*-1.

In conclusion, we have shown a high prevalence of *mcr-1* in chicken meat with a large and unexplained variation between supermarket chains. The approach, specifically targeting the presence of *mcr-1*, resulted in a much higher prevalence than previous studies that did not specifically target colistin resistance. These findings warrant further studies to elucidate the underlying mechanisms of spread and the genetic location of the *mcr*-1 gene. Moreover, continued monitoring of the potential reservoirs for this plasmid-mediated colistin resistance is of utmost importance.

LIST OF ABBREVIATIONS

ESBL=extended-spectrum beta-lactamase
COO=country of origin
DDD=defined daily dose

DECLARATIONS

Ethics approval and consent to participate
Not applicable

Consent for publications
Not applicable

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

Competing interest
The author(s) declare(s) that they have no competing interests

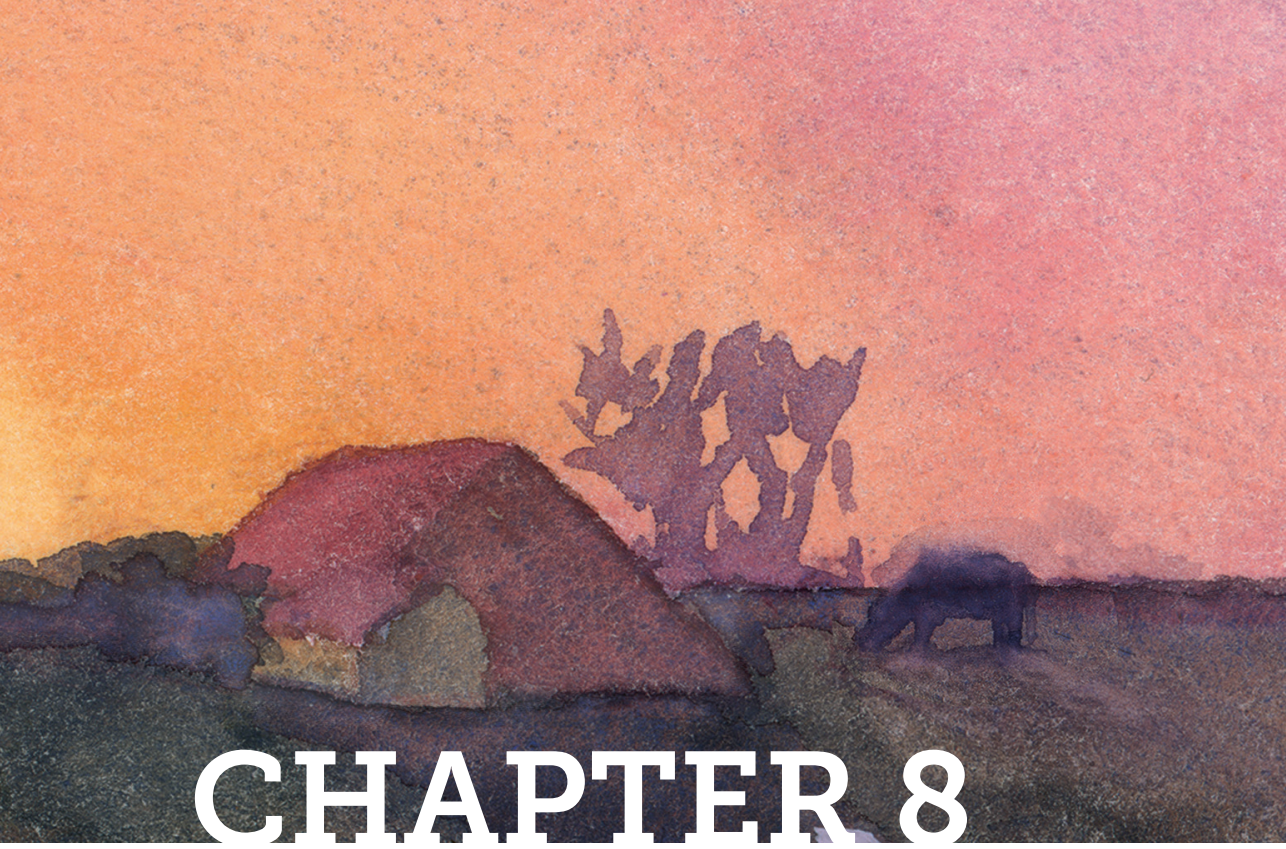
Authors' contributions
The study was planned and designed by ES, JK, MK, NS, CV and PH. PH collected the samples. NS and CV conducted the experiments. The interpretation of the results was done by ES, JK, MK and PH. The manuscript was prepared by ES and JK. All authors contributed to and commented on the manuscript.

Acknowledgement
We would like to thank Prof. Dr. Surbhi Malhorta-Kumar for providing the *mcr-2* positive *Escherichia coli*. In addition, we would like to thank Dr. Tanja Geelen for helpful discussions and Renee Ladestein for the purchase of the retail chicken meat samples.

REFERENCES

1. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. Lancet Infect Dis. Elsevier Ltd; 2016;16: 161–168. doi:10.1016/S1473-3099(15)00424-7
2. Skov RL, Monnet DL. Plasmid-mediated colistin resistance (mcr-1 gene): Three months later, the story unfolds. Eurosurveillance. 2016;21: 1–6. doi:10.2807/1560-7917.ES.2016.21.9.30155
3. Schwarz S, Johnson AP. Transferable resistance to colistin: A new but old threat. J Antimicrob Chemother. 2016;71: 2066–2070. doi:10.1093/jac/dkw274
4. Yang YQ, Zhang AY, Ma SZ, Kong LH, Li YX, Liu JX, et al. Co-occurrence of mcr-1 and ESBL on a single plasmid in Salmonella enterica. J Antimicrob Chemother. 2016;71: 2336–2338. doi:10.1093/jac/dkw243
5. Mediavilla JR, Patrawalla A, Chen L, Chavda KD, Mathema B, Vinnard C, et al. Colistin- and carbapenem-resistant Escherichia coli harboring mcr-1 and blaNDM-5, causing a complicated urinary tract infection in a patient from the United States. MBio. 2016;7: 5–8. doi:10.1128/mBio.01191-16
6. Kluytmans–van den Bergh MF, Huizinga P, Bonten MJ, Bos M, De Bruyne K, Friedrich AW, et al. Presence of mcr-1 -positive Enterobacteriaceae in retail chicken meat but not in humans in the Netherlands since 2009. Euro Surveill. Sweden; 2016;21: 30149. doi:10.2807/1560-7917.ES.2016.21.9.30149
7. Wang Y, Zhang R, Li J, Wu Z, Yin W, Schwarz S, et al. Comprehensive resistome analysis reveals the prevalence of NDM and MCR-1 in Chinese poultry production. Nat Microbiol. Nature Publishing Group; 2017;16260. doi:10.1038/nmicrobiol.2016.260
8. Nijhuis RHT, Veldman KT, Schelfaut J, Van Essen-Zandbergen A, Wessels E, Claas ECJ, et al. Detection of the plasmid-mediated colistin-resistance gene mcr-1 in clinical isolates and stool specimens obtained from hospitalized patients using a newly developed real-time PCR assay. J Antimicrob Chemother. 2016;71: 2344–2346. doi:10.1093/jac/dkw192
9. Bonten MJM. Antimicrobial resistance: is it really all going wrong now? Ned Tijdschr Geneeskd. Netherlands; 2016;160: D81.
10. EUCAST. Recommendations for MIC determination of colistin (polymyxin E) as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group.
11. Monte DF, Mem A, Fernandes MR, Cerdeira L, Esposito F, Galvão JA, et al. Chicken meat as a reservoir of colistin-resistant Escherichia coli strains carrying mcr-1 genes in South America. Antimicrob Agents Chemother. 2017;61: 2–5. doi:10.1128/AAC.02718-16
12. Irrgang A, Roschanski N, Tenhagen BA, Grobbel M, Skladnikiewicz-Ziemer T, Thomas K, et al. Prevalence of mcr-1 in E. coli from livestock and food in Germany, 2010-2015. PLoS One. 2016;11: 2010–2015. doi:10.1371/journal.pone.0159863
13. de Greef S, Mouton J. NethMap 2015, consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands in 2014 [Internet]. 2015. Available: http://www.swab.nl/swab/cms3.nsf/uploads/4F5A0D8E6F0DD139C1257E6E0051833A/$FILE/NethmapMaran2015 _webversie.pdf

14. Du H, Chen L, Tang Y-W, Kreiswirth BN. Emergence of the mcr-1 colistin resistance gene in carbapenem-resistant Enterobacteriaceae. Lancet Infect Dis. Elsevier Ltd; 2016;16: 287–8. doi:10.1016/S1473-3099(16)00056-6
15. Haenni M, Métayer V, Gay E, Madec JY. Increasing trends in mcr-1 prevalence among extended-spectrum-β-lactamase-producing Escherichia coli isolates from French calves despite decreasing exposure to colistin. Antimicrob Agents Chemother. 2016;60: 6433–6434. doi:10.1128/AAC.01147-16
16. Falgenhauer L, Waezsada SE, Yao Y, Imirzalioglu C, Käsbohrer A, Roesler U, et al. Colistin resistance gene mcr-1 in extended-spectrum β-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany. Lancet Infect Dis. 2016;16: 282–283. doi:10.1016/S1473-3099(16)00009-8
17. Poirel L, Jayol A, Nordmann P. Polymyxins: Antibacterial Activity, Susceptibility Testing, and Resistance Mechanisms Encoded by Plasmids or Chromosomes. Clin Microbiol Rev. 2017;30: 557–596. doi:10.1128/CMR.00064-16



CHAPTER 8

Summary and general discussion

Pepijn Huizinga

SUMMARY AND GENERAL DISCUSSION

Introduction

This thesis looked into different topics around the central theory that antimicrobial-resistant microorganisms (AMR) in food could be a source of AMR in humans. The first aim of this thesis was to quantify the frequency of contamination of different food items with Extended-Spectrum Beta-Lactamase producing Enterobacteriaceae (ESBL-E) or plasmid-mediated colistin resistance. With food being a potential source of ESBL-E for humans a second hypothesis was formed that medication decreasing natural barriers of bacteria, such as proton pump inhibitors decrease the natural gastric acid barrier, could increase the risk of becoming a carrier with ESBL-E. As such, a second aim was to investigate the effect of proton-pump inhibitor use as a risk factor for ESBL-E carriage. Thirdly, ESBL-E isolates from human perianal cultures, blood cultures and retail chicken meat were genetically compared to investigate whether genetic overlap between the human and chicken meat domains exists. Finally, the prevalence of *mcr*-1, a mobile colistin resistance gene, in humans and chicken meat, was investigated in two studies. This summary and discussion combine the main outcomes from the studies from this thesis and put them in a broader perspective.

