Transmission of antibiotic resistance from animals to humans:

Broilers as a reservoir of ESBL-producing bacteria

Patricia M.C. Huijbers

Transmission of antibiotic resistance from animals to humans:

Broilers as a reservoir of ESBL-producing bacteria

Patricia M.C. Huijbers

Thesis committee

Promotor
Prof. Dr M.C.M. de Jong
Professor of Quantitative Veterinary Epidemiology
Wageningen University

Co-promotors
Dr E.A.M. Graat
Assistant professor, Quantitative Veterinary Epidemiology Group
Wageningen University

Dr E. van Duijkeren
Senior scientist, National Institute for Public Health and the Environment, Bilthoven

Other members
Prof. Dr H. Smidt, Wageningen University
Prof. Dr J. Dewulf, Ghent University, Belgium
Prof. Dr D.J.J. Heederik, Utrecht University, The Netherlands
Prof. Dr A. Voss, Radboud University Nijmegen, The Netherlands

This research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences (WIAS).

Transmission of antibiotic resistance from animals to humans:

Broilers as a reservoir of ESBL-producing bacteria

Patricia M.C. Huijbers

Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr A.P.J. Mol,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 15 January 2016
at 1.30 p.m. in the Aula.

Patricia M.C. Huijbers
Transmission of antibiotic resistance from animals to humans: Broilers as a reservoir of ESBL-producing bacteria,
156 pages.

PhD thesis, Wageningen University, Wageningen, NL (2016)
With references, with summary in English

ISBN 978-94-6257-621-6

Abstract

Huijbers, P.M.C. (2016). Transmission of antibiotic resistance from animals to humans: Broilers as a reservoir of ESBL-producing bacteria. PhD thesis, Wageningen University, Wageningen, the Netherlands.

Antibiotic resistance in animals becomes a public health issue when there is transmission of antibiotic resistant bacteria, or their resistance genes, from animals to humans. β-lactam antibiotics are critically important for the treatment of human bacterial infections. Resistance to this class of antibiotics, mediated by extended-spectrum β-lactamases (ESBL) has emerged. Broilers might contribute to transmission to humans due to the high prevalence of ESBL-producing Enterobacteriaceae among their intestinal biome, compared to other livestock species, companion animals, and wildlife. Transmission to humans might occur via the food chain, by direct contact or via the environment. The aim was to investigate transmission of antibiotic resistant bacteria between animals and humans, and more specifically transmission of ESBL-producing *E. coli* between broilers, and between broilers and humans in varying degrees of contact with these animals. Systematically collected and categorised evidence from literature showed that clinically relevant antibiotic resistant bacteria were present in the natural environment, that is in soil, water, air and wildlife. It was therefore hypothesised that humans in areas with high broiler densities might have an increased risk for carriage of ESBL-producing Enterobacteriaceae. This hypothesis was rejected, as the observed risk was lower for these individuals. The situation might be different for individuals living on broiler farms as ESBL-producing *E. coli* were detected on all investigated farms. Among broilers, the within farm prevalence approached 100%, and there was no difference between conventional and organic farms at five weeks, i.e. just before slaughter on conventional farms. On organic farms, the prevalence decreased to 80.0% at 70 days, i.e. slaughter age. Not only transmission to humans via the farm environment, but close physical contact with broilers might, therefore, lead to increased risk for carriage. Prevalence among farmers, their family members and employees on both conventional (19.1%) and organic (18.5%) broiler farms was higher compared to humans in the general population (5.1%). Moreover, people in close contact with live broilers showed the highest risk (27.1 vs. 14.3%). Evidence for clonal transmission of ESBL-producing *E. coli* between humans and broilers was found on conventional farms, and horizontal gene transfer was suspected on both conventional and organic farms. Even without selection pressure from antibiotics ESBL-producing *E. coli* were able to transmit and persist in an organic broiler flock, which shows that broilers form a reservoir of antibiotic resistance genes. This leads to an increased risk of carriage of humans on farms through direct contact with broilers and possibly via the direct farm environment. As only a very small percentage of the general population is exposed to live broilers, direct contact with broilers does not appear to be important for carriage in the general human population.

Table of contents

Chapter 1

General Introduction

Introduction

The emergence of antibiotic resistant bacteria in both humans and animals requires urgent attention. Antibiotic resistance in animals becomes a public health issue when there is transmission of antibiotic resistant bacteria, or their resistance genes, from animals to humans. Infections caused by antibiotic resistant bacteria are difficult to treat, and are associated with excess mortality, prolonged hospital stays, and increased costs [1]. Antibiotic resistance genes are not restricted to pathogens, however. They are widely distributed among human and animal commensal bacteria, and bacteria in the natural environment [2,3]. Limiting the spread of antibiotic resistance genes is of utmost importance, and requires an approach that takes into account transmission between humans, between animals, between animals and humans, and the possible role of the environment in all these transmissions.

This chapter begins by examining the origin, evolution and transmission of antibiotic resistant bacteria. β-lactam antibiotics are critically important for the treatment of human bacterial infections [4], and resistance to this class of antibiotics has emerged in Gram-negative bacteria. The chapter, therefore, proceeds with an introduction to β-lactamase mediated resistance, and the occurrence and transmission of β-lactamase-producing Enterobacteriaceae in humans, animals and the environment. Finally, the scope and outline of this thesis are given.

Origin, evolution and transmission of antibiotic resistant bacteria

Origin

Antibiotic resistance is a naturally occurring phenomenon, widespread in the environment that predates the modern selective pressure of clinical antibiotic use [5]. Metagenomic analyses of DNA from 30,000-year-old permafrost sediments from Alaska yielded a diverse collection of genes encoding resistance to β-lactam, tetracycline and glycopeptide antibiotics [5], providing evidence for this concept. The apparently ancient and ubiquitous character of antibiotic resistance genes in the natural environment raises questions about their function in nature. Finding an answer to this question begins with the definition of an antibiotic, which is "a chemical substance, produced by micro-organisms, which has the capacity to inhibit the growth of and even to destroy bacteria and other micro-organisms" [6]. Evidence is accumulating, however, that antibiotics and antibiotic resistance might have other functions besides bacterial inhibition and protection. At low (i.e. sub-inhibitory) concentrations, antibiotics affect the transcription of many cellular functions [7,8]. This effect on transcription is also seen for synthetic antibiotics, suggesting that their modes of action are analogues to those of antibiotics that occur in the natural environment [9]. It has subsequently been hypothesised that antibiotics may act as

signalling molecules in the natural environment [9,10]. The question then follows whether antibiotic resistance is a self-protection process, a mechanism to modulate the signalling activity of antibiotics, or perhaps it has another function that remains to be elucidated [10].

Evolution

Since antibiotic resistance genes seem to have their origins in environmental bacteria, changes affecting microbiota in the environment may have an impact on antibiotic resistance. These changes could be brought about by the release of antibiotics used for prophylaxis and curative treatment in human and veterinary medicine, and for growth promotion in animal husbandry, but also the release of human and animal related microbiota containing resistance genes [11]. The evolution of genes encoding β-lactamases can be used to illustrate this. It has been suggested that plasmid-encoded β-lactamases have their origin in penicillin-binding proteins, which are enzymes involved in cross-linking peptidoglycan to form bacterial cell walls, and are the target for β-lactam antibiotics [12,13]. It is also speculated that these genes encoding β-lactamases originate from soil-dwelling actinomycetes such as *Streptomyces*, and have spread to other Gram-positive bacteria, and then to Gram-negative bacteria through horizontal gene transfer [14]. Selection pressure from antibiotics used in human and veterinary medicine might have influenced the spread of these β-lactamases, and might also have led to the accumulation of mutations, resulting, for example, in genes that encode β-lactamases with an extended spectrum in Gram-negative Enterobacteriaceae ([15]; see section 2).

Transmission

Transmission can involve transfer of whole bacteria from one host to the other i.e. clonal transfer, or transfer of resistance genes, located on mobile genetic elements, between bacterial species i.e. horizontal gene transfer, including both pathogenic and non-pathogenic strains. These processes are influenced by antibiotic use in human and veterinary medicine [16]. Antibiotic resistant bacteria are excreted by humans and animals via e.g. faeces, leading to contamination of the environment (Figure 1). In the environment, antibiotic resistant bacteria of human and animal origin come into contact with environmental bacteria, potentially resulting in the exchange of resistance genes.

Humans and animals may be exposed to antibiotic resistant bacteria by direct physical contact or indirect contact via the environment, that is fomites and the natural environment (Figure 1). Exposure to these bacteria could lead to infection, carriage or disease in both humans and animals. Infection is defined as the acquisition of an antibiotic resistant bacterium by a host. Carriage, or colonisation, is defined as an outcome of infection whereby an antibiotic resistant bacterium is recovered from a non-sterile site (e.g. intestine) at which no damage is clinically apparent. Disease is defined as a state of infection where host damage occurs as a result of host-microbe interaction and is sufficient to disturb host homeostasis [17].

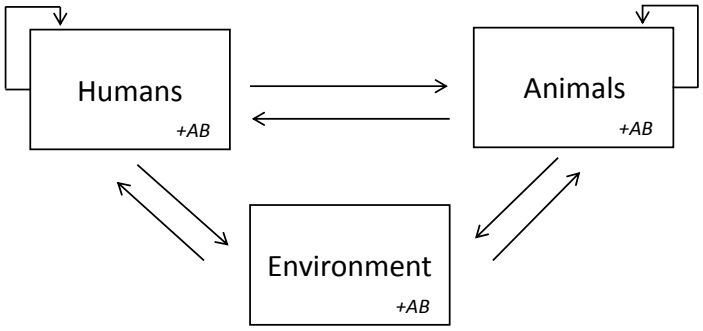

Figure 1. Schematic representation of transmission of antibiotic resistant bacteria between humans, animals and the environment. Antibiotics (+AB) are used for treatment and prophylaxis in human and veterinary medicine, and these antibiotics are disseminated to the environment e.g. via faeces.

β-lactamase-producing Enterobacteriaceae

Resistance to β-lactam antibiotics has emerged in Gram-negative Enterobacteriaceae, which is mainly due to the production of β-lactamases, especially the extended-spectrum β-lactamases (hereafter referred to as ESBLs), but also AmpC β-lactamases [2]. The mechanism by which β-lactamases confer resistance is by antibiotic modification; they are able to hydrolyse the characteristic β-lactam ring, thereby inactivating the antibiotic [18]. ESBLs cause resistance to various types of newer beta-lactam antibiotics, including penicillins, third-generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime) and monobactams (aztreonam), but not cephamycins (cefoxitin, cefotetan), and carbapenems (imipenem, meropenem, ertapenem) (reviewed by [19]). AmpC β-lactamases have a broader spectrum of resistance. In addition to penicillins, third-generation cephalosporins and monobactams, they also confer resistance to cephamycins, and are not inhibited by clavulanic acid (reviewed by [20]).

In Enterobacteriaceae, ESBLs are generally encoded by plasmid-located genes. The most common ESBL-gene families are bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA} (www.lahey.org/studies). bla_{TEM} and bla_{SHV} are derived from bla_{TEM-1}, bla_{TEM-2} and bla_{SHV-1}, which lack an extended-spectrum, and differ in as few as one amino acid from their progenitors, leading to a changed substrate profile. Certain bla_{CTX-M} types seem to have emerged from environmental bacteria of the *Kluyvera* spp. [21]. bla_{TEM}, bla_{SHV} and bla_{CTX-M} have been found most commonly in *Escherichia coli* and *Klebsiella pneumoniae*, but also in other members of the Enterobacteriaceae such as *Citrobacter* spp., *Proteus* spp., *Salmonella* spp., *Morganella morganii* and *Enterobacter* spp. bla_{OXA} have mainly been found in *Pseudomonas aeruginosa* (reviewed by [22]).

AmpC-enzymes are encoded by both chromosomal and plasmid-located genes in Enterobacteriaceae. In species such as *Enterobacter* spp. and *Citrobacter* spp., AmpC enzymes

are inducible upon exposure to β-lactam antibiotics and can be expressed at high levels by mutation. Overexpression confers resistance to broad-spectrum cephalosporins including cefotaxime and ceftazidime. In bacterial species lacking or poorly expressing chromosomal AmpC-genes such as *K. pneumonia* and *E. coli*, transmissible plasmids have acquired genes for AmpC-enzymes. AmpC-genes include bla_{CMY}, bla_{ACT}, bla_{DHA}, bla_{FOX} and bla_{MIR}. Some of these varieties are determined by chromosomal genes, and represent possible progenitors for the plasmid-determined enzymes (reviewed by [20]).

β-lactamase genes are associated with transposable elements, which facilitate their movement between chromosome and plasmid, and between plasmids within a bacterial cell. For movement of genes between bacterial cells a conjugative element (i.e. plasmid or conjugative transposon) or bacteriophage is required (reviewed by [16]). It is speculated that mobile genetic elements and bacteriophages have contributed to the successful selection and dispersion of β-lactamase genes within and between bacterial species [23,24].

Occurrence and transmission of β-lactamase-producing Enterobacteriaceae

Occurrence and risk factors in humans and animals

In Europe from 2010 to 2013, a 9.5% to 12.6% significant increase in resistance to third-generation cephalosporins in *E. coli*, which is an indicator for ESBL-production, was observed in invasive human isolates (blood and cerebrospinal fluid). In the Netherlands, the percentage of third-generation cephalosporin resistant *E. coli* (3GCREC) was 5.1% out of 3387 invasive *E. coli* isolates in 2010 and 5.8% out of 4740 in 2013, with 88.3% of 3GCREC isolates being ESBL-positive in 2013. In comparison, the lowest and highest percentages of 3GCREC in 2013 were found in Iceland (5.0% out of 121; 73.6% ESBL) and Bulgaria (39.6% out of 187; 90.9% ESBL), respectively [25]. Risk factors associated with hospital-acquired infections with ESBL-producing Enterobacteriaceae include female gender, previous antibiotic therapy, use of a nasogastric tube, use of invasive procedures (i.e. arterial or urinary catheters), and a longer period of hospitalisation [26-28]. Information about prevalence of ESBL-producing Enterobacteriaceae carriage in the general population is limited to specific studies, as no systematic (international) surveillance network has been set up to monitor ESBL-producing Enterobacteriaceae in the community. In the Netherlands, the prevalence of ESBL- and AmpC-carriage among individuals registered at five general practitioners in the area of Amsterdam (n=550) was 9.5% and 1.3%, respectively [29]. Risk factors for community-acquired infections with ESBL-producing Enterobacteriaceae include recent antibiotic-use, residence in a long term care facility, recent hospitalisation, age ≥65 years, and male gender [30]. Travel abroad has been reported as a risk factor in multiple studies [31-33].