Prevalence of ESBL-E in retail chicken meat

After a rise in clinically detected ESBL-E in humans, the question of the source of the ESBL-E arose. A high prevalence of ESBL-E in farm animals and retail (chicken) meat was detected and investigated as a potential source [1–4]. From 2009, programs were initiated to decrease veterinary antimicrobial use. From 2009 to 2013, the latter being the start of the study described in Chapter 2, the antimicrobial use in the veterinary sector was decreased with 57.7% [1]. Specifically for meat chickens, this decrease was 56% between 2009 and 2014 [5]. The large-scale reduction in antimicrobial use was followed by a decrease in ESBL-E in chicken faeces. Whether the decrease in ESBL-E in chicken faeces would translate into an equal decline of ESBL-E contamination of retail chicken meat was unknown. Many different factors could be of influence; method of slaughter and processing of the meat could neutralize the effects of decreased ESBL-E in chickens. Moreover, the effect of imported meat on the community's actual exposure is not considered when only looking at animals reared in the Netherlands. To have a better picture of the source that the general Dutch population is exposed to, the prevalence and genetic characteristics of ESBL-E in retail chicken meat were investigated in the Netherlands.

The first finding of the study described in **Chapter 2** was that the prevalence of contamination of retail chicken meat with ESBL-E decreased from 68.3% in 2014 to 44.6% in 2015, absolute risk difference of 23.7% (95% confidence interval (CI): 12.6% - 34.1%).

There was also a difference in ESBL-E prevalence between conventional chicken meat (61.5%) and free-range chicken meat (36.4%), absolute risk difference of 25.2% (95% CI: 12.9% - 36.5%). The difference in prevalence of contamination with ESBL-E between the different supermarket chains stood out; 76.5% (95% CI: 66.2 – 84.5%) in the supermarket chain with the highest prevalence and 37.8% (95% CI: 28.1 – 48.6%) in the lowest. Chapter 2 showed that the decreasing ESBL-E prevalence in chicken faeces presented in the MARAN reports (Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands, yearly reports) was also detectable on retail chicken meat. From 2014 on the MARAN reports included the prevalence of ESBL-E on retail chicken meat and have reported similar findings as presented in chapter 2.

A decrease in antimicrobial use in the veterinary sector was followed by a decrease in antimicrobial-resistant microorganisms in livestock and retail chicken meat. Since 2015 the ESBL-E prevalence has further decreased in retail chicken meat from 41.9% (547/1304) in 2015 to 13.7% (36/262) in 2019 [6,7]. Decreased antimicrobial use in the veterinary sector did not lead to a decrease in animal health or in the farms' economic performances. Attention to other health strategies at the farm level and the use of slower growing chickens were essential factors to allow the decrease in antibiotic use [8]. It is not possible to make any causal inferences based only on our observational study. However, trials decreasing antimicrobial use in food-producing animals show decreased antimicrobial resistance rates in animals and humans [9]. An integral effort within the veterinary sector and all adjacent sectors have led to a decrease in contamination with ESBL-E on retail chicken meat.

It remains a complicated problem where the food sector, government and individual consumers play essential roles and where vision and responsibility for both problems with antimicrobial resistance rates and the sustainability of our ecosystem will be important points of discussion in the coming decades.

Prevalence of ESBL-E in bean sprouts

Previous studies showed that bean sprouts were contaminated with ESBL-E [10,11]. Bean sprouts contaminated with ESBL-E potentially create a larger risk of transmission to humans than other food products as they are often consumed raw or only lightly

cooked [12]. With this in mind, a more in-depth investigation was performed on the frequency and genetic characteristics of ESBL-E contamination in retail bean sprouts.

The study described in **Chapter** 3 showed that 19.1% (95% CI: 13.2% - 26.7%) of bean sprout samples were contaminated with ESBL-E between 2013 – 2016. All of the isolates were *Klebsiella spp.* with the majority (80.8%) being *K. pneumoniae*. Batch contaminations, multiple samples containing clonally related ESBL-EC isolates tightly related in time of purchase and as such likely contaminated from the same source, were frequently found. Samples purchased from supermarkets were positive for ESBL-E in 25.3% compared to 5.0% of samples from non-supermarket retailers.

Bean sprouts have always created a potential risk for food poisoning due to the combination of how they are produced, in a warm, humid environment, combined with raw or near raw consumption. Many outbreaks worldwide have been reported usually with *Salmonella* or pathogenic *E. coli* strains (German EHEC outbreak in 2011), but other bacteria such as *Bacillus cereus* and *Listeria monocytogenes* have also been described [13–19]. Outbreaks caused by these bacteria are found because acute illness occurs shortly after ingesting contaminated food. This is different for the ESBL-producing *Klebsiella* isolates described in this chapter. An increase in carriage of an often-unnoticed resistant microorganism with low temporal relation to the moment of transmission is not easily detected. Because of this insidious transmission, a proactive approach to identifying and reducing sources of ESBL-E or resistant microorganisms in a broader sense is of vital importance.

Proton-pump inhibitors

The body has different barriers against bacteria entering the body. One of these protection mechanisms is the gastric acid barrier. It is known that a decreased gastric acid barrier increases the risk of gastrointestinal infections with *Salmonella, Campylobacter* spp. and *Clostridium difficile* [20–24]. In Chapter 4, the hypothesis was that disrupting the gastric acid barrier could also increase the risk of becoming a carrier with an antimicrobial-resistant microorganism.

In studies combining the different medication classes to affect the gastric-acid barrier, no apparent effects were described [25–27]. When specifically looking at PPIs, which have the most significant effect on gastric pH, conflicting results have been reported [28,29].

In the cross-sectional study in **Chapter 4,** the use of medication altering the gastric acid barrier and the prevalence of ESBL-E carriage in recently admitted patients (exclusion if admitted for >2 days) were investigated. The study showed that 8.5% (95% CI: 5.6% -

12.6%) of patients using proton pump inhibitors carried ESBL-E, this was 2.9% (95% CI: 1.5% - 5.5%) for patients not using proton pump inhibitors. In a multivariable model, the odds ratio for patients using a PPI was 3.89 (95% CI: 1.65 – 9.19) to be colonised with ESBL-E at admission to the hospital.

Shortly after the publication of this paper, multiple studies were published confirming the association between proton-pump inhibitor use and increased risk of rectal carriage with ESBL-E. One of the latest being a systematic review and meta-analysis that shows that in 12 published studies the effect of proton-pump inhibitors is relatively stable and is estimated to increase the odds of intestinal carriage with antimicrobial-resistant microorganisms, either Enterobacteriaceae or vancomycin-resistant enterococci with roughly 75% (OR = 1.74; 95% CI, 1.40-2.16; I^2 = 68%)[30]. Although much of the data is from observational studies, the fact that the effect is now found consistently among the studies and a plausible hypothesis leaves little doubt as to the validity of this effect.

With increasing knowledge of proton-pump inhibitors' infectious and non-infectious side effects, the indications for proton-pump inhibitors should be re-evaluated. It is the most frequently prescribed group of medication in the world [31]. This is also the case in the Netherlands, with over three million individuals receiving one or more prescriptions in 2016 [32]. There are many indications where proton-pump inhibitor should be prescribed as the advantages outweigh the potential complications, but overprescribing remains a severe issue with reports showing 40 – 70% of proton-pump inhibitors are prescribed off-label [33–35]. Actions should be continued to decrease unnecessary prescribing of proton-pump inhibitors. Furthermore, selling proton-pump inhibitors over the counter at drugstores should be reconsidered in light of the new evidence.

Comparative genomics of ESBL-E isolates from human perianal and blood cultures and retail chicken meat

Extended-Spectrum Beta-Lactamase-producing *E. coli* (ESBL-EC) are regularly isolated from retail chicken meat. The Netherlands had high antimicrobial use in the veterinary sector a decade ago. This was contrasted with low antimicrobial use in human medicine. These circumstances created interesting conditions to investigate whether and to what extend the transmission of ESBL-EC from retail chicken meat to humans occurred. This remained a topic of debate. Studies using conventional typing methods have frequently identified overlap in Multi-Locus Sequence Type (MLST) and ESBL genes between the domains, but studies using whole-genome sequencing have questioned these conclusions. In this study the genetic similarities between ESBL-EC derived from human perianal cultures and blood cultures and ESBL-EC derived from retail chicken meat are investigated using whole-genome sequencing.

Chapter 5 shows that some human-derived ESBL-EC isolates are genetically so similar to chicken meat-derived ESBL-EC isolates based on genes likely to be present on mobile-genetic elements that they cannot be distinguished from one another. Based on these mobile genetic elements alone the current study found that 6.7% (95% CI: 2.8% – 14.1%) of ESBL-EC isolates from blood cultures and 8.2% (95% CI: 3.8% – 16.3%) of ESBL-EC isolates from perianal cultures were mispredicted as ESBL-EC isolates derived from chicken meat in a discriminant analysis of principal components (DAPC). This suggests a chicken meat-related origin for these isolates. Chicken meat-derived ESBL-EC isolates were almost never mispredicted as human-derived ESBL-EC isolates based on mobile genetic elements. Three human-derived ESBL-EC isolates (4%) were found in wgMLST clades specific to chicken meat-derived ESBL-EC isolates. These isolates were also mispredicted as chicken meat-derived isolates based on their mobile genetic elements. One carriage-derived ESBL-EC isolate was clonally related to a chicken meat-derived ESBL-EC isolate, both of ST1304 and containing $bla_{\text{CTX-M-1}}$.

Chapter 5 shows that it is plausible that a few per cent of ESBL-EC isolates found in humans could have their origin in retail chicken meat, though that it is not the main source of ESBL-E in humans. Transmission of mobile genetic elements probably occurs more frequently than clonal transfer. Other articles based on whole-genome sequencing have been sceptical on the possibility of clonal transmission. In my opinion, sampling strategies are key. Superficial sampling of potential sources will not give enough information on the present genetic variability, especially when genetic variability is as large as it is using whole-genome sequencing. In line with Hanage, who described this in a reaction to a paper by Ludden *el al.*: "*the absence of closely related bacteria in the sample may not be good evidence for their absence in nature.*"[36,37]

Causal inference on transmission is difficult based on cross-sectional data. Longitudinal data could lead to more definitive proof of transmission of antimicrobial-resistant microorganisms. However, this may not be necessary; outbreaks of pathogenic Enterobacteriaceae have already provided direct proof that transmission of Enterobacteriaceae occurs from food products to humans [38,39]. It may be more useful to investigate the transmission of plasmids with new available whole-genome sequence techniques and intensify active preventative strategies. Examples that could be tested are rapid screenings for ESBL-E and carbapenemase-producing Enterobacteriaceae followed by active interventions (up and downstream) targeted to interrupt transmission potential.

An observation from the data presented in this chapter that is hopeful and potentially rewarding, is the decrease in the proportion of $bla_{\text{CTX-M-1}}$ in human carriage and blood cultures compared to data from 2009. An initial investigation showed that the

proportion of ESBL genes that are *bla*$_{CTX\text{-}M\text{-}1}$ decreased with two-thirds to around 20% in carriage isolates and 13% of blood culture isolates, whilst no large difference in absolute numbers occurred. Decreasing the burden of ESBL-E in chickens and chicken meat followed by a decreased share of ESBL-E in humans with the specific type of ESBL gene most prevalent in chickens and chicken meat is suggestive for a relation between the two occurrences.

In short, there is evidence that a small proportion of ESBL-E found in humans originated from retail chicken meat. However, most of the ESBL-E will have come from other humans and other, potentially still unknown, sources. The global SARS-CoV-2 pandemic with its mass decrease in human-human interactions and (international) travel will give some indication of the actual burden of ESBL-E caused by these factors. If the ESBL-E prevalence will decrease rapidly during the year 2020 and increase again with decreasing COVID-19 measures in 2021, this may give us important information about the importance of some sources for ESBL-E in humans.

Plasmid mediated colistin resistance in retail chicken meat and humans in the Netherlands

In a world of increasing antimicrobial resistance, including ESBL- and carbapenemase-producing Gram-negative bacteria, colistin is seen as a last resort antimicrobial. In countries with a relatively low prevalence of resistance, colistin is used rarely due to toxic side effects. Until 2015 all known colistin-resistance mechanisms were chromosomally mediated, and spread could only occur through clonal transmission. In 2015 *mcr*-1 was discovered, a plasmid-mediated colistin resistance gene [40]. This changed the way of thinking about the risk of colistin resistance, as the combination of resistance to carbapenems and colistin together in one bacterial strain due to horizontal spread of resistance genes became a real risk.

After the discovery of *mcr*-1 in China, the question of how far the gene had spread through the world was highly sought after, and the following two studies share some light on this question for the Netherlands. The combination of the two studies shows both the strengths and weaknesses of whole-genome sequencing in research and diagnostic practice.

Shortly after the discovery of the genetic code for *mcr*-1, isolates in whole-genome sequence databases were screened for the presence of the newly discovered gene. The study in **Chapter 6** mainly looked at selectively cultured ESBL-E isolates and using *in silico* screening to detect co-resistance of ESBL and *mcr*-1 mediated colistin resistance. A selection of isolates related to different outbreaks with antimicrobial

resistant Enterobacteriaceae were also included in the study. The study found the *mcr*-1 gene in 2% of ESBL-producing *E. coli* isolates cultured from chicken meat in the Netherlands in 2009 and 2013 – 2014. One isolate was cultured in 2009 and one pair of isolates was cultured in 2014. The isolates from the pair from 2014 are epidemiologically related to each other; same lot number and purchased on the same day at the same supermarket chain and hence, were most likely contaminated from the same source. *mcr*-1 was not detected in any of the 2275 isolates from humans, collected from either ESBL-E carriage isolates, clinical isolates or isolates from several outbreaks with antimicrobial-resistant Enterobacteriaceae in Dutch hospitals. The speed with which these isolates were digitally screened for the presence of *mcr*-1 is a great strength of whole-genome sequencing databases. The potential to reanalyse data for genes previously unknown is of great value. With this functionality, it was possible to show that *mcr*-1 was already present in ESBL-E isolates in the Netherlands in 2009. It also shows the first limitation: if the gene is not in the database, it will not be found.

The second study on mobile colistin resistance, **Chapter 7**, shows that analyses from Chapter 6 does not reveal the complete picture. In Chapter 7 the prevalence of *mcr*-1 on retail chicken meat from the Netherlands in 2015 was investigated using selective PCRs performed on non-selective enrichment broths and selective cultures on the non-selective enrichment broths. The study found that 24.8% (53/214 samples) of the meat samples contained the *mcr*-1 gene as detected with PCR. The selective cultures found 34 colistin-resistant isolates that were also confirmed to contain *mcr*-1. Comparing these findings, the selective cultures found a *mcr*-1 containing Enterobacteriaceae isolate in 64.2% (34/53 samples) of the samples. In practice, the samples where the PCR was positive but the culture did not confirm presence of a *mcr*-1 containing Enterobacteriaceae isolate, this was usually caused by overgrowth of intrinsically colistin-resistant Gram-negatives such as *Serratia* and *Proteus* species. This study showed a considerably higher prevalence of *mcr*-1 in retail chicken meat compared to the study that screened the isolates in the database. The difference is the fact that the first study mainly looked at co-occurrence of ESBL and *mcr*-1, which is shown to occur only in a small number (3%) of isolates based on the results of Chapter 7.Haenni *et al.* described co-resistance to ESBL and *mcr*-1 in 7% of isolates in veal calves and less frequent in humans in France [41].

How important is the finding of *mcr*-1 in the Netherlands? There is no simple answer to this question. *mcr*-1 is frequently found in retail chicken meat (24.8%) but relatively scarce in human carriage, 0.4% [42]. In the current resistance landscape of the Netherlands, there is no acute problem as colistin is not used for the treatment of acute infections. The resistance mechanisms that lead to colistin use are also scarce,

further decreasing any direct threat. How resistance levels will evolve, both nationally and globally, remains important for the situation in the Netherlands. Low levels of carbapenemase and colistin resistance in the Netherlands will not be guaranteed, as long as the global situation does not change. The interconnectivity, global travel movements, are simply too vast to think that only looking at the Dutch situation can provide solutions.

The combination of chapters 6 and 7 show that one must be careful when reanalyzing data how the findings are presented, and it is of vital importance to confirm the results using a sound microbiological and epidemiological approach.

REFERENCES

1. Mevius D, Dierikx C, Wit B, van Pelt W, Heederik D. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2013 (MARAN 2014) [Internet]. 2014. Available: https://www.wur.nl/upload_mm/4/a/4/46c626bf-5ccb-4996-a294-3e9ef4843355_NethMap-MARAN2014.pdf
2. Cohen Stuart J, van den Munckhof T, Voets G, Scharringa J, Fluit A, Hall ML Van. Comparison of ESBL contamination in organic and conventional retail chicken meat. Int J Food Microbiol. 2012; doi:10.1016/j.ijfoodmicro.2011.12.034
3. Leverstein-van Hall MA, Dierikx CM, Cohen Stuart J, Voets GM, van den Munckhof MP, van Essen-Zandbergen A, et al. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. Clin Microbiol Infect. 2011;17: 873–880. doi:10.1111/j.1469-0691.2011.03497.x
4. Overdevest ITMA, Willemsen I, Elberts S, Verhulst C, Kluytmans JAJW. Laboratory detection of extended-spectrum-beta-lactamase-producing Enterobacteriaceae: evaluation of two screening agar plates and two confirmation techniques. J Clin Microbiol. 2011;49: 519–22. doi:10.1128/JCM.01953-10
5. AVINED. ANTIBIOTICUMGEBRUIK PLUIMVEESECTOR IN 2015 en de trends van afgelopen jaren. 2016; 1–21.
6. Veldman K, Mevius D. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2015 (MARAN 2016) [Internet]. 2016. doi:10.21945/RIVM-2017-0056
7. Veldman KT, Wit B, Franz E, Wullings B, Heederik D, Mevius DJ. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2019 (MARAN 2020) [Internet]. 2020. Available: https://www.rivm.nl/publicaties/nethmap-2020-consumption-of-antimicrobial-agents
8. Bergevoet R, van Asseldonk M, Bondt N, van Horne P, Hoste R, de Lauwere C, et al. Economics of antibiotic usage on Dutch farms - The impact of antibiotic reduction on economic results of pig and broiler farms in the Netherlands [Internet]. Den Haag; 2019. Available: https://edepot.wur.nl/475403
9. Tang KL, Caffrey NP, Nóbrega DB, Cork SC, Ronksley PE, Barkema HW, et al. Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. Lancet Planet Heal. 2017;1: e316–e327. doi:10.1016/S2542-5196(17)30141-9
10. Reuland EA, Al Naiemi N, Raadsen SA, Savelkoul PHM, Kluytmans JAJW, Vandenbroucke-Grauls CMJE. Prevalence of ESBL-producing Enterobacteriaceae in raw vegetables. Eur J Clin Microbiol Infect Dis. 2014;33: 1843–6. doi:10.1007/s10096-014-2142-7
11. Kim HS, Chon JW, Kim YJ, Kim DH, Kim M sang, Seo KH. Prevalence and characterisation of extended-spectrum-beta-lactamase-producing Escherichia coli and Klebsiella pneumoniae in ready-to-eat vegetables. Int J Food Microbiol. Elsevier B.V.; 2015;207: 83–86. doi:10.1016/j.ijfoodmicro.2015.04.049
12. Evers EG, Pielaat A, Smid JH, van Duijkeren E, Vennemann FBC, Wijnands LM, et al. Comparative Exposure Assessment of ESBL-Producing Escherichia coli through Meat Consumption. PLoS One. 2017;12: e0169589. doi:10.1371/journal.pone.0169589
13. Sugiyama A, Iwade Y, Akachi S, Nakano Y, Matsuno Y, Yano T, et al. An outbreak of Shigatoxin-producing Escherichia coli O157:H7 in a nursery school in Mie Prefecture. Jpn J Infect Dis. 2005;58: 398–400.