ESBL-producing Enterobacteriaceae are also reported in livestock, companion animals, and wildlife. In the European programme for monitoring antimicrobial resistance, cefotaxime-resistance (i.e. indicator for ESBL) was shown among *E. coli* isolates randomly selected from broilers at the slaughterhouse (6.6%), from pigs (1.3%), and from cattle (i.e. veal calves, beef cattle, dairy cows; 1.2%; [34]). In the Dutch monitoring programme, these percentages were 2.9% resistance among broilers, 2.0% in white veal calves, 0.5% in pigs and 0.4% among dairy cows. No positive isolates were recovered from rosé veal calves (0.0%; [35]). When selective enrichment was applied, prevalences of 66.0% in broilers, 17.9% in white veal calves, 12.3% in pigs, 11.3% in rosé veal calves, and 6.0% in dairy cows were found [35]. Unlike in livestock, prevalences of ESBL-producing Enterobacteriaceae in companion animals, such as dogs, cats and horses, are only available from specific studies. Without selective enrichment, between 2.6% (1/39) and 6.9% (14/204) of dogs, and between 0.0% (0/36) and 3.3% (2/61) of cats were ESBL-positive [36,37]. With selective enrichment, and taking into account that samples originated from non-diarrheic and diarrheic animals, 45.0% (9/20) of non-diarrheic dogs, 55.0% (11/20) of diarrheic dogs, 0.0% (0/20) of non-diarrheic cats, and 25.0% (5/20) of diarrheic cats were ESBL-positive [38]. Doljeska et al. [39] isolated *E. coli* after selective enrichment from horses at a riding centre and an equine clinic, and found 11.4% (5/44) ESBL-positive horses at a riding centre and 34.2% (13/38) at an equine clinic. The first study on ESBL-producing bacteria in wildlife dates from 2006, where 16% (9/56) of animals sampled from national parks were found to be colonised by ESBL-producing *E. coli* after selective enrichment. Positive isolates were recovered from birds of prey (n=5), deer (n=2), fox (n=1) and owl (n=1; [40]). Since then, studies have focussed mainly on wild birds, wild boar and rodents (reviewed by [41]). When focussing on those studies where selective enrichment was used, the prevalence of carriage ranged from 0.5% (2/396) to 26.9% (32/119) in wild birds [42,43], 1.7% (5/293) to 10.4% (8/77) in wild boar [44,45], and 4.2% (19/456) to 14.2% (8/56) in rodents [46,47]. Studies looking at risk factors for infection with or carriage of ESBL-producing bacteria in animals are few and limited to the veterinary hospital setting. A study in dogs from a veterinary teaching hospital in Australia showed that hospitalisation, treatment with a fluoroquinolone, and diagnostic imaging within 42 days prior to admission increased the risk of carriage of multi-drug resistant *E. coli* at admission [48]. In a study at the same veterinary hospital, it was also shown that hospitalisation for >6 days, treatment with cephalosporins prior to admission, treatment with cephalosporins for >1 day, and treatment with metronidazole while hospitalised were associated with increased risk of rectal carriage of multi-drug resistant *E. coli* during hospitalisation [49].

Transmission between humans, animals and the environment

It is clear from the previous section that β-lactamase-producing Enterobacteriaceae are widely distributed among human and animal populations, and that there are a number of risk

factors associated with infection or carriage. Understanding more about transmission within, and between human and animal populations is an important starting point for intervention. When spatiotemporally-related isolates from two individuals are the same with respect to bacterial species, plasmid type, and/or ESBL-gene this is an indication that transmission has occurred. Using this measurement it can be said that transmission of ESBL-producing Enterobacteriaceae has been shown between humans in hospitals [50,51], and between humans in households [52,53]. Furthermore, *E. coli* strain sharing has been shown between pets, and between humans and pets (i.e. dog, cat) within the same household [54], which suggests that strains of ESBL-producing Enterobacteriaceae might also be shared. Studies looking at transmission of β-lactamase-producing Enterobacteriaceae between humans and animals on livestock farms are scarce and limited to pig farms, but results suggest that working with positive pigs is associated with an increased risk of carriage [55-57]. There do not seem to be any studies looking at on-farm transmission of ESBL-producing bacteria between animals. Transmission between livestock is plausible, as animals are usually housed together in groups where frequent contact with other group members, as well as with the shared environment (e.g. faecal matter), occurs. Further research is needed to determine, and preferably quantify, transmission rates and routes between livestock, between humans and livestock on farms, and between humans living in proximity to the farm environment.

Aim and outline of the thesis

Poultry, in particular broilers, might play a role in the transmission of resistance genes to humans due to the high prevalence of ESBL-producing Enterobacteriaceae among their intestinal biome [58], compared to other livestock species, companion animals, and wildlife [34-36,41]. Poultry meat has been identified as a possible source for human disease [59], and transmission to humans via the food chain is hypothesised [60], as a high degree of genetic similarity among ESBL-*E. coli* isolates from chicken meat and humans based on the mobile genetic elements, virulence genes, and genomic backbone was shown [61]. This is only one possible route, however, by which ESBL-producing Enterobacteriaceae might be exchanged between humans and animals. Transmission by close physical contact and via the environment should also be considered as broiler farms, their immediate surroundings, but also the wider natural environment could be contaminated with ESBL-producing Enterobacteriaceae, potentially leading to transmission at sites relevant for human exposure. The aim of this thesis is, therefore, to investigate transmission of antibiotic resistant bacteria between animals and humans, and more specifically the transmission of ESBL-producing *E. coli* between broilers, and between broilers and humans in varying degrees of contact with broilers. In order to gain insight into transmission routes, studies were conducted answering key questions on (1)

the role of the natural environment in the transmission of clinically relevant antimicrobial resistant (AMR) bacteria to humans; (2) prevalence of, and risk factors for, carriage of ESBL-producing Enterobacteriaceae in the general human population living in areas with high and low broiler densities, and humans on conventional and organic broiler farms; and (3) prevalence of carriage, and transmission dynamics among broilers on a broiler farm under field conditions.

Clinically relevant AMR bacteria, present among the intestinal biome of humans and animals, might be disseminated to the natural environment i.e. soil, water, and air/dust, via human and animal faeces i.e. wastewater and manure. Relevant literature was therefore systematically collected and categorised to elucidate the role of the natural environment in the transmission of these bacteria to humans (*Chapter 2*). The occurrence of clinically relevant AMR bacteria in the environment leads to the hypothesis that individuals in areas with high broiler densities might have an increased risk for carriage. The prevalence of, and risk factors for carriage of ESBL-producing Enterobacteriaceae are therefore determined for humans living in municipalities with either a high or low broiler density (*Chapter 3*). Not only transmission via the environment, but close physical contact with broilers might lead to increased risk for carriage in humans. The prevalence of ESBL/AmpC-producing *E. coli* carriage among farmers, their family members and employees on a conventional broiler farm are therefore estimated. Furthermore, risk factors for carriage, with an emphasis on contact with live broilers are identified and quantified. To gain further insight into transmission routes, isolates from humans and broilers within farms are compared with respect to molecular characteristics (*Chapter 4*). Due to differences in management practices between conventional and organic farms, especially antibiotic use, the prevalence of ESBL/AmpC-producing *E. coli* carriage among broilers, and humans living and/or working on organic broiler farms are estimated, and compared to results from the conventional farms. In addition, molecular typing results of isolates from humans and broilers are presented (*Chapter 5*). More information about transmission of ESBL-producing *E. coli* between broilers could provide valuable insights into the extensive dissemination of these bacteria among animals, resulting in a starting point for on-farm intervention. Transmission rates and routes of ESBL-producing *E. coli*, and specific phylogenetic groups, are therefore quantified in an organic broiler flock where no antibiotics were used (*Chapter 6*). This thesis concludes with a discussion of the contribution of ESBL-producing *E. coli* from broilers to human carriage, and the relative contribution of other sources and pathways in carriage of humans in the general population. Furthermore, recommendations for future research and main conclusions from this thesis are given (*Chapter 7*).

References

1. de Kraker, M.E.A., Davey, P.G., Grundmann, H. 2011. Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteraemia: Estimating the burden of antimicrobial resistance in Europe. *PLoS Medicine*. 8:e1001104.
2. Hawkey, P.M., Jones, A.M. 2009. The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy*. 64:i3-i10.
3. Allen, H.K., Donato, J., Wang, H.H., *et al*. 2010. Call of the wild: antibiotic resistance genes in natural environments. *Nature Reviews Microbiology*. 8:251-259.
4. WHO (World Health Organisation). 2012. Critically important antimicrobials for human medicine – 3rd revision. Geneva, Switzerland. http://www.who.int/foodsafety/publications/ antimicrobial-resistance/en/ (accessed 29 September 2015).
5. D'Costa VM, King CE, Kalan L, Morar M, Sung WWL, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, Golding GB, Poinar HN, Wright GD. 2011. Antibiotic resistance is ancient. *Nature*. 477: 457-461.
6. Waksman, S.A. 1947. What is an antibiotic or an antibiotic substance? *Mycologia*. 39:565-569.
7. Goh, E.B., Yim, G., Tsui, W., *et al*. 2002. Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proceedings of the National Academy of Sciences of the United States of America*. 99:17025-17030.
8. Linares, J.F., Gustafsson, I., Baquero, F., *et al*. 2006. Antibiotics as intermicrobial signaling agents instead of weapons. *Proceedings of the National Academy of Sciences of the United States of America*. 103:19484-19489.
9. Yim, G., Wang, H.H., Davies, J. 2007. Antibiotics as signalling molecules. *Philosophical Transactions of the Royal Society B: Biological Sciences*. 362:1195-1200.
10. Davies, J. 2006. Are antibiotics naturally antibiotics? *Journal of Industrial Microbiology and Biotechnology*. 33:496-499.
11. Martinez, J.L. 2009. The role of natural environments in the evolution of resistance traits in pathogenic bacteria. *Proceedings of the Royal Society B*. 276:2521-2530.
12. Massova, I., Mobashery, S. 1998. Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. *Antimicrobial Agents and Chemotherapy*. 42:1-17.
13. Meroueh, S.O., Minasov, G., Lee, W., *et al*. 2003. Structural aspects for evolution of β-lactamases from penicillin-binding proteins. *Journal of the American Chemical Society*. 125:9612-9618.
14. Kirby, R. 1992. Evolutionary origin of the class A and class C β-lactamases. *Journal of Molecular Evolution*. 34:345-350.
15. Hall, B.G., Barlow, M. 2004. Evolution of the serine β-lactamases: past, present and future. *Drug Resistance Updates*. 7:111-123.
16. Smet, A., Martel, A., Persoons, D., *et al*. 2009. Broad-spectrum beta-lactamases among *Enterobacteriaceae* of animal origin: molecular aspects, mobility and impact on public health. *FEMS Microbiology Reviews*. 34:295-316.
17. Pirofski, L.A., Casadevall, A. 2002. The meaning of microbial exposure, infection, colonisation, and disease in clinical practice. *The Lancet Infectious Diseases*. 2:628-635.
18. Hawkey, P.M. 1998. The origins and molecular basis of antibiotic resistance. *British Medical Journal*. 317:657-660.
19. Livermore, D.M. 2008. Defining an extended-spectrum β-lactamase. *Clinical Microbiology and Infection*. 14:3-10.
20. Jacoby, G.A. 2009. AmpC β-lactamases. *Clinical Microbiology Reviews*. 22:161-182.
21. Bonnet, R. 2003. Growing group of extended-spectrum-lactamases: the CTX-M enzymes. *Antimicrobial Agents and Chemotherapy*.48:1-14.
22. Bradford, P.A. 2001. Extended-spectrum β-lactamases in the 21st century: Characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews*. 14:933-951.
23. Karim, A., Poirel, L., Nagarajan, S., *et al*. 2001. Plasmid-mediated extended-spectrum β-lactamase (CTX-M-3 like) from India and gene association with insertion sequence ISEcp1. *FEMS Microbiology Letters*. 201:237-241.
24. Cloeckaert, A., Praud, K., Doublet, B., *et al*. 2007. Dissemination of an extended-spectrum-β-lactamase *bla*TEM-52 gene-carrying IncI1 plasmid in various *Salmonella enterica* serovars isolated from poultry and humans in Belgium and France between 2001 and 2005. *Antimicrobial Agents and Chemotherapy*. 51:1872-1875.
25. ECDC (European Centre for Disease Prevention and Control). 2014. Antimicrobial resistance surveillance in Europe 2013. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm, Sweden.