14. Watanabe Y, Ozasa K, Mermin JH, Griffin PM, Masuda K, Imashuku S, et al. Factory outbreak of Escherichia coli O157:H7 infection in Japan. Emerg Infect Dis. 1999;5: 424–428. doi:10.3201/eid0503.990313
15. Terajima J, Izumiya H, Wada A, Tamura K, Watanabe H. Molecular epidemiological investigation of enterohaemorrhagic Escherichia coli isolates in Japan. J Appl Microbiol Symp Suppl. 2000; 99S-105S. Available: http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=med4&NEWS=N&AN=10880184
16. Puohiniemi R, Heiskanen T, Siitonen A. Molecular epidemiology of two international sprout-borne Salmonella outbreaks. J Clin Microbiol. 1997;
17. Taormina PJ, Beuchat LR, Slutsker L. Infections associated with eating seed sprouts: An international concern. Emerg Infect Dis. 1999;5: 626–634. doi:10.3201/eid0505.990503
18. Dechet AM, Herman KM, Chen Parker C, Taormina P, Johanson J, Tauxe R V., et al. Outbreaks caused by sprouts, United States, 1998-2010: Lessons learned and solutions needed. Foodborne Pathog Dis. 2014;11: 635–644. doi:10.1089/fpd.2013.1705
19. Buchholz U, Bernard H, Werber D, Böhmer MM, Remschmidt C, Wilking H, et al. German Outbreak of Escherichia coli O104:H4 Associated with Sprouts. N Engl J Med. 2011;365: 1763–1770. doi:10.1056/NEJMoa1106482
20. Hassing R-J, Verbon A, de Visser H, Hofman A, Stricker BH. Proton pump inhibitors and gastroenteritis. Eur J Epidemiol. Springer Netherlands; 2016; doi:10.1007/s10654-016-0136-8
21. Howell MD. Iatrogenic Gastric Acid Suppression and the Risk of Nosocomial Clostridium difficile Infection. Arch Intern Med. 2010;170: 784. doi:10.1001/archinternmed.2010.89
22. Kwok CS, Arthur AK, Anibueze CI, Singh S, Cavallazzi R, Loke YK. Risk of Clostridium difficile infection with acid suppressing drugs and antibiotics: meta-analysis. Am J Gastroenterol. Nature Publishing Group; 2012;107: 1011–1019. doi:10.1038/ajg.2012.108
23. Bouwknegt M, van Pelt W, Kubbinga ME, Weda M, Havelaar AH. Potential association between the recent increase in campylobacteriosis incidence in the Netherlands and proton-pump inhibitor use - An ecological study. Euro Surveill. 2014;19: 1–6. doi:10.1016/S0140-6736(13)61276-3
24. Leonard J, Marshall JK, Moayyedi P. Systematic review of the risk of enteric infection in patients taking acid suppression. Am J Gastroenterol. 2007;102: 2047–2056. doi:10.1111/j.1572-0241.2007.01275.x
25. Leung W, Malhi G, Willey BM, McGeer AJ, Borgundvaag B, Thanabalan R, et al. Prevalence and predictors of MRSA, ESBL, and VRE colonisation in the ambulatory IBD population. J Crohn's Colitis. European Crohn's and Colitis Organisation; 2012;6: 743–749. doi:10.1016/j.crohns.2011.12.005
26. Jans B, Schoevaerdts D, Huang TD, Berhin C, Latour K, Bogaerts P, et al. Epidemiology of Multidrug-Resistant Microorganisms among Nursing Home Residents in Belgium. PLoS One. 2013;8: e64908. doi:10.1371/journal.pone.0064908
27. Rodriguez-Bano J, Lopez-Cerero L, Navarro MD, de Alba PD, Pascual A. Faecal carriage of extended-spectrum beta-lactamase-producing Escherichia coli: Prevalence, risk factors and molecular epidemiology. J Clin Microbiol. 2010;48: 1726–1731. doi:10.1128/JCM.02353-09
28. Ben-Ami R, Schwaber MJ, Navon-Venezia S, Schwartz D, Giladi M, Chmelnitsky I, et al. Influx of Extended-Spectrum β-Lactamase-Producing Enterobacteriaceae into the Hospital. Clin Infect Dis. 2006;42: 925–934. doi:10.1086/500936

29. Reuland EA, Naiemi N, Kaiser AM, Heck M, Kluytmans JAJW, Savelkoul PHM, et al. Prevalence and risk factors for carriage of ESBL-producing Enterobacteriaceae in Amsterdam. J Antimicrob Chemother. 2016;71: 1–7. doi:10.1093/jac/dkv441
30. Willems RPJ, Van Dijk K, Ket JCF, Vandenbroucke-Grauls CMJE. Evaluation of the Association between Gastric Acid Suppression and Risk of Intestinal Colonization with Multidrug-Resistant Microorganisms: A Systematic Review and Meta-analysis. JAMA Intern Med. 2020;180: 561–571. doi:10.1001/jamainternmed.2020.0009
31. IMS Institute for Healthcare Informatics . The Global Use of Medicines : Outlook Through 2015. Outlook. 2011; 28. Available: http://www.google.co.uk/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0CFIQFjAA&url=http://www.imshealth.com/deployedfiles/ims/Global/Content/Insights/IMS Institute for Healthcare Informatics/Global_Use_of_Medicines_Report.pdf&ei=6czUT8nYEKuX0QXVxdisBA&usg
32. Stichting Farmaceutische Kengetallen. Aantal gebruikers maagmedicatie stijgt naar ruim drie miljoen. In: Pharmaceutisch Weekblad [Internet]. 2017 [cited 10 Jan 2021]. Available: https://www.sfk.nl/publicaties/PW/2017/gebruikers-maagmedicatie-stijgt-naar-ruim-3-miljoen
33. Katz MH. Failing the Acid Test. Arch Intern Med. 2010;170: 747. doi:10.1001/archinternmed.2010.64
34. van Vliet EPM, Otten HJAM, Rudolphus A, Knoester PD, Hoogsteden HC, Kuipers EJ, et al. Inappropriate prescription of proton pump inhibitors on two pulmonary medicine wards. Eur J Gastroenterol Hepatol. 2008;20: 608–12. doi:10.1097/MEG.0b013e3282f52f95
35. Lanas A. We Are Using Too Many PPIs, and We Need to Stop: A European Perspective. Am J Gastroenterol. Nature Publishing Group; 2016;111: 1085–6. doi:10.1038/ajg.2016.166
36. Ludden C, Raven KE, Jamrozy D, Gouliouris T, Blane B, Coll F, et al. One Health Genomic Surveillance of Escherichia coli Demonstrates Distinct Lineages and Mobile Genetic Elements in Isolates from Humans versus Livestock. Sansonetti PJ, editor. MBio. 2019;10: 1–12. doi:10.1128/mBio.02693-18
37. Hanage WP. Two health or not two health? That is the question. MBio. 2019;10: 1–4. doi:10.1128/mBio.00550-19
38. Morton VK, Kearney A, Coleman S, Viswanathan M, Chau K, Orr A, et al. Outbreaks of Salmonella illness associated with frozen raw breaded chicken products in Canada, 2015–2019. Epidemiol Infect. 2019;147. doi:10.1017/S0950268819001432
39. (CDC) C for DC and P. Outbreak of Salmonella Heidelberg Infections Linked to a Single Poultry Producer — 13 States, 2012–2013. In: Morbidity and Mortality Weekly Report [Internet]. 2013. Available: https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6227a3.htm
40. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: A microbiological and molecular biological study. Lancet Infect Dis. Elsevier Ltd; 2016;16: 161–168. doi:10.1016/S1473-3099(15)00424-7
41. Haenni M, Poirel L, Kieffer N, Châtre P, Saras E, Métayer V, et al. Co-occurrence of extended spectrum β lactamase and MCR-1 encoding genes on plasmids. Lancet Infect Dis. 2016;16: 281–282. doi:10.1016/S1473-3099(16)00007-4
42. Terveer EM, Nijhuis RHT, Crobach MJT, Knetsch CW, Veldkamp KE, Gooskens J, et al. prevalence of colistin resistance gene (mcr-1) containing Enterobacteriaceae in feces of patients attending a tertiary care hospital and detection of a mcr-1 containing, colistin susceptible E. coli. PLoS One. 2017;12: e0178598. doi:10.1371/journal.pone.0178598

CLOSING PAGES

Nederlandse samenvatting

Pepijn Huizinga

NEDERLANDSE SAMENVATTING

Inleiding

Infecties die veroorzaakt worden door bacteriën kunnen vaak behandeld worden met antibiotica. Als bacteriën niet meer gedood worden door antibiotica die meestal wel effect zouden hebben op die bacteriën, is er sprake van antimicrobiële resistentie. In dit proefschrift wordt er gekeken naar een bepaalde groep bacteriën, de Enterobacteriaceae. Deze groep bacteriën bevat de meest voorkomende verwekkers van onder andere urineweginfecties, maar ook infecties vanuit de darm en bijvoorbeeld de galwegen. Voorbeelden van bacteriën uit deze groep zijn *Escherichia coli* en *Klebsiella pneumoniae.*

In het ziekenhuis worden bacteriële infecties vaak behandeld met derde generatie cefalosporines. Als er binnen de groep Enterobacteriaceae resistentie is tegen derde generatie cefalosporines, kan dat komen door de productie van 'extended-spectrum beta-lactamases', die de derde generatie cefalosporines afbreken. In de Nederlandse bevolking is ongeveer 5% van de mensen gekoloniseerd met een extended-spectrum beta-lactamase-producerende Enterobacteriaceae (ESBL-E). Gekoloniseerd, ook wel dragerschap, houdt in dat de bacterie aanwezig is in bijvoorbeeld de darm, maar dat er op dat moment geen infectie mee is. Als er infecties ontstaan met ESBL-E, is de kans groter dat het empirisch gestarte antimicrobiële middel (empirisch, dus nog zonder dat er bekend is welke bacterie de infectie veroorzaakt) niet het juiste antimicrobiële middel is. Dit kan een negatief effect hebben op de behandeling. Om dit zo snel mogelijk op te sporen, worden er kweken verricht. Van de bacteriën die gevonden worden, wordt een gevoeligheidsbepaling verricht om te onderzoeken of de infectie met het juiste antimicrobiële middel behandeld wordt.

Behalve dat het belangrijk is om snel vast te stellen of er sprake is van antimicrobiële resistentie, is het belangrijk om te voorkómen dat mensen deze ESBL-E bij zich dragen. Daarvoor is kennis nodig over de herkomst van de resistente micro-organismen. Het wordt steeds duidelijker, dat er vaak sprake is van overdracht van mens naar mens. Ook zijn mensen die terugkomen uit het buitenland vaker drager van ESBL-E, waarbij de frequentie van acquisitie van ESBL-E dragerschap afhankelijk is van de regio waar ze geweest zijn.