26. Lucet, J.C., Chevret, S., Decré, D., *et al*. 1996. Outbreak of multiply resistant *Enterobacteriaceae* in an intensive care unit: epidemiology and risk factors for acquisition. *Clinical Infectious Diseases*. 22:430-436.
27. Peña, C., Gudiol, C., Tubau, F., *et al*. 2006. Risk-factors for acquisition of extended-spectrum β-lactamase-producing *Escherichia coli* among hospitalised patients. *Clinical Microbiology and Infection*. 12:279-284.
28. Tumbarello, M., Spanu, T., Sanguinetti, M., *et al*. 2006. Bloodstream infections caused by extended-spectrum-β-lactamase-producing *Klebsiella pneumoniae*: risk factors, molecular epidemiology, and clinical outcome. *Antimicrobial Agents and Chemotherapy*. 50:498-504.
29. Reuland, E.A., Halaby, T., Hays, J.P., *et al*. 2015. Plasmid-mediated AmpC: Prevalence and community-acquired isolates in Amsterdam, the Netherlands, and risk factors for carriage. *PLoS One*. 10:e0113033.
30. Ben-Ami, R., Rodriguez-Bano, J., Arslan, H., *et al*. 2009. A multinational survey of risk factors for infection with extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in nonhospitalized patients. *Clinical Infectious Diseases*. 49:682-690.
31. Laupland, K.B., Church, D.L., Vidakovich, J., *et al*. 2008. Community-onset extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli*: importance of international travel. *Journal of Infection*. 57:441-448.
32. Tängdén, T., Cars, O., Melhus, Å., *et al*. 2010. Foreign travel is a major risk factor for colonization with *Escherichia coli* producing CTX-M-type extended-spectrum beta-lactamases: a prospective study with Swedish volunteers. *Antimicrobial Agents and Chemotherapy*. 54:3564-3568.
33. Wickramasinghe, N.H., Xu, L., Eustace, A., *et al*. 2012. High community faecal carriage rates of CTX-M ESBL-producing *Escherichia coli* in a specific population group in Birmingham, UK. *Journal of Antimicrobial Chemotherapy*. 67:1108-1113.
34. EFSA/ECDC (European Food Safety Authority / European Centre for Disease Prevention and Control). 2015. EU summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013. *EFSA Journal*. 13:4036.
35. MARAN. 2015. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2014. http://www.cvi.wur.nl/NL/publicaties/rapporten/maranrapportage/ (accessed 29 September 2015).
36. Carattoli, A., Lovari, S., Franco, A., *et al*. 2005. Extended-spectrum β-lactamases in *Escherichia coli* isolated from dogs and cats in Rome, Italy, from 2001 to 2003. *Antimicrobial Agents and Chemotherapy*. 49:833-835.
37. Costa, D., Poeta, P., Sáenz, Y., *et al*. 2008. Prevalence of antimicrobial resistance and resistance genes in faecal *Escherichia coli* isolates recovered from healthy pets. *Veterinary Microbiology*. 127:97-105.
38. Hordijk, J., Schoormans, A., Kwakernaak, M., *et al*. 2013. High prevalence of fecal carriage of extended spectrum β-lactamase/AmpC-producing *Enterobacteriaceae* in cats and dogs. *Frontiers in Microbiology*. 4:242.
39. Doljeska, M., Duskova, E., Rybarikova, J., *et al*. 2011. Plasmids carrying $bla_{CTX\text{-}M\text{-}1}$ and *qnr* genes in *Escherichia coli* isolates from an equine clinic and horseback riding centre. *Journal of Antimicrobial Chemotherapy*. 66:757-764.
40. Costa, D., Poeta, P., Sáenz, Y., *et al*. 2006. Detection of *Escherichia coli* harbouring extended-spectrum β-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild animals in Portugal. *Journal of Antimicrobial Chemotherapy*. 58:1311-1312.
41. Guenther, S., Ewers, C., Wieler, L.H. 2011. Extended-spectrum beta-lactamases producing *E. coli* in wildlife, yet another form of environmental pollution? *Frontiers in Microbiology*. 2:246.
42. Pinto, L., Radhouani, H., Coelho, C., *et al*. 2010. Genetic detection of extended-spectrum β-lactamase-containing *Escherichia coli* isolates from birds of prey from Serra da Estrela natural reserve in Portugal. *Applied and Environmental Microbiology*. 76:4118-4120.
43. Garmyn, A., Haesebrouck, F., Hellebuyck, T., *et al*. 2011. Presence of extended-spectrum β-lactamase-producing *Escherichia coli* in wild geese. *Journal of Antimicrobial Chemotherapy*. 66:1643-1644.
44. Poeta, P., Radhouani, H., Pinto, L., et al. 2009. Wild boars as reservoirs of extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli* of different phylogenetic groups. *Journal of Basic Microbiology*. 49:584-588.
45. Literak, I., Dolejska, M., Radimersky, T., *et al*. 2010. Antimicrobial-resistant faecal *Escherichia coli* in wild mammals in central Europe: multiresistant *Escherichia coli* producing extended-spectrum beta-lactamases in wild boars. *Journal of Applied Microbiology*. 108:1702-1711.
46. Ho, P.L., Chow, K.H., Lai, E.L., *et al*. 2011. Extensive dissemination of CTX-M-producing *Escherichia coli*

with multidrug resistance to 'critically important' antibiotics among food animals in Hong Kong, 2008-10. *Journal of Antimicrobial Chemotherapy*. 66:765-768.

47. Guenther, S., Wuttke, J., Bethe, A., *et al*. 2013. Is fecal carriage of extended-spectrum β-lactamase-producing *Escherichia coli* in urban rats a risk for public health? *Antimicrobial Agents and Chemotherapy*. 57:2424-2425.
48. Gibson, J.S., Morton, J.M., Cobbold, R.N., *et al*. 2011a. Risk factors for multidrug-resistant *Escherichia coli* rectal colonization of dogs on admission to a veterinary hospital. *Epidemiology and Infection*. 139:197-205.
49. Gibson, J.S., Morton, J.M., Cobbold, R.N., *et al*. 2011b. Risk factors for dogs becoming rectal carriers of multidrug-resistant *Escherichia coli* during hospitalization. *Epidemiology and Infection*. 139:1511-1521.
50. Harris, A.D., Kotetishvili, M., Shurland, S., *et al*. 2007a. How important is patient-to-patient transmission in extended-spectrum β-lactamase *Escherichia coli* acquisition. *American Journal of Infection Control*. 35:97-101.
51. Harris, A.D., Perencevich, E., Johnson, J.A., *et al*. 2007b. Patient-to-patient transmission is important in extended-spectrum β-lactamase–producing *Klebsiella pneumoniae* acquisition. *Clinical Infectious Diseases*. 45:1347-1350.
52. Valverde, A., Grill, F., Coque, T.M., *et al*. 2008. High rate of intestinal colonization with extended-spectrum beta-lactamase-producing organisms in household contacts of infected community patients. *Journal of Clinical Microbiology*. 46:2796-2799.
53. Hilty, M., Betsch, B., Bögli-Stuber, K., *et al*. 2012. Transmission dynamics of extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae* in the tertiary care hospital and the household setting. *Clinical Infectious Diseases*. 55:967-975.
54. Johnson, J.R., Owens, K., Gajewski, A., *et al*. 2008. *Escherichia coli* colonization patterns among human household members and pets, with attention to acute urinary tract infection. *Journal of Infectious Diseases*. 197:218-224.
55. Moodley, A., Guardabassi, L. 2009. Transmission of IncN plasmids carrying $bla_{CTX\text{-}M\text{-}1}$ between commensal *Escherichia coli* in pigs and farm workers. *Antimicrobial Agents and Chemotherapy*. 53:1709-1711.
56. Hammerum, A.M., Larsen, J., Andersen, V.D., *et al*. 2014. Characterization of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* obtained from Danish pigs, pig farmers and their families from farms with high or no consumption of third- or fourth-generation cephalosporins. *Journal of Antimicrobial Chemotherapy*. 69:2650-2657.
57. Dohmen, W., Bonten, M.J.M., Bos, M.E.H., *et al*. 2015. Carriage of extended-spectrum β-lactamases in pig farmers is associated with occurrence in pigs. *Clinical Microbiology and Infection*. 21:917-923.
58. Dierikx, C.M., van der Goot, J., Fabri, T., *et al*. 2013. Extended-spectrum-β-lactamase- and AmpC-β-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers. *Journal of Antimicrobial Chemotherapy*. 68:60-67.
59. Collignon, P., Aarestrup, F.M., Irwin, R., *et al*. 2013. Human deaths and third-generation cephalosporin use in poultry. *Emerging Infectious Diseases*. 19:1339-1340
60. Overdevest, I.T.M.A., Willemsen, I., Rijnsburger, M., *et al*. 2011. Extended-spectrum β-lactamase genes of *Escherichia coli* in chicken meat and humans, the Netherlands. *Emerging Infectious Diseases*. 17:1216-1222.
61. Kluytmans, J.A.J.W., Overdevest, I.T.M.A., Willemsen, I., *et al*. 2013. Extended-spectrum β-lactamase-producing *Escherichia coli* from retail chicken meat and humans: Comparison of strains, plasmids, resistance genes, and virulence factors. *Clinical Infectious Diseases*. 56:478-487.

Chapter 2

Role of the environment in the transmission of antimicrobial resistance to humans: a review

Patricia M.C. Huijbers
Hetty Blaak
Mart C.M. de Jong
Elisabeth A.M. Graat
Christina M.J.E. Vandenbroucke-Grauls
Ana Maria de Roda Husman

Environmental Science and Technology (2015) 49:11993-12004.

Abstract

To establish a possible role for the natural environment in the transmission of clinically relevant AMR bacteria to humans, a literature review was conducted to systematically collect and categorize evidence for human exposure to extended-spectrum β-lactamase-producing Enterobacteriaceae, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant *Enterococcus* spp. in the environment. In total, 239 datasets adhered to inclusion criteria. AMR bacteria were detected at exposure-relevant sites (35/38), including recreational areas, drinking water, ambient air, and shellfish, and in fresh produce (8/16). More datasets were available for environmental compartments (139/157), including wildlife, water, soil, and air/dust. Quantitative data from exposure-relevant sites (6/35) and environmental compartments (11/139) were scarce. AMR bacteria were detected in the contamination sources (66/66) wastewater and manure, and molecular data supporting their transmission from wastewater to the environment (1/66) were found. The abundance of AMR bacteria at exposure-relevant sites suggests risk for human exposure. Of publications pertaining to both environmental and human isolates, however, only one compared isolates from samples that had a clear spatial and temporal relationship, and no direct evidence was found for transmission to humans through the environment. To what extent the environment, compared to the clinical and veterinary domains, contributes to human exposure needs to be quantified. AMR bacteria in the environment, including sites relevant for human exposure, originate from contamination sources. Intervention strategies targeted at these sources could therefore limit emission of AMR bacteria to the environment.

Introduction

The occurrence and spread of antimicrobial resistant (AMR) bacteria are pressing public health problems worldwide. Recently, in its first global report on surveillance of antimicrobial resistance, the World Health Organization (WHO) reported very high rates of resistance in bacteria (*e.g.*, *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*) that cause common healthcare-associated and community-acquired infections in people in all WHO regions [1]. Infections caused by AMR bacteria are associated with excess mortality, prolonged hospital stays, and increased costs [2]. In order to formulate effective intervention strategies to combat intractable infections caused by AMR bacteria, it is important to discern which fraction of the total disease burden and costs are attributable to different sources, including animal reservoirs and vehicles such as foods, or the environment [3].

Enteric bacteria are introduced into the environment with human and animal feces, and people may be exposed to these bacteria through, *e.g.*, recreation in contaminated surface water, consumption of contaminated drinking water, fresh produce, or (shell) fish, and inhalation of bioaerosols. Previous studies indicate that the risk of contracting *Salmonella* spp. or *Campylobacter* spp. in recreational waters is higher than or equal to the risk of contracting the organisms through chicken consumption [4-6]. In the United States of America, 9% of all outbreaks with the pathogenic *E. coli* O157 are waterborne [7]. Based on global WHO risk assessments, it was estimated that there are over 120 million cases annually of gastrointestinal disease from exposure to coastal waters via recreation or by eating raw or lightly cooked shellfish [8]. Multiple studies have described outbreaks of infections with Enterobacteriaceae associated with consumption of fresh produce [9]. Examples include the 2011 outbreak of *E. coli* O104:H4 associated with sprouts in Europe and Northern America [10], and the 2006 outbreak of *E. coli* O157:H7 associated with spinach in the United States [11].

The natural environment has been identified as a pathway by which transmission of AMR bacteria to humans might occur [12]. The extent to which this occurs remains unknown, however. It is important to establish the relative role of the environment in the transmission of AMR bacteria to humans, compared with the spread of AMR bacteria through contact with animal carriers, consumption of food of animal origin, (international) travel, and their spread in healthcare and community settings. The aim of the current study was to establish a possible role for the natural environment, defined as soil, water, air/dust, and wildlife, in the transmission of clinically relevant AMR bacteria to humans by systematically reviewing the peer-reviewed literature. For this purpose three AMR bacteria were selected: extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae (ESBL-Ent), methicillin-resistant *S. aureus* (MRSA), and vancomycin-resistant *Enterococcus* spp. (VRE). Additionally, the contribution of fecal contamination sources (human wastewater and animal manure) to the burden of the AMR bacteria in the environment was explored.

Methods

Search strategy

Two databases (Medline and Scopus) were searched on April 10th, 2014, to identify publications describing one or more of three AMR bacteria in relation to the environment. The bacterial species were selected to represent Gram-negative and Gram-positive species, and species of fecal and non-fecal origin. Moreover, each of the bacterial species colonizes both animals and humans, has a clinically relevant type of resistance, and is sufficiently prevalent in humans and/or animals that its presence in the environment may be anticipated. Accordingly, ESBL-Ent (*i.e.*, *E. coli*, *Enterobacter* spp*., K. pneumoniae*), MRSA and VRE were selected, and carbapenemase-producing Enterobacteriaceae were not selected.

For the purpose of the present review, environment was defined as natural environment (or 'outdoor' environment). Consequently, indoor environments such as hospitals or livestock housing were excluded. Four environmental compartments were distinguished: soil, water, air/dust, and wildlife (Figure 1). Wildlife was categorized as an environmental compartment because these animals are not treated with antibiotics, and their carriage of AMR bacteria is most likely explained by uptake from the natural environment during foraging and drinking. Wildlife can therefore be considered as an extension of the specified environmental compartments, as well as a vehicle for multiplication and spread of these bacteria. Within the environmental compartments, sites relevant for human exposure were defined and included in the search strategy: recreational areas (*i.e.*, beach sand and recreational water), irrigation water, drinking water, urban water (*e.g.*, fountains), shellfish, and ambient air. Fresh produce, *i.e.*, food of plant origin such as vegetables and fruits, may be contaminated with AMR bacteria during growth in contaminated soil and/or irrigation with contaminated water. Consequently this was also included as a 'site' relevant for human exposure. Finally, the main sources of fecal contamination of the environment, wastewater and manure, were included in the search strategy. For the purpose of the current study, manure was defined as animal feces that is intentionally (*e.g.*, for use as fertilizer) or unintentionally (*e.g.*, droppings of free-range animals) introduced into the natural environment, or that is stored, presumably for use as fertilizer. Consequently, only measurements of AMR bacteria in animal feces introduced in natural environments (*e.g.*, not fecal swabs or droppings sampled in stables) were included.

Combinations of key search terms for AMR bacteria and defined environmental compartments, contamination sources, and exposure-relevant sites were used to interrogate the online databases (Table 1). Specifically, the titles, abstracts, and key words of publications included in the online databases were screened for these key search terms.

Table 1. Combinations of key search terms used for the identification of literature on AMR bacteria from environmental compartments, contamination sources, and exposure-relevant sites.

Key subjects	**Medline search terms**
Bacteria	(enterobacter* or escherichia coli? or e coli? or klebsiella? or enterococc* or staphylococcus aureus or s aureus).af
	AND
Resistance types	(extended spectrum beta lactamase? or esbl? or (methicillin adj2 resistan*) or mrsa? or (vancomycin adj2 resistan*) or vre?).af.
	AND
Environmental compartments	(water* or freshwater? or seawater or aquatic or coastal or beach* or lake? or river? or soil? or land? or pasture? or sediment? or air or airborne or dust or wildlife or wild animal? or bird? or mammal? or rodent?).mp.
	OR
Contamination sources	(manure or dropping? or slurry or sludge or lagoon? or compost or fertili?er? or sewage or effluent? or wastewater?).mp
	OR
Exposure-relevant sites[a]	(crop? or vegetable? or fresh produce or sprout? or shellfish or fish).mp
Key subjects	**Scopus search terms**
Bacteria	(TITLE-ABS-KEY(enterobacter* OR escherichia-coli OR e-coli OR klebsiella OR enterococc* OR staphylococcus-aureus OR s-aureus))
	AND
Resistance types	((TITLE-ABS-KEY(extended-spectrum-beta-lactamase OR esbl OR mrsa OR vre) OR TITLE-ABS-KEY(vancomycin W/1 resistan*) OR TITLE-ABS-KEY(methicillin W/1 resistan*)))
	AND
Environmental compartments	(TITLE-ABS-KEY(*water OR water* OR aquatic OR coastal OR beach OR lake OR river OR soil OR land OR pasture OR sediment OR air OR airborne OR dust OR wildlife OR wild-animal OR bird OR mammal OR rodent))
	OR
Contamination sources	(TITLE-ABS-KEY(manure OR dropping OR slurry OR sludge OR lagoon OR compost OR fertili?er OR sewage OR effluent))
	OR
Exposure-relevant sites[a]	(TITLE-ABS-KEY(crop OR vegetable OR fresh-produce OR sprout OR shellfish OR fish))

[a] The exposure-relevant sites recreational water, drinking water, urban water, irrigation water, and ambient air are captured by search terms included under environmental compartments.

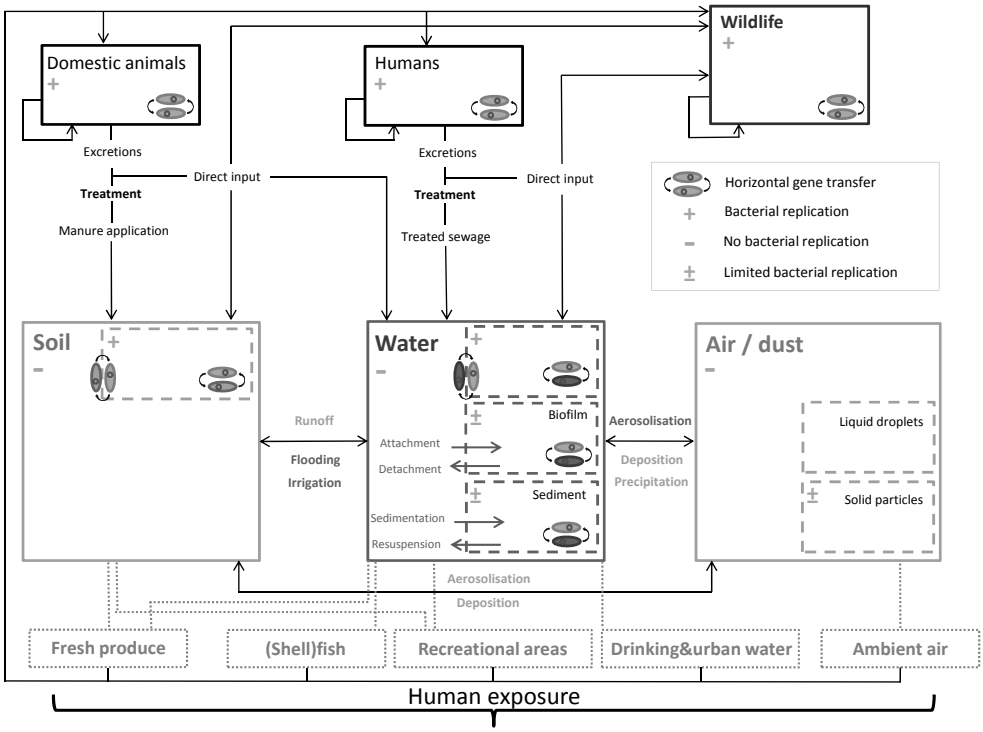

Figure 1. Schematic diagram of environmental compartments, contamination sources, exposure-relevant sites, and processes affecting survival and spread of bacteria.

Selection criteria

Two reviewers independently screened titles and abstracts, resulting from the automated database search, for exclusion criteria. Publications were excluded if they were in a language other than English, Dutch, or German, contained non-original research (*e.g.*, reviews), or did not investigate the specified compartments or AMR bacteria (Figure 2). Publications solely describing resistance genes (*i.e.*, genes encoding ESBL or resistance to methicillin or vancomycin), independently of bacterial hosts, were excluded. In case of conflict of opinion about inclusion of an article based on title or abstract, this was discussed by both reviewers until consensus was reached. Full texts were retrieved for included publications, and for publications where the decision for inclusion or exclusion could not be based on the contents of title and abstract, or where abstracts were not available. Full texts were further assessed for compliance with the inclusion and exclusion criteria (Figure 2A).

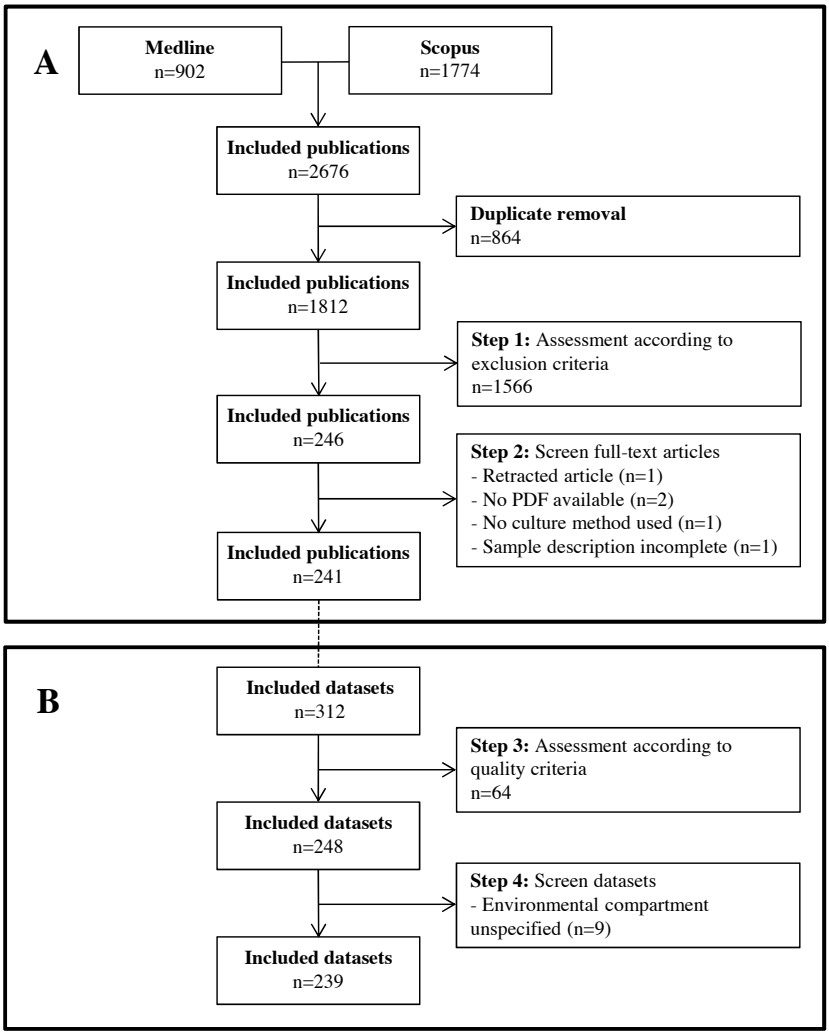

Figure 2. Flowchart of the (A) publication and (B) dataset selection process.

Data extraction

Study characteristics and data from included publications were extracted and exported to a database using a custom-made form. Full details of the extraction fields are available in Table S1 (Supporting Information). In case multiple publications described the same or overlapping sets of samples or isolates, these publications were considered as one data source, *i.e.*, one publication. Publications were categorized on the basis of the type of investigated compartment (contamination sources, environmental compartments, exposure-relevant sites) and the type of AMR bacteria. Individual publications could be included within more than one category.

To ensure the quality of the dataset, the method used to isolate AMR bacteria was assessed. First, it was determined whether selective culture methods were used to detect the AMR bacteria. Selective culture was defined as culture (pre-enrichment or direct plating) in the presence of relevant antibiotics: third-generation cephalosporin-supplemented medium or commercial screening medium for the isolation of ESBL-Ent; methicillin-, oxacillin-, or cefoxitin-supplemented medium or commercial screening medium for MRSA; and vancomycin-supplemented medium for VRE. The rationale for this assessment is the lack of sensitivity of non-selective culturing methods for the detection of AMR bacteria, and the resulting unreliability for drawing conclusions on the basis of lack of detection when non-selective culturing was used. Second, it was determined whether the antibiotic resistance was confirmed using phenotypic and/or molecular methods. Phenotypic confirmation tests deemed valid included growth of isolates on media containing concentrations equal to or above clinical breakpoints, established minimal inhibitory concentrations (MICs) equal to or above clinical breakpoints, and, in the case of disc diffusion assays, inhibition zones smaller than clinical breakpoints as described in the Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. VanB-carrying VRE variants with MICs below the current CLSI breakpoint concentration (32 µg/mL) have been described [14]; therefore growth at concentrations >16 µg/mL was also considered a valid phenotypic confirmation. Polymerase chain reactions (PCRs) detecting mecA and vanA or vanB genes were considered valid molecular tests for MRSA and VRE, respectively. For ESBL-Ent, PCRs detecting CTX-M genes, PCRs detecting TEM, SHV, and OXA genes in combination with sequencing to establish subtypes, or PCRs detecting other ESBL genes were considered valid molecular confirmation tests. Sequencing of TEM, SHV, and OXA genes is required since non-ESBL-encoding alleles exist of these genes. Studies in which AMR bacteria were investigated but not detected using only non-selective culture methods, or where resistance of isolates was not confirmed appropriately, were excluded from analysis.

For each publication, it was assessed whether AMR bacteria were enumerated, and whether environmental isolates were compared with isolates from fecal contamination sources or with human isolates for phenotypic or genetic relatedness. From the perspective of the current review, *i.e.*, study of documentation on transmission from the environment to humans and on emission from contamination sources to the environment, only comparisons between isolates from samples with a spatiotemporal relationship were considered relevant. Tests frequently used for establishing relatedness of isolates included pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), antibiotic resistance profiling, *Staphylococcus* protein A gene (spa)-typing (specifically for MRSA), and PhenePlate (PhP) biochemical fingerprinting (specifically for VRE). Additional tests included those identifying resistance genes and their genetic environment, *e.g.*, identification of ESBL-genotype and plasmid characterization for ESBL-Ent, and staphylococcal cassette chromosome SCC*mec*-typing for MRSA.

Results

General

The selection process yielded 241 publications that met the inclusion criteria (Figure 2A), and from these publications data were extracted. Fifteen publications described the same or overlapping samples or isolates, and these were combined to represent seven publications. Some publications described multiple AMR bacteria, environmental compartments, contamination sources, and/or exposure-relevant sites. For further study, data from included publications were divided into datasets, with each dataset describing one type of AMR bacteria (*i.e.*, ESBL-Ent, MRSA, VRE) in combination with one type of environmental compartment (*i.e.*, soil, water, air/dust, wildlife), contamination source (*i.e.*, wastewater, manure), or fresh produce. The 241 included publications resulted in 312 datasets (Figure 2B). After applying the defined quality criteria, 64 datasets were excluded. For 9 datasets, the results could not be interpreted due to lack of specification in which of the studied compartment(s) AMR bacteria were found. These had not been not excluded earlier in the selection process because the publications also contained other, valid datasets. This left 239 datasets for further study. Details on publications describing these datasets are available in Table S2 (Supporting Information). The majority of datasets described wildlife (n=71), wastewater (mostly municipal or urban sewage) (n=60), and water (n=56), followed by soil (n=25), fresh produce (n=16), manure (n=6), and air/dust (n=5). ESBL-Ent (n=102) and VRE (n=88) were described more often compared to MRSA (n=49).

All continents were represented; however more than half of the datasets described isolates collected in Europe (n=139), followed by North America (n=39), Asia (n=38), Africa (n=9), South America (n=8), Oceania (n=3), and the Antarctic (n=2). One dataset contained isolates that were collected from both Europe and Asia.

Exposure-relevant sites and fresh produce

Of 157 datasets concerning environmental compartments (Table 2), 24% (38/157) included sites that are relevant for human exposure (Table 3). Of these, almost two-thirds were about recreational water (n=15) or beach sand (n=12). The remaining datasets were about drinking water (n=5), ambient air (n=3), shellfish (n=2), and irrigation water (n=1). No datasets pertaining to urban water (*e.g.*, fountains) were identified. Furthermore, 16 datasets concerned fresh produce such as vegetables, fruits, sprouts, and herbs (Table 3). Overall, AMR bacteria were detected in 92% (35/38) of datasets about exposure-relevant sites, and in 50% (8/16) of datasets about fresh produce.

Only six datasets concerning exposure-relevant sites included quantitative data on the AMR bacteria (Table 4). Five datasets concerned concentrations in recreational areas, ranging from $10^{0.1}$-10^{3} CFU/100mL [15-18]. One dataset provided information on concentrations in blue mussels, which was <10 CFU/g [19].

Table 2. Detection of AMR bacteria in environmental compartments.

Environmental compartment	AMR bacteria[a]	Geographic regions investigated	Detection[b]		Description of samples	
			n/N	%	Detected	Not detected
Soil	ESBL-Ent	Europe, Asia, S. America	4/5	80	Manure-amended soil, pasture soil, river bank sediment, farm-related soil	Agricultural soil
	MRSA	Europe, N. America, Asia	12/12	100	Beach sand, manure-amended soil, soil unknown origin, soil around farm	-
	VRE	Europe, N. America, Asia	7/8	88	Beach sand, farmland without manure, (non) agricultural soils, freshwater/coastal sediment	Soil receiving/not receiving pig slurry
	Sub total		23/25	92		
Water	ESBL-Ent	Europe, Africa, N. and S. America, Asia	24/26	92	Marine/fresh surface waters, well water, water bags, public tap water	Fresh surface water, irrigation water
	MRSA	N. America, Asia	10/11	91	Marine surface waters	Fresh surface waters
	VRE	Europe, Africa, N. and S. America, Asia	17/19	89	Marine/fresh surface waters, ground/well water wells	Marine/fresh surface waters, well water
	Sub total		51/56	91		
Air/dust	ESBL-Ent	Europe	2/2	100	Air around wastewater treatment plants	-
	MRSA	Europe	3/3	100	Air around livestock farms	-
	VRE	-	-	-	-	-
	Sub total		5/5	100		
Wildlife	ESBL-Ent	Europe, Asia, N. and S. America, Africa, Antarctic	29/34	85	Multiple wild bird/mammal species, fish	Multiple wild bird species
	MRSA	Europe, N. America, Asia	14/17	82	Multiple wild bird/mammal species	Multiple wild mammal species
	VRE	Europe, N. America	17/20	85	Multiple wild bird/mammal species, shellfish, fish	Multiple wild bird/mammal species, amphibians, reptiles
	Sub total		60/71	85		
Total			**139/157**	**89**		

[a] Abbreviations used: AMR bacteria, antimicrobial resistant bacteria; ESBL-Ent, extended-spectrum β-lactamase-producing Enterobacteriaceae; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus* spp. [b] n/N, number of datasets where at least one positive sample was detected divided by the total number of datasets; %, percentage of datasets where AMR bacteria were detected.