In Nederland kwamen dragerschap van en infecties met ESBL-E tot de eeuwwisseling weinig voor, waarna dit redelijk plots toenam. Ook veranderde het type ESBL genen. Dit waren vooral bla_{SHV} en bla_{TEM} ESBL genen, wat in de loop van de tijd veranderde

naar *bla*$_{CTX-M}$ genen. Tevens kwam ESBL-E dragerschap ook voor bij mensen die geen duidelijk contact met de gezondheidszorg hadden. Vóór de toename ESBL-E dragerschap kwamen infecties met ESBL-E eigenlijk alleen voor bij mensen met intensief zorgcontact.

Bij de toename van ESBL-E kwam ook de vraag: waar komt het vandaan? In Nederland wordt er al lange tijd spaarzaam gebruik gemaakt van antimicrobiële middelen bij de mens. Dit in tegenstelling tot de veehouderij. Om dit concreet te maken: Nederland behoorde qua gebruik van antimicrobiële middelen per persoon structureel tot de laagste van Europa. Dit terwijl het voor het gebruik in veehouderij tot de hoogste gebruikers van Europa hoorde. Uit een aantal onderzoeken kwam naar voren, dat er overlap bestond tussen de ESBL genen die gevonden werden bij de ESBL-E die gekweekt werden bij de mens en de ESBL-E die gekweekt werden uit kippenvlees. Uit deze punten tezamen, kwam de hypothese voort dat ESBL-E uit kippenvlees, of in bredere zin uit de voedselketen, een bron kon zijn voor de toename van ESBL-E bij de mens.

Dit proefschrift

Dit proefschrift kijkt naar verschillende vragen rondom het centrale thema: kan voedsel dat besmet is met resistente bacteriën een bron zijn voor deze micro-organismen bij mensen? Verschillende aspecten worden bekeken, zoals: hoe vaak komen ESBL-E voor in kippenvlees en taugé? Hebben mensen die maagzuur remmende medicijnen gebruiken in de vorm van protonpompremmers een verhoogde kans om drager te zijn van ESBL-E? Er worden genetische analyses uitgevoerd op ESBL-E uit rectumkweken, bloedkweken en uit kweken van kippenvlees, om de vraag te beantwoorden of er overeenkomsten zijn die erop kunnen wijzen dat de bacteriën die bij de mens gekweekt zijn, afkomstig zouden kunnen zijn van het kippenvlees, of juist niet. Naast het resistentie mechanisme ESBL, wordt er ook in twee hoofdstukken gekeken naar de aanwezigheid van een gen wat resistentie tegen colistine kan veroorzaken. Colistine is een antimicrobieel middel wat vaak als laatste redmiddel gezien wordt, als er ook resistentie is tegen veel andere antimicrobiële middelen. Colistine heeft meer bijwerkingen dan de middelen die meestal gebruikt worden, maar bij ernstige infecties waar behandeling noodzakelijk is en waar resistentie tegen veel andere antimicrobiële middelen speelt, kan het wel van belang zijn. Er wordt gekeken naar de aanwezigheid van dit *mcr*-1 gen bij de mens en in kippenvlees.

Prevalentie van ESBL-E in kippenvlees

Na een toename van de uit klinische monsters gedetecteerde ESBL-E bij mensen, rees de vraag waar de toename vandaan kwam. Er werd een hoge prevalentie van ESBL-E in vee en (kippen)vlees gevonden en onderzocht als mogelijke bron voor de ESBL-E

bij mensen. Vanaf 2009 werden er landelijke programma's geïnitieerd om het gebruik van antibiotica in de veehouderij te verminderen. Tussen 2009 en 2013 werd het totale gebruik van antimicrobiële middelen in de veehouderij verminderd met 57,7%. Ook bij kippen was de afname in het gebruik van antimicrobiële middelen 56% tussen 2009 en 2014. Deze afname werd gevolgd door een afname van ESBL-E bij de kippen zelf. Of deze afname van ESBL-E bij de kippen ook vertaald kon worden naar een afname van ESBL-E in kippenvlees, was nog niet bekend. Het is mogelijk dat veel andere factoren hierop van invloed zouden kunnen zijn, zoals de methode van slachten of het proces van verpakken. Dit zou een reductie, die wel zichtbaar was bij de kippen zelf, teniet kunnen doen bij het vlees. Ook wordt er vlees geïmporteerd uit het buitenland, wat misschien gecontamineerd is met andere bacteriën. Hier zouden andere ESBL genen in gevonden kunnen worden dan die aanwezig zijn in de Nederlandse (pluim)veestapel. Om een zo goed mogelijk beeld te krijgen van aan welke ESBL-E de Nederlandse bevolking blootgesteld wordt bij het consumeren van kippenvlees, is er onderzoek gedaan naar welke ESBL-E er voorkomen op kipfilet uit een aantal grote Nederlandse supermarkten en hoe vaak het kippenvlees hiermee gecontamineerd is.

In hoofdstuk 2 wordt beschreven dat de prevalentie van besmetting van kippenvlees met ESBL-E is afgenomen van 68,3% in 2014 naar 44,6% in 2015, een absolute risico reductie van 23,7% (95% betrouwbaarheidsinterval (BI): 12,6% - 34,1%). Ook is er een verschil in de mate van contaminatie met ESBL-E gevonden tussen conventioneel kippenvlees (61,5%) en scharrelkippenvlees (36,4%), absolute risicoverschil 25,2% (95% BI: 12,9% - 36,5%). Tussen de verschillende supermarktketens waren er ook verschillen in de mate van contaminatie van kippenvlees met ESBL-E; 76,5% (95% BI: 66,2% - 84,5%) in de supermarktketen met de hoogste prevalentie en 37,8% (95% BI: 28,1 – 48,6%) bij de keten met de laagste prevalentie. Deze resultaten bevestigden dat de afname van ESBL-E dragerschap bij kippen ook terug te vinden was in het verminderd voorkomen van contaminatie van kippenvlees met ESBL-E. Na 2014 werd de mate van contaminatie met ESBL-E van kippenvlees structureel weergegeven in de jaarlijks MARAN rapporten (Monitoring of Antimicrobial Resistance and Antibiotic Usage in Animals in the Netherlands), waarin ook al eerder de afname van ESBL-E in pluimvee weergegeven was.

Een forse afname in het gebruik van antimicrobiële middelen in de veehouderij, werd gevolgd door een afname van resistente micro-organismen op kippenvlees. De laatste monsters van deze studie zijn in 2015 verzameld. Sinds die tijd heeft de afname van contaminatie van kippenvlees met ESBL-E verder doorgezet. In de MARAN rapporten werd in 2015 een ESBL-E prevalentie van 41,9% (547/1304) op kippenvlees gevonden wat inmiddels gedaald is naar 13,7% (35/262) in 2019. Wat nog belangrijk

is om hierbij mee te nemen, is dat de afname van het gebruik van antimicrobiële middelen niet gepaard ging met een afname van de gezondheid van de dieren of van de economische opbrengsten van de boerderijen. Het gebruik van andere strategieën voor de gezondheid van de dieren en het gebruik van een trager groeiend kippenras, waren essentiële factoren om het gebruik van antimicrobiële middelen veilig te kunnen reduceren. Een integrale aanpak door de veterinaire sector, met alle aanpalende sectoren, hebben geleid tot een significante afname van de besmetting van kippenvlees met ESBL-E.

Antimicrobiële resistentie in de voedselketen, of misschien wel de duurzaamheid van ons ecosysteem, zal een belangrijk onderwerp van discussie zijn het komende decennium, waar zowel de voedselproductie sector, de overheid en de individuele consumenten belangrijke rollen in vervullen.

Prevalentie van ESBL-E in taugé

In eerdere studies werd aangetoond dat taugé soms gecontamineerd is met ESBL-E. Dit kan extra risicovol zijn, omdat taugé vaak rauw of slechts kort gekookt gegeten wordt. Het risico op transmissie van het voedsel naar de mens is daardoor groter. Op basis van deze gegevens werd er een uitgebreider onderzoek verricht naar de frequentie van het voorkomen van contaminatie van taugé met ESBL-E en werd er gekeken naar de genetische samenstelling van deze ESBL-E.

In hoofdstuk 3 wordt beschreven dat 19,1% (95% BI: 13,2% – 26,7%) van de taugémonsters gecontamineerd was met ESBL-E in de periode tussen 2013 en 2016. Alle gekweekte ESBL-E bacteriën vielen onder het genus *Klebsiella,* waarvan het merendeel, 80,8%, *Klebsiella pneumoniae* was. Er werden meerdere malen in meerdere monsters, die qua tijdsperiode en locatie nauw aan elkaar verwant waren, clonale isolaten gevonden. Dit wekt de suggestie dat ze vanuit één bron besmet zijn. Taugé uit supermarkten was vaker gecontamineerd met ESBL-E (25,3%), vergeleken met monsters die in andere type winkels gekocht waren (5,0%).

Taugé is relatief risicovol als bron voor voedselinfecties door de manier waarop het geproduceerd wordt. Ontkieming in vochtige en warme omstandigheden waarin ook bacteriën goed kunnen groeien, en consumptie van rauwe of bijna rauwe taugé creëren gecombineerd een risico. Er zijn behoorlijk wat uitbraken geassocieerd aan kiemgroente gemeld in de wereld, met name met *Salmonella* of bepaalde pathogene varianten van *E. Coli.* De Duitse EHEC uitbraak is hier een duidelijk voorbeeld van. Andere verwekkers worden minder vaak gemeld, maar komen wel degelijk voor, zoals die met *Bacillus cereus* en *Listeria monocytogenes*. Dit type

uitbraken komt aan het licht door het pathogene karakter van deze bacteriën. Er is een cluster van mensen met hetzelfde ziektebeeld en men gaat zoeken waar dit vandaan komt. Dit is echt anders bij de ESBL-producerende *Klebsiella* isolaten die gevonden werden in de taugé. Mensen worden er niet direct ziek van en dit kan dus lange tijd onder de radar een bron van verspreiding zijn. Door het sluipende karakter van deze transmissie, is het mijns inziens van belang om een proactieve houding te hebben in het opzoeken van de bronnen van resistentie en te proberen transmissie te voorkomen.

Protonpompremmers

Het lichaam kent verschillende barrières tegen potentieel binnendringende bacteriën. Eén van deze barrières is het maagzuur in de maag. Het is bekend dat een verminderde maagzuurbarrière, dus een verhoogde pH, leidt tot een groter risico op gastro-intestinale infecties met bacteriën als *Salmonella, Campylobacter* en *Clostridium difficile.* In hoofdstuk 4 wordt de hypothese onderzocht, dat een vermindering van de maagzuurbarrière ook met een verhoogd risico gepaard gaat om drager te zijn van ESBL-E.