Table 3. Detection of AMR bacteria at exposure-relevant sites and in fresh produce.

Exposure-relevant site	AMR bacteria[a]	Countries investigated	Detection[b]	
			n/N	%
Beach sand	ESBL-Ent	Portugal	1/1	100
	MRSA	United States	8/8	100
	VRE	Malaysia, United States	3/3	100
	Sub total		12/12	100
Recreational water	ESBL-Ent	Algeria, Netherlands	2/2	100
	MRSA	United States	9/10	90
	VRE	Malaysia, United States	3/3	100
	Sub total		14/15	93
Drinking water	ESBL-Ent	Bangladesh, Democratic Republic of Congo, Nicaragua	3/3	100
	MRSA	-	-	-
	VRE	Germany, South Africa	1/2	50
	Sub total		4/5	80
Ambient air	ESBL-Ent	Poland	2/2	100
	MRSA	Germany	1/1	100
	VRE	-	-	-
	Sub total		3/3	100
Shellfish	ESBL-Ent	-	-	-
	MRSA	-	-	-
	VRE	Denmark, United Kingdom	2/2	100
	Sub total		2/2	100
Irrigation water	ESBL-Ent	Netherlands	0/1	0
	MRSA	-	-	-
	VRE	-	-	-
	Sub total		0/1	0
Total			**35/38**	**92**
Fresh produce[c]	ESBL-Ent	Japan, Netherlands, Portugal, Saudi Arabia, Spain	4/7	57
	MRSA	Iran, South Korea	2/2	100
	VRE	Denmark, Germany, Italy, Portugal, South Africa, Spain, Sweden, Turkey, United Kingdom	2/7	29
Total			**8/16**	**50**

[a] Abbreviations used: AMR bacteria, antimicrobial resistant bacteria; ESBL-Ent, extended-spectrum β-lactamase-producing Enterobacteriaceae; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus* spp. [b] n/N, number of datasets where at least one positive sample was detected divided by the total number of datasets; %, percentage of datasets where AMR bacteria were detected. [c] AMR bacteria were detected in vegetables, fruits, (imported) herbs, sprouts, mixed salad, and pre-packaged fruit juice. They were not detected in other vegetables, lettuce from a farm, mixed salad, sprouts, and crops from a farm.

Genetic relatedness between environmental and human isolates

Of all publications where AMR bacteria were detected in environmental samples, 12 included information on the relationship between human and environmental isolates by PFGE, PhP biochemical fingerprinting, MLST, spa-typing, or SCC*mec*-typing (ESBL-Ent, n=4; MRSA, n=6; VRE, n=2). Together these publications contained six datasets on wildlife, six on surface water, and four on soil. Two of the publications on soil and surface water concerned exposure-relevant sites (*i.e.*, recreational waters and beach sand). Of the publications pertaining to environmental and human isolates, however, only one compared isolates from samples that had a clear spatial and temporal relationship [20]. Human nasal cultures, beach sand samples, and marine water samples were collected on the same day and stranded pilot whale (*Globicephala macrorhynchus*) samples were collected within two months at a marine mammal conservancy in the United States. MRSA isolates from whales, human volunteers attending these animals, and the beach sand and marine water associated with the marine mammal conservancy showed a high degree of genetic relatedness (≥95% similar PFGE patterns and same SCC*mec*-type) [20].

Environmental compartments

AMR bacteria were detected in at least one of the samples in 89% (139/157) of the datasets concerning environmental compartments (Table 2). The majority of these datasets were about ESBL-Ent (n=67), followed by VRE (n=47) and MRSA (n=43). High prevalences, defined as the percentage of datasets with at least one positive sample for the AMR bacteria, were observed in all environmental compartments and ranged from 85-100% (Table 2). All air/dust datasets concerned AMR hotspots (*i.e.*, wastewater treatment plants and livestock farms). In the case of soil, the majority of datasets were obtained at recreational beaches and from agriculturally related soils. By contrast, datasets concerning wildlife and water were obtained from a variety of species and locations, not necessarily related to AMR hotspots.

Bacterial concentrations were described in 8% (11/139) of datasets where AMR bacteria were detected: three soil datasets from Germany, Denmark, and United States; five water datasets from the Netherlands, United States, and Denmark; two air datasets from Germany; and one wildlife dataset from Denmark (Table 4).

In three publications, birds and mammals were investigated in conjunction with at least one other environmental compartment, at the same time and geographic location, to establish the relation between isolates from different compartments [20-22]. Doljeská *et al*. [21] showed that ESBL-Ent were present in pond water and in black-headed gulls (*Larus ridibundus*) nesting on the same pond. However, based on AMR profiles, ESBL genes, and macro-restriction profiles, isolates from wildlife and surface water were not related. Hernandez *et al.* [22] isolated ESBL-Ent from surface water but not from penguin (*Pygoscelis papua*) feces. Hower *et al*. [20] found MRSA isolates with 99% similar PFGE profiles and the

same SCC*mec*- and *spa*-type in short-finned pilot whales (*G. macrorhynchus*), water, and beach sand.

Table 4. Concentrations of AMR bacteria in environmental compartments, including exposure-relevant sites.

Environmental compartment	AMR bacteria	Type (source)	Concentration[d]	Country	Reference
Soil	MRSA	Agricultural[a] (around turkey and broiler farm)	$2.3x10^3$-$2.7x10^5$ CFU/ pair bootswabs	Germany	[55]
		Sand (beach)[b,c]	2.0-66.2 MPN/100mL	United States	[17]
	VRE	Agricultural[a] (research station)	ND, <10 CFU/g[e]	Denmark	[19]
		Non-agricultural[a] (research station)	ND, <10 CFU/g[e]		
Water	ESBL-Ent	Fresh/marine (river, lake, North Sea)[b]	0.15-15 CFU/100mL	Netherlands	[15]
		Fresh (not under influence of WWTP)	10 CFU/100mL		
		Fresh (river at discharge WWTP)	10^2-10^3 CFU/100mL		
	MRSA	Marine (Pacific Ocean)[b,c]	2.0-66.2 MPN/100mL	United States	[17]
		Marine (Pacific Ocean)[b]	0.65 CFU/100mL	United States	[16]
		Marine (Atlantic Ocean, bather related)[b]	<2-780 CFU/100mL	United States	[18]
		Marine (Atlantic Ocean, ambient)[b]	<2-260 CFU/100mL		
	VRE	Marine (at outlet WWTP)	$\leq 10^{-2}$ CFU/mL	Denmark	[19]
Air/dust	MRSA	50m outside stable (turkey farm)[b]	7-93 CFU/m^3	Germany	[55]
		150m outside stable (turkey farm)[b]	11-23 CFU/m^3		
		Directly outside stable (pig farm)	$3.2x10^1$- $4.0x10^1$ CFU/m^3	Germany	[56]
Wildlife	VRE	Shellfish (at outlet WWTP)[b]	<10 CFU/g	Denmark	[19]

[a] 'agricultural', soil exposed to animal manure; 'non-agricultural', soil not exposed to animal manure. [b] Exposure-relevant site. [c] Marine water, freshwater, and sand samples from two marine beaches were pooled to give this result. [d] Abbreviations used: CFU, colony-forming units; MPN, most probable number; ND, not detected. [e] VRE were not detected by direct plating on Slanetz-Bartley agar with vancomycin.

Contamination sources

AMR bacteria were detected in at least one of the samples in all (60/60) datasets concerning wastewater (Table 5). The majority of these datasets concerned VRE (n=33), followed by ESBL-Ent (n=23) and MRSA (n=4). Three types of wastewater could be distinguished: (1)

community wastewater, or water derived from sewer systems and wastewater treatment plants (WWTP); (2) hospital wastewater; and (3) industrial wastewater, or water derived from slaughterhouses, factories, and farms. AMR bacteria were detected in at least one of the samples in all (6/6) datasets concerning manure. These datasets most often concerned ESBL-Ent (n=5), followed by VRE (n=1). No datasets on MRSA in manure were identified in the current review (Table 5).

Table 5. Detection of AMR bacteria in datasets describing contamination sources**.**

Contamination source	AMR bacteria[a]	Geographic regions investigated	Detection[b]		Description of samples
			n/N	%	
Wastewater	ESBL-Ent	Africa, Europe, S. America, Oceania	23/23	100	Untreated, secondary effluent, sludge, other
	MRSA	Europe, N. America, Oceania	4/4	100	Untreated, secondary effluent, tertiary effluent, sludge, other
	VRE	Asia, Europe, N. America	33/33	100	Untreated, secondary effluent, tertiary effluent, sludge, other, unspecified
	Sub total		60/60	100	
Manure	ESBL-Ent	Europe, Asia	5/5	100	Broiler, laying hen, pig, duck, dairy cow
	MRSA	-	-	-	-
	VRE	Europe	1/1	100	Pig
	Sub total		6/6	100	
Total			**66/66**	**100**	

[a] Abbreviations used: AMR bacteria, antimicrobial resistant bacteria; ESBL-Ent, extended-spectrum β-lactamase-producing Enterobacteriaceae; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus* spp. [b] n/N, number of datasets where at least one positive sample was detected divided by the total number of datasets; %, percentage of datasets where AMR bacteria were detected.

Eighteen percent (12/66) of datasets about contamination sources included quantitative data: 11 of the wastewater datasets and one of the manure datasets (Table 6). The effect of wastewater treatment on concentrations of ESBL-Ent and VRE was investigated in seven publications originating from Algeria [23], Spain [24], Denmark [19,25], United States [26,27], and Portugal [28]. The majority of these publications described only mechanical (*e.g.*, sedimentation) and biological (*e.g.*, activated sludge) treatment of wastewater. A reduction of 1-4 $\log_{10}$-units was observed during secondary treatment. In three publications [26-28], concentrations of VRE in tertiary effluent were additionally described. Tertiary treatment in these publications entailed UV treatment, chlorination, lagooning and sand filtration, respectively. A further reduction of 2-3 $\log_{10}$-units was observed relative to secondary treated effluents. UV treatment, chlorination and lagooning brought VRE concentrations below the detection limit, but VRE could still be detected after sand filtration [26-28]. No information was available on the effect of manure treatment on AMR bacterial concentrations.

Three publications about wastewater included bacterial concentrations in community wastewater receiving wastewater from AMR hotspots as well as hotspot wastewater, *i.e.*, from a hospital [28], and drug production plants [19,25]. Concentrations of AMR bacteria were highest in AMR hotspot wastewater, followed by WWTP influent and WWTP effluent. When investigated [19,25], WWTP influents or sewage not containing AMR hotspot wastewater had lower concentrations of AMR bacteria compared to the counterparts receiving hotspot wastewater (Table 6).

Table 6. Concentrations of AMR bacteria in contamination sources.

Contamination source	AMR bacteria	Type (source)[a]	Concentration[b]	Country	Reference
Wastewater	ESBL-Ent	Influent (WWTP)	$4.6x10^3$-$1.6x10^5$ CFU/100mL	Algeria	[23]
		Effluent (WWTP)	$5.1x10^2$-$1.3x10^3$ CFU/100mL		
		Rinse water (poultry farm)	$3.9x10^6$-$5.8x10^7$ CFU/L	Netherlands	[57]
		Effluent (WWTP)	10^2-10^3 CFU/100mL	Netherlands	[15]
		Hospital	10^4-10^5 CFU/mL	Poland	[58]
	VRE	Influent (WWTP)	2-140 CFU/100mL	United Kingdom	[59]
		Influent (WWTP)	10^3-10^4 CFU/100ml	Spain	[24]
		Effluent (WWTP)	10-10^2 CFU/100mL		
		Factory	$0.9x10^5$-$5.1x10^6$ CFU/mL	Denmark	[25]
		Influent (WWTP, inlet 1[c])	$1.5x10^2$-$5.8x10^3$ CFU/mL		
		Influent (WWTP, inlet 2[d])	$6.9x10^1$-$6.4x10^2$ CFU/mL		
		Sludge (WWTP)	$4.2x10^2$-$6.1x10^4$ CFU/mL		
		Effluent (WWTP)	$3.6x10^{-1}$-$2.8x10^0$ CFU/mL		
		Sludge (Factory)	10^9 CFU/mL	Denmark	[19]
		Effluent (Factory)	10^3 CFU/mL		
		Sewage[d] (sewer upstream)	<10-10^3 CFU/mL		
		Sewage[c] (sewer downstream)	10^2-10^4 CFU/mL		
		Influent (WWTP[d], inlet 1[c])	10^3-10^4 CFU/mL		
		Influent (WWTP[d], inlet 2[d])	10^2-10^4 CFU/mL		
		Influent (WWTP[c])	10^3 CFU/mL		
		Sludge (WWTP[d])	10^3-10^4 CFU/mL		
		Sludge (WWTP[c])	10^3 CFU/mL		

Table 6. Continued

Contamination source	AMR bacteria	Type (source)[a]	Concentration[b]	Country	Reference
		Effluent (WWTP[d])	10^{-1}-10^{0} CFU/mL		
		Effluent (WWTP[c])	10^{-1} CFU/mL		
		Influent (WWTP)	$2.5x10^{3}$-$8.6x10^{4}$ CFU/100mL	United States	[27]
		Secondary effluent (WWTP)	$9.6x10^{1}$-$1.0x10^{3}$ CFU/100mL		
		Tertiary effluent (WWTP)	ND-3.3 CFU/100mL[e]		
		Other (WWTP)	ND-$1.9x10^{5}$ CFU/100mL		
		Influent (WWTP)	ND (winter), 10^{4}-10^{5} CFU/100mL (spring, summer)	United States	[26]
		Secondary effluent (WWTP)	ND (winter), 400-5200 CFU/100mL (spring, summer)		
		Tertiary effluent (WWTP)	ND		
		Hospital	$1.6x10^{1}$-$2.2x10^{3}$ CFU/mL	Portugal	[28]
		Influent[c] (WWTP)	$6.7x10^{0}$-$4.1x10^{2}$ CFU/mL		
		Effluent (WWTP)	~10^{0} CFU/mL		
Manure	ESBL-Ent	Dung heap (poultry farm)	≥0.1 CFU/g	Netherlands	[57]
		Storage tank (poultry farm)	<0.1 CFU/g		
		Free-range area (poultry farm)	$3.1x10^{3}$-$9.3x10^{3}$ CFU/g		

[a] WWTP, wastewater treatment plant. [b] CFU, colony-forming units; ND, not detected. [c] Receiving and [d] not receiving wastewater from the specified hotspot (*e.g.*, hospital or factory wastewater). [e] Only detected when chlorination was not operational.