In studies die naar de gecombineerde effecten gekeken hebben van alle maagzuur remmende producten, werden geen duidelijk effecten gevonden op het voorkomen van ESBL-E dragerschap. In studies die specifiek naar protonpompremmers gekeken hebben, de meest effectieve middelen in deze categorie van medicijnen, werden conflicterende resultaten beschreven.

Hoofdstuk 4 beschrijft een onderzoek naar de associatie tussen protonpompremmer gebruik en dragerschap van ESBL-E in patiënten die maximaal twee dagen opgenomen lagen in het ziekenhuis. De studie liet zien dat 8,5% (95% BI: 5,6% - 12,6%) van de patiënten met protonpompremmers drager was van ESBL-E vergeleken met 2,9% (95% BI: 1,5% - 5,5%) van de patiënten die geen protonpompremmers gebruikten. Een multivariabele analyse liet zien, dat mensen die protonpompremmers gebruikten een hogere kans hadden om drager te zijn van ESBL-E bij of kort na opname in het ziekenhuis, odds ratio 3,89 (95% BI: 1,65 – 9,19).

Kort na de publicatie van dit onderzoek zijn er verschillende studies gepubliceerd die de associatie tussen het gebruik van protonpompremmers en een verhoogde kans op rectaal dragerschap met ESBL-E bevestigden. Er is ook een systematische review en meta-analyse van dit onderwerp verricht, waarin het effect van protonpompremmers op het risico om drager te zijn van resistente micro-organismen onderzocht werd. Voor de associatie tussen protonpompremmer gebruik en dragerschap met ESBL-E werd een odds ratio van 1,4 (95% BI: 1,20 – 1,70) gevonden. Ondanks het feit dat veel van deze

data van observationele aard zijn, zorgt het feit dat het effect inmiddels consistent gevonden wordt en er een plausibele hypothese aan ten grondslag ligt, dat ik nog weinig twijfel heb aan de validiteit van dit gevonden effect.

Met de toegenomen kennis van de infectieuze en niet-infectieuze bijwerkingen van protonpompremmers, zouden de indicaties voor protonpompremmer gebruik opnieuw geëvalueerd moeten worden. Het zijn de meest frequent voorgeschreven middelen in de wereld. Dit is ook het geval in Nederland. Er werden in 2016 aan meer dan drie miljoen individuele mensen recepten voor protonpompremmers verstrekt. Er zijn veel indicaties waarvoor het gebruik van protonpompremmers nog steeds zeer belangrijk is en de gezondheidswinst ruim afsteekt tegenover de risico's, maar het teveel voorschrijven is ook een groot probleem, wat in verschillende rapporten beschreven wordt. Tussen de 40 en 70% van het protonpompremmergebruik is off-label. Er zou actie ondernomen moeten worden om het onnodig voorschijven van protonpompremmers terug te dringen, of waar mogelijk te vervangen door minder sterke middelen. Ook zou heroverwogen moeten worden of protonpompremmers vrij verkrijgbaar zouden moeten blijven bij drogisterijen.

Vergelijkende genomica van ESBL-E isolaten uit perianale kweken, bloedkweken en kweken van kippenvlees

Kippenvlees is vaak gecontamineerd met extended-spectrum beta-lactamase-producerende *E. coli* (ESBL-EC). In het verleden werden er in Nederland veel antimicrobiële middelen gebruikt in de veehouderij. Dit stond in sterk contrast met het spaarzame gebruik van antimicrobiële middelen bij de mens. Deze tegenstelling, gecombineerd met de klinische toename van ESBL-E bij mensen, maakte het relevant om te onderzoeken of er sprake was van transmissie van ESBL-EC van kippenvlees naar de mens, en zo ja, hoeveel dit zou kunnen zijn. Studies met conventionele typeermethodes zoals Multi Locus Sequence Typing (MLST) en het typeren van de ESBL genen, hebben overlap laten zien tussen de types ESBL-EC die gekweekt werden bij de mens en uit kippenvlees. Recentere studies die gebruik maakten van whole-genome sequencing (WGS) hebben, door de verhoogde resolutie bij het vergelijken van de isolaten, een aantal eerdere conclusies in twijfel getrokken. In deze studie wordt er gekeken naar verschillen en overlap in de genomica van ESBL-EC isolaten uit perianale kweken, bloedkweken en kweken van kippenvlees.

Hoofdstuk 5 laat zien dat een aantal ESBL-EC isolaten gekweekt uit de mens genetisch niet te onderscheiden zijn van ESBL-EC isolaten gekweekt van kippenvlees, gebaseerd op genen die meestal op mobiele genetische elementen liggen. Dit was zo bij 6,7% (95% BI: 2,8% - 14,1%) van de ESBL-EC isolaten uit bloedkweken en 8,2% (95% BI: 3,8% -

16,3%) van de ESBL-EC isolaten uit perianale kweken. Dit suggereert dat kippenvlees een mogelijke oorsprong is voor deze isolaten die uit de mens gekweekt werden. De ESBL-EC isolaten uit kippenvlees leken vrijwel nooit op ESBL-EC isolaten gekweekt uit de mens. Drie ESBL-EC isolaten gekweekt uit de mens (4%) werden gevonden in whole-genome MLST clades waar in deze dataset vrijwel alleen kippenvlees isolaten in zaten. Deze drie isolaten werden ook op basis van de mobiele genetische elementen ingedeeld bij de ESBL-EC isolaten die uit kippenvlees gekweekt waren. Ook was er één ESBL-EC isolaat uit een perianale kweek clonaal verwant aan een ESBL-EC isolaat uit kippenvlees.

Hoofdstuk 5 maakt het plausibel dat een paar procent van de ESBL-EC isolaten bij de mens hun oorsprong in kippenvlees zouden kunnen hebben. Verder zal transmissie van mobiele genetische elementen vaker voorkomen dan clonale verspreiding van isolaten. In andere studies wordt er scepsis geuit over het voorkomen van clonale tranmissie tussen de verschillende domeinen. Wat mij betreft gaat het erom hoe en welke samples er genomen worden. Het nemen van slechts een paar monsters van verschillende bronnen zal eigenlijk nooit voldoende informatie geven over de aanwezige genetische variabiliteit om het vóórkomen van clonale transmissie uit te kunnen sluiten. Dit wordt ook geschreven door Hanage in een reactie op een paper van Ludden *et al.*: "*The absence of closely related bacteria in the sample may not be good evidence for their absence in nature*."

Causale interferentie over transmissie is niet vast te stellen op basis van cross-sectionele data. Longitudinale data zouden tot hardere conclusies kunnen leiden met betrekking tot transmissie. Het is echter de vraag of die onderzoeken nodig zijn. Er is voor verschillende pathogene *E. coli* soorten al aangetoond dat er uitbraken vanuit voedsel ontstaan zijn, waardoor het principe van clonale transmissie feitelijk al is aangetoond. Meer kennis over de mate van transmissie zou nuttig zijn. Echter, het zou misschien beter zijn om te focussen op de mate van transmissie van plasmiden met de steeds verbeterende whole-genome sequencing technieken en meer te investeren in preventieve strategieën. Hierbij kan gedacht worden aan snelle screeningstechnieken voor ESBL-E en carbapenemase-producerende Enterobacteriaceae, gevolgd door actieve interventies, zoals up- en downstream gericht proberen de transmissieketens te doorbreken en eventuele bronnen structureel weg te nemen.

Een observatie van de data uit dit hoofdstuk, die best hoopvol is met betrekking tot de transmissie vanuit kippenvlees, is de afname van het aandeel van het specifieke ESBL-gen $bla_{\text{CTX-M-1}}$ in zowel perianale kweken als bloedkweken van de mens, vergeleken met data uit 2009. Een verkennende analyse laat zien, dat het aandeel van $bla_{\text{CTX-M-1}}$

met ongeveer twee derde is afgenomen tot 20% van de ESBL-E isolaten uit perianale kweken en 13% uit bloedkweken. Dit zonder dat er grote veranderingen geweest zijn in absolute aantallen. De afname van contaminatie van ESBL-E in kippenvlees, welke gevolgd werd door een afname van dragerschap van ESBL-E bij de mens van het specifieke ESBL-gen $bla_{CTX\text{-}M\text{-}1}$, dat het meeste voorkomt in kippenvlees, zou mogelijk aan elkaar te correleren zijn.

Samenvattend, er zijn aanwijzingen dat een klein deel van de ESBL-EC die gevonden worden bij de mens, zijn oorsprong uit kippenvlees zou kunnen hebben. Echter, de meeste van de bij de mens gevonden ESBL-E komen van andere mensen of van andere, eventueel nog onbekende, bronnen. De wereldwijde SARS-CoV-2 pandemie zal op het gebied van de ESBL-E epidemiologie ook kunnen leiden tot nieuwe inzichten. Er is een grote afname van mens-mens interacties en ook het internationale vliegverkeer is enorm afgenomen in 2020. Dit zal naar verwachting in de loop van 2021 weer gaan toenemen. Deze pandemie zou dus nieuwe inzichten kunnen geven over hoe groot de bijdrage is van een aantal van deze factoren op het vóórkomen van ESBL-E in de Nederlandse bevolking.

Het voorkomen van plasmide-gemedieerde colistine resistentie in kippenvlees en bij mensen in Nederland

In een wereld met toegenomen antimicrobiële resistentie, waaronder ESBL- en carbapenemase-producerende Gram-negatieve bacteriën, wordt colistine gezien als een laatste redmiddel. In landen met relatief lage antimicrobiële resistentie wordt colistine weinig gebruikt, omdat het relatief veel bijwerkingen heeft. Tot 2015 waren alle mechanismen die tot colistine resistentie leidden chromosomaal gemedieerd, en kon er dus slechts transmissie plaatsvinden via clonale verspreiding. In 2015 werd het mobiele colistine resistentiegen 1, *mcr*-1, ontdekt. Hierdoor veranderde in één klap de manier waarop er naar colistine resistentie gekeken werd. Nu was de mogelijkheid om via verticale transmissie, dus zelfs tussen verschillende soorten bacteriën, dit gen uit te wisselen zeer reëel.