Relation between isolates from environmental compartments and contamination sources

Twenty publications included datasets describing both wastewater and environmental compartments. In only nine of these, however, was it specified that the investigated wastewater and environmental compartments were geographically connected and sampled at the same time [15,19,24,29-34]. All of these publications investigated surface water in conjunction with wastewater and were performed in multiple European countries. Two of the datasets additionally included air at a Polish WWTP in which ESBL-Ent were detected, and another included Danish wildlife (mussels) in which VRE were detected [19,30,31]. In eight of nine publications describing surface water receiving wastewater from WWTP (all except ref 31), ESBL-Ent and VRE were detected in both wastewater and surface water. In three of

these eight publications, the relation between isolates from wastewater and environmental isolates was not investigated [24,32,34]. In three other publications, isolates were compared with respect to PFGE profiles or ESBL genotype [19,30,31]. Isolates from wastewater and surface water had different PFGE profiles in the Danish study [19], while similar ESBL genotypes were seen in wastewater, river water, and air at the Polish WWTP [30,31]. In the two remaining studies, isolates were compared with respect to multiple characteristics. Novais *et al.* [33] showed that isolates from wastewater and surface water had different PFGE profiles, while resistance genes, virulence traits, and AMR profiles were similar, suggesting horizontal gene transfer. Blaak *et al.* [15] conducted multiple analyses on spatiotemporally related ESBL-Ent isolates obtained from Dutch wastewater and downstream surface water (including recreational water), and demonstrated identical isolates with respect to sequence type, phylogenetic group, AMR profile, and ESBL genotype. A contribution of WWTP/sewage to the presence of AMR bacteria in surface water is further supported by quantitative data from two studies: Blaak *et al.* [15] showed similar ESBL-Ent concentrations in surface water at WWTP effluent discharge points and in effluents (10^2-10^3 CFU/100mL), and Gómez *et al.* [24] demonstrated that VRE concentrations were higher closer to the WWTP (10^4 CFU/100mL), compared to upstream or further downstream (10^2-$10^{2.5}$ CFU/100mL).

In five publications, manure was investigated in relation to environmental compartments [35-39]. In two of these direct deposition of feces in the environment was investigated. Hasan *et al.* [37] detected ESBL-*E. coli* in wild birds inhabiting the same lakeshore as poultry and ducks from surrounding households. Isolates were spatially related, but their temporal relationship was unclear [37]. Ma *et al.* [38] detected ESBL-*E. coli* in water from ponds on a duck farm. Isolates from the ponds and ducks were spatiotemporally related and had similar PFGE profiles, phylogenetic groups, and/or ESBL-genes [38]. The three remaining publications concerned livestock manure in conjunction with application to agricultural land. Friese *et al.* [35] detected ESBL-*E. coli* in samples taken from previously fertilized fields (within the last 6 weeks) around (unspecified distance) pig and broiler houses, and in slurry samples. It was not indicated, however whether pig slurry from the farms was used to fertilize the fields [35]. Hartmann *et al.* [36] detected ESBL-*E. coli* in cultivated soil amended one year before with liquid cow manure from one farm and in pasture soil on another farm. No information was provided, however, on the spatiotemporal relationship between these isolates and those from fresh and composted manure [36]. Manero *et al.* [39] investigated whether VRE was present in crops receiving pig slurry and soils receiving or not receiving pig slurry, but VRE were not detected.

Discussion

Transmission of clinically relevant AMR bacteria to humans by exposure to the natural environment, *e.g.*, recreational water and beach sands, drinking water, ambient air, and shellfish, is plausible. Quantitative data available in a small proportion of the included datasets describing environmental compartments support this. There were no publications, however, providing direct evidence for transmission of AMR bacteria to humans resulting from exposure to the environment. In the current review, the highest level of evidence for transmission was considered when genetic relatedness was shown between bacterial strains through molecular typing of spatiotemporally related human and environmental isolates collected at exposure-relevant sites. Although a number of studies performed molecular typing of human and environmental isolates, only one obtained this level of evidence [20]. In this study, the direction of transmission could not be determined (environment transmitting AMR bacteria to humans or vice versa), however, nor could transmission via a common source be excluded. Tools to further investigate transmission of clinically relevant AMR bacteria to humans by exposure to the natural environment include risk assessments, microbial source tracking, and epidemiological studies. Ideally, these tools should be combined to place environmental exposure in context with exposures in the clinical and veterinary/agricultural domains. Attribution of different sources and pathways that play a role in the transmission of AMR bacteria to humans can help to identify and prioritize intervention strategies.

Using Quantitative Microbial Risk Assessment (QMRA), the risk of human exposure to AMR bacteria in the environment can be quantified [40]. This approach requires knowledge on the concentrations of clinically relevant AMR bacteria at exposure-relevant sites. Furthermore, it requires dose estimates following human consumption of fresh produce or shellfish, ingestion of surface water, and inhalation of bioaerosols, together with the frequency and duration of these events. Although scarce, datasets including quantitative data from exposure-relevant sites were identified in the current review. Where AMR bacteria were not enumerated at exposure-relevant sites, additional aspects to be considered for risk assessment include survival in, and transport of, AMR bacteria to sites of exposure, and changes in bacterial concentration between the measured site (*i.e.*, environmental compartment, contamination source) and site of exposure. Horizontal gene transfer rates between clinically relevant AMR bacteria and environmental bacteria must also be addressed for more accurate estimates of exposure [41]. Another aspect to be considered for risk assessment is *in vivo* fitness of AMR bacteria in the human host following ingestion; however, quantitative data are lacking.

Microbial source tracking might be used to gain further understanding of how clinically relevant AMR bacteria emitted from contamination sources are transmitted to humans via the environment [42]. This method involves phenotypic and genotypic characterization of AMR bacterial isolates of human, animal, and environmental origin for the purpose of identifying

differences between groups of bacteria that can be used to ascertain the source from which they were derived [3]. Phenotypic characterization methods include serotyping and antibiotic susceptibility profiling [3]. Among genotypic methods, pulsed-field gel electrophoresis (PFGE) is considered the 'gold standard', but microarrays, multilocus sequence typing (MLST), multilocus variable-number tandem repeat analysis (MLVA), and whole genome sequencing (WGS) are becoming widespread [43]. The current systematic review did not include articles that investigated the prevalence of antibiotic resistance genes without relating them to a specific bacterial species. This was based on the assumption of a more direct risk associated with exposure to AMR bacteria that are capable of colonizing or infecting humans. However, taking into account the spread of whole bacteria alone might underestimate transmission of AMR, as horizontal gene transfer also plays an important role in the dissemination of antibiotic resistance [44]. Compared to culture-dependent techniques, metagenomic approaches and next-generation sequencing could provide more insight into the prevalence and diversity of antibiotic resistance determinants in the environment, while quantitative PCR might be helpful in collecting information about their distribution [45]. Microbial source tracking to investigate transmission of AMR bacteria to humans at exposure-relevant sites should preferably take into account transfer of both bacteria and their resistance determinants.

Epidemiological approaches can be used to identify possible exposure routes responsible for carriage of, or infection with, AMR bacteria. For example, Frank *et al.* [46] describe an outbreak of an infection with Shiga-toxin-producing *E. coli* O104:H4 harboring an ESBL gene (CTX-M-15) in Germany, where sprouts were identified as the most likely vehicle of infection [47]. In a study population comprising 100 cases and 190 controls, Søraas *et al.* [48] showed that recreational freshwater swimming was an independent risk factor for ESBL-positive urinary tract infections in people in Norway, along with travel to Asia, the Middle East, or Africa during the past six weeks to 24 months, recent use of fluoroquinolones and β-lactams, and diabetes mellitus. In a cross-sectional study by Huijbers *et al.* [49], however, swimming in a river, lake, or pond was not identified as a risk factor for ESBL-Ent carriage in 1025 Dutch adults. Furthermore, Rosenberg Goldstein *et al.* [50] found no significant difference in the odds of methicillin-susceptible *S. aureus* (MSSA), multidrug resistant MSSA, and vancomycin-susceptible enterococci colonization among spray irrigation workers using reclaimed water (n=19) and controls not routinely exposed to reclaimed water (n=24). Also, none of the sampled individuals were positive for MRSA or VRE [50]. These particular studies were not included in the current review, as they did not actually investigate the presence of ESBL-Ent, MRSA, and/or VRE in the environment. This might have led to the exclusion of studies that support the role of the environment in transmission of clinically relevant AMR bacteria. A search for epidemiological studies reporting a relation between environmental exposure and infection with AMR bacteria showed that these were scarce, however (data not shown). It might also be possible to consider data on susceptible variants of selected bacterial species

and assume that a proportion of these infections were caused by resistant variants. This approach does not take into account that the survival and spread of AMR bacteria might be different compared to those of susceptible strains both in the environment and upon entering the human body. Furthermore, for the purpose of risk assessment and attribution of different transmission routes, which provide targets for interventions, information about prevalence, concentration, and types of AMR bacteria in the environment is imperative.

Quantitative and molecular data provide evidence for dissemination of AMR bacteria from contamination sources to the environment, including to exposure-relevant sites. AMR bacteria were detected in all publications investigating wastewater. Moreover, where AMR bacteria were enumerated, high concentrations were observed, also in wastewater that is discharged onto surface water. This, together with molecular typing results, demonstrates the contribution of sewage and WWTP effluent to the presence of AMR bacteria in surface water. AMR bacteria were also detected in the six publications concerning manure in relation to the environment. There are currently no studies investigating the prevalence and concentration of AMR bacteria in manure or slurry prior to soil application. There are also no studies demonstrating the effect of manure or slurry application on the prevalence and concentration of AMR bacteria in soil and on fresh produce. Studies investigating the effect of manure application on resistance genes in soil have been conducted, however. For example, Fahrenfeld *et al.* [51] showed significant increases in soil gene copy numbers of antibiotic resistance genes (*sul1*, *sul2*, and *ermF*) after manure application, and dissipation of these genes to background levels within 2 months. The role of manure application on environmental contamination with clinically relevant AMR bacteria needs to be addressed further and placed into perspective relative to environmental contamination from human sources. An aspect not addressed by this review, but also important to consider here, is the dissemination of antibiotic residues to the environment through wastewater and manure [40]. For example, half-lives of five days for β-lactams to 100 days for tetracyclines and sulfonamides have been reported in manure [52], suggesting that when applied to land they might act as selective agents to help propagate AMR bacteria or resistance genes.

It is clear that, in order to estimate exposures and risks associated with environmental pathways of AMR bacteria further investigation is necessary. Management options exist, however, that can work synergistically with existing policies and goals, and could be put into effect immediately [53]. Dissemination via wastewater to exposure-relevant sites is suspected; therefore, an effective target for intervention could be at wastewater collection points and wastewater treatment plants. It has been shown that concentrations of AMR bacteria were reduced by advanced wastewater treatment processes such as ozone, UV, ultrafiltration, and chlorination [26,27]. In addition, membrane bioreactor processes have been shown to be very effective in reducing bacterial numbers by over 6 $\log_{10}$ [54], and might prove useful in diminishing AMR bacteria. Another intervention measure could be treatment of manure;

however, data on AMR bacteria in manure are scarce, and there are no studies investigating the effect of manure treatment on concentrations of AMR bacteria in the environment. The efficiency of reducing AMR bacteria by composting and other digestion processes should be evaluated.

In conclusion, the abundance of AMR bacteria at exposure-relevant sites suggests that risk of human exposure to AMR bacteria in the environment is plausible. To what extent the environment contributes to human exposure, also compared to the clinical and veterinary/agricultural domains, needs to be quantified. Important knowledge gaps have been identified that should be addressed in future studies. AMR bacteria in the environment, including sites relevant for human exposure, originate from wastewater and probably manure. Intervention strategies targeted at these sources could therefore limit emission of AMR bacteria to the environment.

Acknowledgements

This work was financially supported by the Netherlands Organization for Health Research and Development (ZonMw) and the Dutch Ministry of Health, Welfare and Sport (VWS). We thank drs. A.M.P. Kersten for her valuable assistance with the literature search and Prof. dr. J.W.M. van der Meer and Prof. dr. T.J.M. Verheij for their helpful suggestions on the manuscript.

References

1. World Health Organization (WHO). 2014. *Antimicrobial resistance: global report on surveillance*; World Health Organization: Geneva, Switzerland. http://www.who.int/drugresistance/documents/surveillancereport/en/ (accessed 3 September 2015).
2. de Kraker, M.E.A., Davey, P.G., Grundmann, H. 2011. Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteraemia: Estimating the burden of antimicrobial resistance in Europe. *PLoS Medicine*. 8:e1001104.
3. Pires, S.M., Evers, E.G., van Pelt, W., *et al*. 2009. Attributing the human disease burden of foodborne infections to specific sources. *Foodborne Pathogens and Disease.* 6:417-424.
4. Domingues A.R., Pires, S.M., Halasa, T., et al. 2012. Source attribution of human campylobacteriosis using a meta-analysis of case-control studies of sporadic infections. *Epidemiology and Infection.* 140:970-981.
5. Domingues, A.R., Pires, S.M., Halasa, T., *et al*. 2012. Source attribution of human salmonellosis using a meta-analysis of case-control studies of sporadic infections. *Epidemiology and Infection.* 140:959-969.
6. Evers, E.G., van der Vels-Klerx, H.J., Nauta, M.J., *et al*. 2008. Campylobacter source attribution by exposure assessment. *International Journal of Risk Assessment and Management.* 8:174-189.
7. Rangel, J.M., Sparling, P.H., Crowe, C., *et al*. 2005. Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. *Emerging Infectious Diseases.* 11:603-609.
8. Shuval, H. 2003. Estimating the global burden of thalassogenic diseases: human infectious diseases caused by wastewater pollution of the marine environment. *Journal of Water and Health.* 1:53-64.
9. Franz, E., van Bruggen, A.H. 2008. Ecology of *E. coli* O157:H7 and *Salmonella enterica* in the primary vegetable production chain. *Critical Reviews in Microbiology.* 34:143-161.
10. Centres for Disease Control and Prevention (CDC). 2013. Outbreak of *Escherichia coli* O104:H4 infections associated with sprout consumption - Europe and North America, May-July 2011. Morbidity and Mortality Weekly Report. 62:1029-1031.
11. Centers for Disease Control and Prevention (CDC). 2006. Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach - United States, September 2006. Morbidity and Mortality Weekly Report. 55:1-2.
12. Davies, J., Davies, J. 2010. Origins and evolution of antibiotic resistance. *Microbiology and Molecular Biology Reviews.* 74:417-433.
13. Clinical Laboratory Standards Institute (CLSI). 2010. *Performance standards for antimicrobial susceptibility testing;* 20th Informational Supplement M100-20; Clinical Laboratory Standards Institute: Wayne, PA.
14. Centres for Disease Control and Prevention (CDC). 2010. *Vancomycin-resistant Enterococci (VRE) and the clinical laboratory*; Centres for Disease Control and Prevention: Atlanta, GA. http://www.cdc.gov/HAI/settings/lab/VREClinical-Laboratory.html (accessed 3 September 2015).
15. Blaak, H., de Kruijf, P., Hamidjaja, R.A., *et al*. 2014. Prevalence and characteristics of ESBL-producing *E. coli* in Dutch recreational waters influenced by wastewater treatment plants. *Veterinary Microbiology.* 171:448-459.
16. Goodwin, K.D., McNay, M., Cao, Y., *et al*. 2012. A multi-beach study of *Staphylococcus aureus*, MRSA, and Enterococci in seawater and beach sand. *Water Research*. 46:4195-4207.
17. Levin-Edens, E., Soge, O.O., No, D., *et al*. 2012. Methicillin-resistant *Staphylococcus aureus* from Northwest marine and freshwater recreational beaches. *FEMS Microbiology Ecology.* 79:412-420.
18. Plano, L.R.W., Shibata, T., Garza, A.C., *et al*. 2013. Human-associated methicillin-resistant *Staphylococcus aureus* from a subtropical recreational marine beach. *Microbial Ecology.* 65:1039-1051.
19. Guardabassi, L., Dalsgaard, A. 2004. Occurrence, structure, and mobility of Tn1546-like elements in environmental isolates of vancomycin-resistant Enterococci. *Applied Environmental Microbiology.* 70:984-990.
20. Hower, S., Phillips, M.C., Brodsky, M., *et al*. 2013. Clonally related methicillin-resistant *Staphylococcus aureus* isolated from short-finned pilot whales (*Globicephala macrorhynchus*), human volunteers, and a bayfront cetacean rehabilitation facility. *Microbial Ecology.* 65:1024-1038.
21. Doljeská, M., Bierošová, B., Kohoutová, L., *et al*. Antibiotic-resistant *Salmonella* and *Escherichia coli* isolates with integrons and extended-