Na de ontdekking van *mcr*-1 in China, was men benieuwd hoe ver dit gen zich al verspreid had door de wereld. In de laatste twee studies van dit proefschrift wordt gekeken naar de verspreiding van *mcr*-1 in Nederland. Whole-genome sequencing (WGS) is een relatief nieuwe techniek, die sterk in opkomst is binnen de microbiologie. In de twee hoofdstukken wordt zowel een kracht van WGS duidelijk, maar ook een potentiele valkuil.

Bij de ontdekking van *mcr*-1 werd de genetische code voor dit gen gedeeld. Met dit gegeven kon er direct in reeds bestaande databases van eerder gesequencete micro-organismen gezocht worden of het *mcr*-1 gen al aanwezig was in deze historische data. Dit is precies wat er gedaan is in hoofdstuk 6. Selectief gekweekte ESBL-E isolaten werden *in-silico* gescreend voor het nieuwe gen. Er wordt hier gezocht naar isolaten die co-resistentie hebben, dus zowel ESBL produceren en mcr-1 gemedieerde colistine resistentie hebben. Ook werden een aantal isolaten onderzocht waar fenotypisch sprake was van colistine resistentie binnen uitbraak settingen.

In hoofdstuk 6 werd *mcr*-1 gevonden in 2% van de ESBL-EC isolaten van kippenvlees die verzameld waren in 2009 en tussen 2013 en 2014 in Nederland. Eén isolaat werd gekweekt in 2009 en één paar isolaten werd gekweekt in 2014. Dit laatste paar had een sterke epidemiologische link, namelijk hetzelfde lotnummer van het vlees, gekocht op dezelfde dag bij dezelfde supermarktketen. Het is dus waarschijnlijk dat deze twee isolaten dezelfde bron hebben. *mcr*-1 werd niet aangetoond in een grote verzameling van ESBL en non-ESBL producerende Gram-negatieve bacteriën met humane afkomst uit Nederlandse ziekenhuizen. De snelheid waarmee deze isolaten digitaal gescreend werden voor de aanwezigheid van *mcr*-1 is een ongelofelijke kracht van WGS. Het potentieel om data te heranalyseren voor nieuw ontdekte genen is echt van groot belang. Er is hiermee aangetoond, met relatief weinig inspanning, dat dit gen dat in 2015 ontdekt is, al in 2009 in Nederland aanwezig was. Het laat ook een beperking van de techniek zien: als het gen niet in de database zit, wordt het niet gevonden.

In hoofdstuk 7, de tweede studie over *mcr*-1, wordt duidelijk dat de gevonden resultaten in hoofdstuk 6 niet het hele plaatje schetsten. In hoofdstuk 7 werd de prevalentie van *mcr*-1 in kippenvlees uit Nederland onderzocht op vleesmonsters uit 2015. Dit werd gedaan door PCRs op niet selectieve vloeibare ophopingsmedia en door gebruik te maken van selectieve kweekmedia. Deze studie liet zien dat in 24,8% (53/214) van de vleesmonsters het *mcr*-1 gen aanwezig was. In de selectieve kweken werden in 15,9% (34/214) van de kweken colistine-resistente Enterobacteriaceae aangetoond, waar met PCR op het isolaat de aanwezigheid van het *mcr*-1 gen moleculair bevestigd werd. Bij het vergelijken van deze twee methodes, werden in 64,2% (34/53) van de monsters waar het *mcr*-1 gen met PCR op vloeibaar medium aangetoond was, een *mcr*-1 bevattend isolaat aangetoond. In de monsters waar dit niet lukte, was er meestal sprake van overgroei door intrinsiek colistine resistente Gram-negatieve bacteriën zoals *Serratia* en *Proteus*. Deze studie liet een hogere prevalentie van *mcr*-1 zien dan in eerste instantie misschien verwacht zou worden op basis van hoofdstuk 6. Dit komt

door het feit dat in hoofdstuk 6 grotendeels naar gecombineerde ESBL productie en *mcr*-1 gemedieerde colistine resistentie werd gekeken. Gebaseerd op de isolaten die gevonden werden in de studie in hoofdstuk 7, was deze co-resistentie slechts aanwezig in 3% (1/34) van de isolaten.

Hoe belangrijk is de vondst van *mcr*-1 in Nederland? Daar is niet één passend antwoord op. *mcr*-1 werd frequent gevonden op kippenvlees, maar lijkt relatief weinig voor te komen bij mensen, namelijk 0.4%. In het huidige resistentielandschap in Nederland ontstaan er geen acute problemen door de gevonden colistine resistentie. Colistine wordt weinig gebruikt, en de resistentiemechanismen waardoor we op colistine over zouden moeten gaan, bijvoorbeeld carbapenemases, zijn gelukkig echt nog zeldzaam. Wel moeten de veranderingen in het resistentielandschap goed in de gaten gehouden worden, zowel nationaal als internationaal. Het is niet mogelijk om Nederland als losstaand land te beschouwen met de hoge mate van internationale mensbewegingen. Er zullen continu nieuwe antimicrobiële resistente bacteriën mee naar Nederland teruggenomen worden en het enige wat hieraan gedaan kan worden, is op een globale schaal naar de resistentie problematiek te kijken en daar naar oplossingen te zoeken.

De combinatie van hoofdstuk 6 en 7 laat goed zien, dat men altijd voorzichtig moet zijn met het trekken van conclusies uit data die opnieuw geanalyseerd zijn, en dat het van essentieel belang is om de resultaten te bevestigen met onderzoek met specifiek voor die vraagstelling opgezette gedegen microbiologische technieken.

CLOSING PAGES

Dankwoord

Pepijn Huizinga

DANKWOORD

Als je toekomstig opleider je vraagt of je interesse hebt om ook onderzoek te doen, wat tot een promotietraject zou kunnen leiden, is het moeilijk om te overzien wat dat voorstel precies inhoudt. Er zijn vele clichés op te schrijven en veel zijn er waar. Het leren onderzoek doen is uitdagend, zowel inhoudelijk als alle zaken er omheen. Het is een traject wat veel impact heeft gehad op mijn leven. Eén van de zaken die ik op den duur het meest ben gaan voelen, is het idee dat je eigenlijk nooit 'vrij' hebt. Natuurlijk heb je dat wel, maar toch heb je altijd het gevoel dat je beter aan je promotie had kunnen gaan zitten. Mogelijk is dit sterker als je het combineert met een medische specialisatie. Ondanks wat heuvels en frustratiemomenten, heb ik er veel van geleerd en heb ik dingen bereikt die ik niet verwacht had ooit te zullen doen. Veel van de zaken die ik geleerd heb tijdens mijn promotie zijn zeer waardevol geweest in mijn eerste jaar als arts-microbioloog, in pandemie tijd... bijvoorbeeld het frequent toepassen van het motto: 'perfect is the enemy of good'.

Beste Jan, zeer veel dank voor het begeleiden van mijn promotietraject. Zowel je gave om ingewikkelde onderwerpen begrijpelijk te maken voor een breder publiek, als het vroegtijdig zien van broodkruimels die je snel om weet te zetten in onderzoekslijnen, zijn bijzonder inspirerend geweest. Je hebt me geleerd om toe te werken naar afronding van projecten en altijd terug te gaan naar de oorspronkelijke vraag. Korte artikelen met een duidelijke boodschap zijn ook veel waard. De roadtrip van 1450 km (Dresden retour) was een bijzondere start aan dit gezamenlijke traject. Veel dank.

Beste Marjolein, ondanks dat je geen formele rol had in mijn promotietraject, heb je er veel tijd en energie in geïnvesteerd. In mijn directe begeleiding en in het sparren over stukken, met altijd het doel voor ogen om de stukken naar een hoger niveau te krijgen. Jouw visie op waar stukken heen moesten en zeer scherpe blik op fouten in analyses en databases, waren essentieel voor mij om deze promotie op een goede en veilige manier af te kunnen ronden. Dit deed je ook nog eens in extreem hoog tempo, op letterlijk ieder moment van de dag. Het genot om een vraag even bij iemand neer te leggen, was bij jou altijd van zeer korte duur. Als het antwoord niet binnen vijf minuten kwam, was het er zeker binnen het uur (ook om 01.00 uur 's nachts overigens). Heel erg veel dank voor je inzet, tijd en energie.

Beste Carlo, ik wil je bedanken voor het zijn van mijn handen in het lab. Je scherpe observatievermogen en de extreme precisie waarmee je de (vaak grote hoeveelheden) kweken uitvoerde, vormen de praktische basis van dit onderzoek. Je was soms moeilijk te vinden in het lab, tussen de enorme stapels platen die je om je heen verzamelt,

maar gelukkig begon je meestal wel te zingen ("Ja de mens is geen konijn, die met een wortel en een biet, zomaar de grond in schiet!") om je positie én aanwezigheid kenbaar te maken! Het was fijn om problemen tijdens de promotie met je te kunnen bespreken. Je uitgebreide ervaring in het lab, gecombineerd met je immer opgewekte noot, zijn goud waard. Je positieve houding en complimenteuze aard hebben mij echt verder geholpen. Ook heb je me geleerd kritisch te kijken naar kweken en daarbij oog te hebben voor de bijzondere en/of gevaarlijkere micro-organismen. Het heeft de eerste vroeg gedetecteerde *Brucella* al opgeleverd!

Veel dank aan de beoordelingscommissie: **Jaap Wagenaar, Christina Vandenbroucke–Grauls, Arjan Stegeman, Jeroen Dewulf en Dick Heederik,** dat jullie in deze hectische tijden zitting hebben willen nemen in de beoordelings- en promotiecommissie.

Het Bredase vrijdagochtend promovendioverleg: **Evert, Joep, Andreas, Veronica, Ina, Carlo, Marjolein, Jan, Suzan, Eefje, Jaco, Jacobien en Suzan.** We hebben veel lol gehad bij de inhoudelijke besprekingen, waar de door Veronica geïnitieerde 'Joep Bingo', immer waardevolle handenalcohol discussies, (eerlijk is eerlijk) volledig onnavolgbare SOS-respons verhandelingen (bril neerleggende Joep: "Snappen jullie het?") en discussies over *mcr*-1, AmpC, DAPC en ATP, toch altijd naar waardevolle artikelen geleid hebben. Het elkaar helpen en uitdagen heeft me verder geholpen. Bij elkaar spuien creëerde ruimte en lucht om door te gaan als er wat tegenzat. Ook de promovendi appgroep bleek waardevol in het delen van successen, tegenslag en heel veel flauwe grappen! Heel veel dank voor een hele leuke en bijzondere tijd.

Mijn **paranimfen Joep en Evert**. Joep, zonder je hulp en uitdaging was ik nooit begonnen met Python programmeren. Het heeft een legio aan mogelijkheden gecreëerd in mijn promotietraject. En naast je jaloersmakende snelheid om stof tot je te nemen en te onthouden ben je ook een hele leuke joviale kerel. Evert, wat heb je veel van iedereen overgenomen, altijd bereid om mee te denken en echt altijd vriendelijk. Een bijzonder slim en warm persoon om in mijn team gehad te hebben. En je briljant droge humor is een gave.

Het sequencen voor de studies is grotendeels in Groningen gedaan, waar het contact met **Silvia García-Cobos, Sigrid Rosema, John Rossen en Alex Friedrich** altijd positief verliep. Heel veel dank voor de leuke samenwerking.

Dank aan de medeonderzoekers van de verschillende studies voor de input en de geleverde commentaren op de studies: **Marc Bonten, Martine Bos, Katrien de Bruyne, Alex Friedrich, Silvia García-Cobos, Jan Kluytmans, Marjolein Kluytmans-van den**

Bergh, Suzan Pas, Miranda van Rijen, Paul Savelkoul, Eefje Schrauwen, Maarten van Smeden, Nick van Spreuwel, John Rossen, Nils van 't Veer, Carlo Verhulst, Ina Willemsen.

De artsen-microbioloog van MAMBZ (Maatschap Artsen-Microbioloog Brabant/ Zeeland) die hebben bijgedragen aan mijn opleiding tot arts-microbioloog en aan deze promotie wil ik ook bedanken: **Anton Buiting, Wouter van den Bijllaardt, Bram Diederen, Jolanda van Hooydonk, Peter Kabel, Peter van Keulen, Jan Kluytmans, Jan Marcelis, Jean-Luc Murk, An-Emmie Nieman, Anne Maijer-Reuwer, Kees Verduin, Jacobien Veenemans, Dik Versteeg, Bas Wintermans, Rob Wintermans en Mireille Wulf.**

De mede-AIOS uit Tilburg en Breda; de groep collega's waar ik elke dag mee samengewerkt heb, waar ik tijdens mijn eerste consulten om de paar minuten met vragen binnen kon lopen zonder me opgelaten te voelen: **Wouter van den Bijllaardt**, **Jacobien Veenemans, Anne Maijer-Reuwer, Els Denie, Judith Beuving, Brigitte van Cleef, Evert den Drijver, Joep Stohr, Karin Gast, Andreas van Arkel, Basia Bergmans, Karen Couderé en Nathalie van der Moeren.** We hebben met elkaar ervaren, beleefd en bediscussieerd wat het vak van arts-microbioloog in zou moeten houden en vele kleine en grotere trajecten doorlopen. De intensieve samenwerking met elkaar heeft mij verder gebracht dan waar ik gedacht had te kunnen komen. Ook hebben we het met elkaar mogelijk gemaakt om promoties naast een medisch specialistenopleiding te doen, wat echt wel veel flexibiliteit heeft gevraagd van iedereen in de groep. Daarvoor ben ik jullie allen zeer dankbaar.

Analisten en deskundigen infectiepreventie uit Breda en Tilburg. Louisa en Geert, dank dat jullie mij de eerste 6 maanden in Breda onder jullie vleugels genomen hebben en me een introductie gegeven hebben over de microbiologie in het lab. Verder heel veel dank aan alle enthousiaste analisten waarmee ik: preparaten bekeken heb, verbaasd was over gekke kweken, een stuk touw uit feces gevist heb (worm?!!), frustratie en blijdschap gedeeld heb over een al dan niet in storing staande Kiestra, samen in de diepe krochten van de oudbouw gedoken ben (ja Gonny! Mooie avonturen), en de kneepjes van het infectiepreventie vak besproken heb. Kortom, te veel om op te noemen, maar wel de basis die nodig is om ook maar te kunnen beginnen om arts-microbioloog te worden en een promotietraject te realiseren. Allen heel erg veel dank!

Renee Ladestein, we konden natuurlijk niet van tevoren bedenken dat kippenvlees kopen spannend zou worden. Dankzij iemand die dreigementen naar een supermarktketen stuurde, moesten we onze plannen om met een grote witte bak

met ijs de supermarkten in te gaan toch een beetje aanpassen. Veel dank voor je hulp! **Nick van Spreuwel, Melissa Konings, Natassia Heezen,** allen studenten die in het lab gewerkt hebben en een bijdrage hebben geleverd aan de totstandkoming van mijn boekje. Veel dank voor jullie inzet.

De Bootcampclub: **Jaco, Wouter, Chantal, Joep** en de mensen die kortere tijd mee gedaan hebben; heel erg veel dank voor vele dinsdag/woensdagavonden waarop we tijdens het opdrukken/planken/squatten/burpees en praten over de tuin, ook uitgebreid over de leuke en ook ronduit frustrerende zaken van het onderzoek gesproken hebben. Heel veel dank voor deze uitlaatklep!

Het plan was om de promotie af te schrijven, een paar avonden per week, in de eerste maanden na mijn start bij **Izore**. And then COVID-19 happened… De avonden vlogen voorbij aan crisismanagement en alles wat tijd vroeg tijdens de COVID-19 pandemie. Ik wil de hele vakgroep van Izore bedanken dat ze toch tijd vrijgemaakt hebben voor mij om wat laatste eindjes af te ronden in de weinige COVID-19 luwten. Veel dank **Jan, Daphne, Karola, Nicolien, Teysir, Afke, Aziz, Loredana, Anne-Marie, Rianne en Theo.**

Jolien en Noach, Wessel, Shoko en Chris, Charliene en Peter, Martine en Rosanne, kortom zeer waardevolle vrienden. Heel veel dank voor de Pep-talks die ik van jullie gehad heb. Het kunnen uitwisselen van ervaringen tijdens het promoveren, en hoe je dat promotietraject in vredesnaam moet combineren met je gezins- en sociale leven, heeft me balans gebracht. Ook als ik die balans wel eens kwijt was, stuurden jullie me snel weer het rechte pad op door even te focussen op de dingen waar het echt om draait in het leven. Ik ben heel erg blij dat ik jullie heb leren kennen en om me heen heb mogen verzamelen.

De PROMSTA-ers; ik wil jullie bedanken voor het zijn van een voorbeeld en een veilige groep om mee op te groeien. Of het nou Mars raketten waren, Pictionary, tennis of volleybal; er werd altijd wat gedaan en iedereen mocht meedoen. Ook de verschillende gesprekken over de complexere onderwerpen gaven mij weer moed. Een groep met een enorme positiviteit en stuk voor stuk voorbeelden hoe in het leven te kunnen staan. Ik kon eigenlijk niet anders dan ook een gooi te doen om te promoveren. Het is gelukt en we gaan het op de volgende barbecue vieren!

Tom, Ineke en Femke, het gezin waarin ik mocht opgroeien. Heel erg veel dank voor een bijzonder goede start. Met eindeloos geduld en liefde werd mij werkelijk iedere kans gegund en gegeven om het maximale uit mijzelf te kunnen halen. Met veel oog voor elkaar en de wereld om ons heen, waar altijd situaties van meerdere kanten

bekeken werden, en uitgegaan werd van het goede in de mens. Ik heb geleerd geen angst te hebben om naar mensen toe te stappen, ik durf hulp te vragen en het aan te bieden. Ik ben jullie hier zeer zeer dankbaar voor.

Marieke, eindelijk, eindelijk, eindelijk, is mijn promotie klaar en niet in de minste plaats door jou. Jouw regel- en plankunsten, je zorgvuldigheid en precisie, en het feit dat je me zo hebt willen ondersteunen, zijn dé redenen dat ik dit traject heb kunnen doorlopen. Het is thuis best wel eens zwaar geweest, als ik weer eens tot laat zat door te werken met een slecht humeur, omdat ik achter een fout in een database was gekomen. Ik heb je best wat te verduren gegeven, dank voor je vergevingsgezindheid. En het meest dankbaar ben ik voor het feit dat je jouw leven met mij wilt delen. Je fantastische lach en vrolijke ogen waarmee je de wereld tegemoet treedt. Ik vind het geweldig en ben je zeer dankbaar dat we naast elkaar kunnen lopen in ons leven.

Fien, je bent nu ongeveer 2,5 jaar bij ons en niet meer weg te denken. Je lacht net zo hard als je moeder (van wie heb je dat??) en weet ieders aandacht te trekken. Je geniet met volle teugen van het leven, tot de batterij even leeg is, je laadt hem op, en begint weer van voren af aan. Ik verheug me erop om, zonder het gevoel aan mijn promotie te moeten werken, in alle rust met je te kunnen spelen en ontdekken.

Loes, we wachten je komst in spanning af. Gisteren was Marieke uitgerekend, dus het kan niet lang meer duren! Jouw verwachte komst heeft me extra motivatie gegeven om moeilijke knopen door te hakken en echt naar afronding toe te werken. Voordat je geboren bent heb je mij al geholpen en ik kan niet wachten om je te leren kennen! Alvast welkom in ons gezinnetje.

CLOSING PAGES

About the author

Pepijn Huizinga

ABOUT THE AUTHOR

Pepijn Huizinga was born on November 19th, 1985 in Maarssen. He graduated from the Cals College in Nieuwegein. He obtained a bachelor degree in Chemistry from the University of Utrecht in 2007, after which he started his medical training at the University Medical Centre Utrecht.

During his medical training he performed a research internship at the division of pediatric infectious diseases and medical microbiology, under the supervision of dr. K. Trzcinski, looking at the effect of *S. pneumoniae* on human monocytes. This adventure further sparked his interest in research. He obtained his medical degree in 2013, after which he worked as a medical doctor in a clinic in Elandsdoorn (South Africa) and in pediatrics and neurology in the Gelderse Vallei Hospital in Ede. After this year, in which he obtained hands-on clinical experience, he started his training as a clinical microbiologist in the Elisabeth TweeSteden Hospital in Tilburg and the Amphia Hospital in Breda in 2014. He combined his medical specialist training with the work that eventually lead to this thesis under the supervision of Prof. dr. J.A.J.W. Kluytmans.

During his specialist training he married Marieke and they received their first child Fien (2018)! In 2019 they moved to Ysbrechtum in Friesland, in the north of the Netherlands, where Marieke works as a pediatrician in the Antonius Hospital in Sneek and Pepijn works at Izore, Center for Infectious Diseases Friesland.

They eagerly await their second child, who is expected in the coming weeks.