spectrum beta-lactamases in surface water and sympatric black-headed gulls. *Journal of Applied Microbiology.* 106:1941-1950.

22. Hernández, J., Stedt, J., Bonnedahl, J., *et al*. 2012. Human-associated extended-spectrum β-lacatamase in the Antarctic. *Applied Environmental Microbiology.* 78:2056-2058.
23. Alouache, S., Estepa, V., Messai, Y., *et al*. 2014. Characterization of ESBLs and associated quinolone resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from an urban wastewater treatment plant in Algeria. *Microbial Drug Resistance.* 20:30-38.
24. Gómez, M., Díaz, M.T., Araujo, M., *et al*. 2010. Waste water treatment plants as redistributors of resistance genes in bacteria. *WIT Transactions on Ecology and the Environment.* 135:83-94.
25. Guardabassi, L., Brønnum, P.T., Danø, R., *et al*. 2002. Dissemination of vancomycin-resistant Enterococci harboring vanA through disposal of waste derived from industrial production of vancomycin. *Microbial Drug Resistance.* 8:401-406.
26. Nagulapally, S.R., Ahmad, A., Henry, A., *et al*. 2009. Occurrence of ciprofloxacin-, trimethoprim-sulfamethoxazole-, and vancomycin-resistant bacteria in a municipal wastewater treatment plant. *Water Environment Research.* 81:82-90.
27. Rosenberg Goldstein, R.E., Micallef, S.A., Gibbs, S.G., *et al*. 2014. Detection of vancomycin-resistant Enterococci (VRE) at four U.S. wastewater treatment plants that provide effluent for reuse. *Science of the Total Environment.* 466-467:404-411.
28. Varela, A.R., Ferro, G., Vredenburg, J., *et al*. 2013. Vancomycin resistant Enterococci: from the hospital effluent to the urban wastewater treatment plant. *Science of the Total Environment.* 450-451:155-161.
29. Blanch, A.R., Caplin, J.L., Iversen, A., *et al*. 2003. Comparison of enterococcal populations related to urban and hospital wastewater in various climatic and geographic European regions. *Journal of Applied Microbiology.* 94:994-1002.
30. Korzeniewska, E., Korzeniewska, A., Harnisz, M. 2013. Antibiotic resistant *Escherichia coli* in hospital and municipal sewage and their emission to the environment. *Ecotoxicology and Environmental Safety.* 91:96-102.
31. Korzeniewska, E., Harnisz, M. 2013. Extended-spectrum beta-lactamase (ESBL)-positive Enterobacteriaceae in municipal sewage and their emission to the environment. *Journal of Environmental Management.* 128:904-911.
32. Kühn, I., Iversen, A., Finn, M., *et al*. 2005. Occurrence and relatedness of vancomycin-resistant Enterococci in animals, humans, and the environment in different European regions. *Applied Environmental Microbiology.* 71:5383-5390.
33. Novais, C., Coque, T.M., Ferreira, H., *et al*. 2005. Environmental contamination with vancomycin-resistant Enterococci from hospital sewage in Portugal. *Applied Environmental Microbiology.* 71:3364-3368.
34. Schwartz, T., Kohnen, W., Jansen, B., *et al*. 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. *FEMS Microbiology Ecology.* 43:325-335.
35. Friese, A., Schulz, J., Laube, H., *et al*. 2013. Faecal occurrence and emissions of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) and ESBL/AmpC-producing *E. coli* from animal farms in Germany. *Berliner und Münchener Tierärztliche Wochenschrift.* 126:175-180.
36. Hartmann, A., Locatelli, A., Amoureux, L., *et al*. 2012. Occurrence of CTX-M producing *Escherichia coli* in soils, cattle, and farm environment in France (Burgundy region). *Frontiers in Microbiology.* 3:e83.
37. Hasan, B., Sandegren, L., Melhus, Å., *et al*. 2012. Antimicrobial drug-resistant *Escherichia coli* in wild birds and free-range poultry, Bangladesh. *Emerging Infectious Diseases.* 18:2055-2058.
38. Ma, J., Liu, J.H., Lv, L., *et al*. 2012. Characterization of extended-spectrum β-lactamase genes found among *Escherichia coli* isolates from duck and environmental samples obtained on a duck farm. *Applied Environmental Microbiology.* 78:3668-3673.
39. Manero, A., Vilanova, X., Cerdà-Cuéllar, M., *et al*. 2006. Vancomycin- and erythromycin-resistant Enterococci in a pig farm and its environment. *Environmental Microbiology.* 8:667-674.
40. Ashbolt, N.J., Amézquita, A., Backhaus, T., *et al*. 2014. Human health risk assessment (HHRA) for environmental development and transfer of antibiotic resistance. *Environmental Health Perspectives.* 121:993-1001.
41. Hunter, P.R., Wilkinson, D.C., Catling, L.A., *et al*. 2008. Meta-analysis of experimental data concerning antimicrobial resistance gene transfer rates during conjugation. *Applied Environmental Microbiology.* 74:6085-6090.
42. Harwood, V.J., Staley, C., Badgley, B.D., *et al*. 2014. Microbial source tracking markers for detection of fecal contamination in environmental waters:

relationships between pathogens and human health outcomes. *FEMS Microbiology Reviews.* 38:1-40.
43. Sabat, A.J., Budimir, A., Nashev, D., *et al*. 2013. Overview of molecular typing methods for outbreak detection and epidemiological surveillance. *Euro Surveillance.* 18:20380.
44. Dröge, M., Pühler, A., Selbitschka, W. 1999. Horizontal gene transfer among bacteria in terrestrial and aquatic habitats as assessed by microcosm studies. *Biology and Fertility of Soils.* 29:221-245.
45. Monier, J.M., Demanèche, S., Delmont, T.O., *et al*. 2011. Metagenomic exploration of antibiotic resistance in soil. *Current Opinion in Microbiology.* 14:229-235.
46. Frank, C., Werber, D., Cramer, J.P., *et al*. 2011. Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. *New England Journal of Medicine*. 365:1771-1780.
47. Buchholz, U., Bernard, H., Werber, D., *et al*. 2011. German outbreak of *Escherichia coli* O104:H4 associated with sprouts. *New England Journal of Medicine*. 365:1763-1770.
48. Søraas, A., Sundsfjord, A., Sandven, I., *et al*. 2013. Risk factors for community-acquired urinary tract infections caused by ESBL-producing Enterobacteriaceae- A case-control study in a low prevalence country. *PloS One.* 8:e69581.
49. Huijbers, P.M.C., de Kraker, M., Graat, E.A.M., *et al*. 2013. Prevalence of extended-spectrum β-lactamase-producing Enterobacteriaceae in humans living in municipalities with high and low broiler density. *Clinical Microbiology and Infection*. 19:E256-E259.
50. Rosenberg Goldstein, R.E., Micallef, S.A., Gibbs, S.G., *et al*. 2014. Occupational exposure to *Staphylococcus aureus* and *Enterococcus* spp. among spray irrigation worker using reclaimed water. *International Journal of Environmental Research and Public Health.* 11:4340-4355.
51. Fahrenfeld, N., Knowlton, K., Krometis, L.A., *et al*. 2014. Effect of manure application on abundance of antibiotic resistance genes and their attenuation rates in soil: Field-scale mass balance approach. *Environmental Science and Technology.* 48:2643-2650.
52. Chee-Sanford, J.C., Mackie, R.I., Koike, S., *et al*. 2009. Fate and transport of antibiotic residues and antibiotic resistance genes following land application of manure waste. *Journal of Environmental Quality.* 38:1086-1108.
53. Pruden, A., Joakim Larsson, D.G., Amézquita, A., *et al*. 2013. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environmental Health Perspectives.*121:878-885.
54. Krüger, U. 2000. Membrane-supported biological wastewater treatment. *Schriftenreihe des Vereins für Wasser-, Boden-, und Lufthygiene.* 105:319-324.
55. Friese, A., Schulz, J., Zimmermann, K., *et al*. 2013. Occurrence of livestock-associated methicillin-resistant *Staphylococcus aureus* in turkey and broiler barns and contamination of air and soil surfaces in their vicinity. *Applied and Environmental Microbiology.* 79:2759-2766.
56. Clauss, M.; Schulz, J.; Stratmann-Selke, J. 2013. Reduction of livestock-associated methicillin-resistant *Staphylococcus aureus* (LA-MRSA) in the exhaust air of two piggeries by a bio-trickling filter and a biological three-step air cleaning system. *Berliner und Münchener Tierärztliche Wochenschrift.* 126:137-142.
57. Blaak, H., Hamidjaja, R.A., van Hoek, A.H.A.M., *et al*. 2014. Detection of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* on flies at poultry farms. *Applied Environmental Microbiology.* 80:239-246.
58. Korzeniewska, E., Harnisz, M. 2013. Beta-lactamase-producing Enterobacteriaceae in hospital effluents. *Journal of Environmental Management.* 123:1-7.
59. Bates, J., Jordens, J.Z., Griffiths, D.T. 1994. Farm animals given as a putative reservoir for vancomycin-resistant enterococcal infection in man. *Journal of Antimicrobial Chemotherapy.* 34:507-514.

Supplementary Table S1. Data extraction form.

column id	explanation	code
refid	name referee	ph=patricia huijbers; hb=hetty blaak
articleid	last name of first author, year of publication, addition if multiple pub one year	nameyyyyabc
title	title article	free text (export from EndNote)
author	authors of article	free text (export from EndNote)
journal	journal title	free text (export from EndNote)
pubyear	year of publication	free text (export from EndNote)
vol	journal volume	free text (export from EndNote)
pubtype	type of publication	free text (export from EndNote)
country	country from which samples were collected	free text
year	year that samples were collected	yyyy
amr	AMR bacteria identified	1=esbl; 2= mrsa; 3=vre
bacteria	specific bacterial genus/species identified if AMR=1	1=*E. coli*; 2=*K. pneumoniae*; 3=*Enterobacter* spp.
compart	which type(s) of compartment(s) were investigated	1=source; 2=environment; 3=exposure
multiplecompart	AMR bacteria identified in more than one compartment	1=yes; 0=no
environment	which environmental compartment(s) were investigated	1=soil; 2=water; 3=air/dust; 4=wildlife
environmentx	short description of environmental compartment	free text
source	which source compartment(s) were investigated	1=manure; 2=wastewater; 3=other; 4=none
sourcex	short description of source compartment	free text
exposure	exposure compartment(s) that were investigated	1=fresh produce/crops; 2=fish/shellfish; 3=drinking water; 4=irrigation water; 5=recreational water; 6=beach sand; 7=urban water; 8=ambient air; 9=none
exposurex	short description of exposure compartment	free text
presence	presence of AMR bacteria in compartment(s)	1=yes; 0=no; 2=unclear
presencex	compartment(s) where AMR bacteria are present	free text
enrichment	selective enrichment during isolation process	1=yes; 0=no
isolation	culture of bacteria during isolation process	1=nonselective; 2=selective; 3= both
resistance	method used to determine resistance mechanism	1=phenotypic; 2=molecular; 3=both; 4=none
gene	resistance gene(s) identified	1=yes; 0=no
genex	name(s) of resistance gene(s)	free text
quantify	concentration of AMR bacteria determined	1=yes; 0=no
human	human isolates investigated	1=yes; 0=no

Supplementary Table S2. Articles included in the systematic review.

		AMR bacteria[a]	detected	first author	year	volume	journal
environmental compartments	soil	ESBL	no	Blaak	2014a	168-169	Int J Food Microbiol
			yes	Friese	2013b	126	Berl Münch Tierärztl Wochenschr
			yes	Hartmann	2012	3	Front Microbiol
			yes	Lu	2010	76	Appl Environ Microbiol
			yes	Nobrega	2013	33	Pesq Vet Bras
		MRSA	yes	Friese	2013a	79	Appl Environ Microbiol
			yes	Friese	2013b	126	Berl Münch Tierärztl Wochenschr
			yes	Goodwin	2009	43	Water Res
			yes	Goodwin	2012	46	Water Res
			yes	Hower	2013	65	Microb Ecol
			yes	Ko	2011	60	J Med Microbiol
			yes	Levin-Edens	2012	79	FEMS Microbiol Ecol
			yes	Plano	2013	65	Microb Ecol
			yes	Roberts	2013	4	Front Microbiol
			yes	Schulz	2012	78	Appl Environ Microbiol
			yes	Soge	2009	64	J Antimicrob Chemother
			yes	Yamahara	2012	78	Appl Environ Microbiol
		VRE	no	Manero	2006	8	Environ Microbiol
			yes	Dada	2013	185	Environ Monit Assess
			yes	Guardabassi	2004	70	Appl Environ Microbiol
			yes	Kühn	2005	71	Appl Environ Microbiol
			yes	Roberts	2009	107	J Appl Microbiol
			yes	Santiago-Rodriquez	2013	11	J Water Health
			yes	Thevenon	2012	86	Chemosphere
			yes	Vignesh	2012	64	Mar Pollut Bull
	water	ESBL	no	Blaak	2014a	168-169	Int J Food Microbiol
			no	Dolejská	2009	106	J Appl Microbiol
			yes	Alouache	2011	27	Microbes Environ
			yes	Amaya	2012	18	Clin Microbiol Infect
			yes	Blaak	2014c	171	Vet Microbiol

AMR bacteria[a]	detected	first author	year	volume	journal
	yes	Chen	2010	158	Environ Poll
	yes	Chouchani	2013	45	Scand J Infect Dis
	yes	Colomer-Lluch	2013	68	J Antimicrob Chemother
	yes	De Boeck	2012a	18	Emerg Infect Dis
	yes	De Boeck	2012b	31	Eur J Clin Microbiol
	yes	Dhanji	2011	66	J Antimicrob Chemother
	yes	Hernandez	2012	78	Appl Environ Microbiol
	yes	Hong	2004	10	Microb Drug Resist
	yes	Hu	2013	79	Appl Environ Microbiol
	yes	Jang	2013	47	Environ Sci Technol
	yes	Kamruzzaman	2013	76	Diagn Micr Infec Dis
	yes	Kim	2008	46	J Microbiol
	yes	Korzeniewska	2013a	91	Ecotox Environ Safe
	yes	Łuczkiewicz	2011b	20	Pol J Environ Stud
	yes	Ma	2012	78	Appl Environ Microbiol
	yes	Machado	2009	63	J Antimicrob Chemother
	yes	Sharma	2008	51	Indian J Pathol Micr
	yes	Tacão /Tacão	2012/2014	78/48	Appl Environ Microbiol /Water Res
	yes	Talukdar	2013	8	PLoS One
	yes	Tissera	2013	20	Malays J Med Sc
	yes	Zurfluh	2013	79	Appl Environ Microbiol
MRSA	no	Kassem	2008	46	J Clin Microbiol
	yes	Enns	2012	46	Water Res
	yes	Goodwin	2009	43	Water Res
	yes	Goodwin	2012	46	Water Res
	yes	Hower	2013	65	Microb Ecol
	yes	Levin-Edens	2012	79	FEMS Microbiol Ecol
	yes	Plano	2011	11	BMC Microbiol
	yes	Plano	2013	65	Microb Ecol
	yes	Roberts	2013	4	Front Microbiol
	yes	Soge	2009	64	J Antimicrob Chemother

	AMR bacteria[a]	detected	first author	year	volume	journal
		yes	Yilmaz	2013	91	B Environ Contam Tox
	VRE	no	Harwood	2001	67	Appl Environ Microbiol
		no	Wilson	2002	79	Int J Food Microbiol
		yes	Ateba	2013	11	J Water Health
		yes	Blanch	2003	94	J Appl Microbiol
		yes	Dada	2013	185	Environ Monit Assess
		yes	Gallert	2005	69	Appl Microbiol Biot
		yes	Guardabassi	2004	70	Appl Environ Microbiol
		yes	Iversen	2002	68	Appl Environ Microbiol
		yes	Kühn	2005	71	Appl Environ Microbiol
		yes	Lata	2009	9	BMC Microbiol
		yes	Mondragón	2011	183	Environ Monit Assess
		yes	Novais/Novais	2005/2008	71/52	Appl Environ Microbiol /Antimicrob Agents Chemother
		yes	Roberts	2009	107	J Appl Microbiol
		yes	Santiago-Rodriquez	2013	11	J Water Health
		yes	Schwartz	2003	43	FEMS Microbiol Ecol
		yes	Talebi/Talebi/Jahangiri	2008a/2008b/2010	74/56/28	Appl Environ Microbiol /Curr Microbiol / Indian J Med Microbiol
		yes	Tansuphasiri	2006	37	SE Asian J Trop Med
		yes	Vignesh	2012	64	Mar Pollut Bull
		yes	Zdragas	2008	42	Water Res
air/dust	ESBL	yes	Korzeniewska	2013a	91	Ecotox Environ Safe
		yes	Korzeniewska	2013c	128	J Environm Manage
	MRSA	yes	Clauss	2013	126	Berl Münch Tierärztl Wochenschr
		yes	Friese	2013a	79	Appl Environ Microbiol
		yes	Schulz	2012	78	Appl Environ Microbiol
	VRE	-	-	-	-	-
wildlife	ESBL	no	Ardiles-Villegas	2011	55	Avian Dis
		no	Hernandez	2012	78	Appl Environ Microbiol
		no	Jarhult	2013	3	Infect Ecol Epidemiol

AMR bacteria[a]	detected	first author	year	volume	journal
	no	Radimersky	2010	109	J Appl Microbiol
	no	Silva	2010	65	J Antimicrob Chemother
	yes	Bonnedahl	2009	4	PLoS ONE
	yes	Bonnedahl	2010	65	J Antimicrob Chemother
	yes	Costa	2006	58	J Antimicrob Chemother
	yes	Dolejská	2009	106	J Appl Microbiol
	yes	Guenther	2010a	65	J Antimicrob Chemother
	yes	Guenther	2010b	2	Environ Microbiol Rep
	yes	Guenther	2012	7	PLoS ONE
	yes	Hasan	2012	18	Emerg Infect Dis
	yes	Hasan	2014	168	Vet Microbiol
	yes	Hernandez	2010	2	Environ Microbiol Rep
	yes	Hernandez	2013	8	PLoS ONE
	yes	Ho/Ho	2011/2013	66/114	J Antimicrob Chemother /J Appl Microbiol
	yes	Janatova	2014	171	Vet Microbiol
	yes	Kmet	2013	20	Ann Agr Env Med
	yes	Literak	2010a	108	J Appl Microbiol
	yes	Literak	2010b	76	Appl Environ Microbiol
	yes	Pinto	2010	76	Appl Environ Microbiol
	yes	Poirel	2012	56	Antimicrob Agents Chemother
	yes	Radhouani	2010	65	J Antimicrob Chemother
	yes	Radhouani	2013	195	Arch Microbiol
	yes	Silva	2011	40	Avian Pathol
	yes	Simões	2010	16	Emerg Infect Dis
	yes	Sousa	2011	8	Foodborne Pathog Dis
	yes	Stephan	2012	154	Schweiz Arch Tierheilk
	yes	Tausova	2012	67	J Antimicrob Chemother
	yes	Veldman	2013	79	Appl Environ Microbiol
	yes	Wallensten	2011	1	Infect Ecol Epidemiol
	yes	Watson	2012	154	Vet Microbiol
	yes	Zurfluh	2013b	41	Int J Antimicrob Ag

	AMR bacteria[a]	detected	first author	year	volume	journal
	MRSA	no	Carson	2012	12	Vector-Borne Zoonot
		no	Meemken	2009	116	Deutsche Tierärztl Wochenschr
		no	Meyer	2014	127	Berl Münch Tierärztl Wochenschr
		yes	Himsworth	2014	9	PLoS ONE
		yes	Ho	2012	50	J Clin Microbiol
		yes	Hower	2013	65	Microb Ecol
		yes	Loncaric	2013	68	J Antimicrob Chemother
		yes	Lozano	2009	64	J Antimicrob Chemother
		yes	Luzzago	2014	170	Vet Microbiol
		yes	Monecke	2013	8	PLoS ONE
		yes	Paterson	2012	67	J Antimicrob Chemother
		yes	Porrero	2013	198	Vet J
		yes	Robb	2013	162	Vet Microbiol
		yes	Schaefer	2009	6	EcoHealth
		yes	Stewart	2014	108	Dis Aquat Organ
		yes	van de Giessen	2009	91	Prev Vet Med
		yes	Wardyn	2012	48	J Wildlife Dis
	VRE	no	Harwood	2001	67	Appl Environ Microbiol
		no	Ishihara	2013	66	Curr Microbiol
		no	Poeta/Poeta	2005a/2005b	55/52	J Antimicrob Chemother /J Vet Med B
		yes	Araújo	2011	17	Microb Drug Resist
		yes	Bahirathan	1998	44	Can J Microbiol
		yes	Barros	2012	27	Microbes Environ
		yes	Figueiredo	2009	49	J Basic Microb
		yes	Guardabassi	2004	70	Appl Environ Microbiol
		yes	Mallon	2002	8	Emerg Infect Dis
		yes	Marrow	2009	45	J Wildlife Dis
		yes	Oravcova	2013	16	Environ Microbiol
		yes	Radhouani	2010b	50	Lett Appl Microbiol
		yes	Radhouani	2010c	8	Proteome Sci
		yes	Radhouani	2011	47	J Wildlife Dis
		yes	Radimersky	2010	109	J Appl Microbiol
		yes	Santos	2013	24	Anaerobe

		AMR bacteria[a]	detected	first author	year	volume	journal
			yes	Sellin	2000	6	Emerg Infect Dis
			yes	Silva	2011	40	Avian Pathol
			yes	Silva	2012	43	Braz J Microbiol
			yes	Wilson	2002	79	Int J Food Microbiol
contamination sources	manure	ESBL	yes	Blaak	2014b	80	Appl Environ Microbiol
			yes	Friese	2013a	79	Appl Environ Microbiol
			yes	Hartmann	2012	3	Front Microbiol
			yes	Hasan	2012	18	Emerg Infect Dis
			yes	Ma	2012	78	Appl Environ Microbiol
		MRSA	-	-	-	-	-
		VRE	yes	Manero	2006	8	Environ Microbiol
	wastewater	ESBL	yes	Alouache	2014	20	Microb Drug Resist
			yes	Amaya	2012	18	Clin Microbiol Infect
			yes	Blaak	2014b	80	Appl Environ Microbiol
			yes	Blaak	2014c	171	Vet Microbiol
			yes	Chagas	2011	111	J Appl Microbiol
			yes	Colomer-Lluch	2013	68	J Antimicrob Chemother
			yes	De Boeck	2012b	31	Eur J Clin Microbiol
			yes	Diallo	2013	47	Water Res
			yes	Diwan	2012	67	J Antimicrob Chemother
			yes	Dolejská	2011	66	J Antimicrob Chemother
			yes	Galvin	2010	76	Appl Environ Microbiol
			yes	Gündoğdu	2013	59	Can J Microbiol
			yes	Korzeniewska	2013a	91	Ecotox Environ Safe
			yes	Korzeniewska	2013b	123	J Environ Manage
			yes	Korzeniewska	2013c	128	J Environ Manage
			yes	Łuczkiewicz	2010	44	Water Res
			yes	Machado	2009	63	J Antimicrob Chemother
			yes	Mesa	2006	58	J Antimicrob Chemother
			yes	Prado	2008	46	Lett Appl Microbiol
			yes	Reinthaler	2010	44	Water Res
			yes	Sabate	2008	159	Res Microbiol
			yes	Usha	2013	6	Asian J Pharm Clin Res

AMR bacteria[a]	detected	first author	year	volume	journal
	yes	Zarfel	2013	173	Environ Pollut
MRSA	yes	Börjesson	2009	43	Water Res
	yes	Börjesson	2010	108	J Appl Microbiol
	yes	Rosenberg Goldstein	2012	120	Environ Health Persp
	yes	Thompson	2013	114	J Appl Microbiol
VRE	yes	Araújo	2010	50	J Basic Microb
	yes	Bates	1994	34	J Antimicrob Chemother
	yes	Blanch	2003	94	J Appl Microbiol
	yes	Borhani	2014	7	Jundishapur J Microbiol
	yes	Da Costa	2006a	41	J Environ Sci Heal B
	yes	Del Campo	2001	45	Antimicrob Agents Chemother
	yes	Gajan	2008	11	Pak J Bio Sci
	yes	Gallert	2005	69	Appl Microbiol Biot
	yes	Gomez	2010	135	WIT Trans Ecol Envir
	yes	Guardabassi	2002	8	Microb Drug Resist
	yes	Guardabassi	2004	70	Appl Environ Microbiol
	yes	Harwood	2001	67	Appl Environ Microbiol
	yes	Iversen	2002	68	Appl Environ Microbiol
	yes	Kühn/Caplin	2005/2008	71/10	Appl Environ Microbiol/ Environ Microbiol
	yes	Talebi/Talebi/Jahangiri	2008a/2008b/2010	74/56/28	Appl Environ Microbiol /Curr Microbiol / Indian J Med Microbiol
	yes	Kotzamanidis	2009	107	J Appl Microbiol
	yes	Łuczkiewicz	2010	44	Water Res
	yes	Łuczkiewicz	2011	64	Water Sci Technol
	yes	Manero	2006	8	Environ Microbiol
	yes	Martins da Costa	2006	40	Water Res
	yes	Nagulapally	2009	81	Water Environ Res
	yes	Novais/Novais	2005/2008	71/52	Appl Environ Microbiol /Antimicrob Agents Chemother
	yes	Poole/Beier	2005/2008	49/80	Antimicrob Agents Chemother /B Environ Contam Tox

		AMR bacteria[a]	detected	first author	year	volume	journal
			yes	Rahimi	2007	11	Iran Biomed J
			yes	Rosenberg Goldstein	2014	466-467	Sci Total Environ
			yes	Sahlstrom	2009	51	Acta Vet Scand
			yes	Schwartz	2003	43	FEMS Microbiol Ecol
			yes	Shaghaghi	2007	186	Water Air Soil Poll
			yes	Torres	1994	33	J Antimicrob Chemother
			yes	Varela	2013	450-451	Sci Total Environ
			yes	Volkmann	2004	56	J Microbiol Meth
			yes	Werner	1997	155	FEMS Microbiology Lett
			yes	Zimermann	1998	16	Antiinfect Drugs Chemother
exposure-relevant sites	beach sand	ESBL	yes	Simões	2010	16	Emerg Infect Dis
		MRSA	yes	Goodwin	2009	43	Water Res
			yes	Goodwin	2012	46	Water Res
			yes	Hower	2013	65	Microb Ecol
			yes	Levin-Edens	2012	79	FEMS Microbiol Ecol
			yes	Plano	2013	65	Microb Ecol
			yes	Roberts	2013	4	Front Microbiol
			yes	Soge	2009	64	J Antimicrob Chemother
			yes	Yamahara	2012	78	Appl Environ Microbiol
		VRE	yes	Dada	2013	185	Environ Monit Assess
			yes	Roberts	2009	107	J Appl Microbiol
			yes	Santiago-Rodriquez	2013	11	J Water Health
	recreational water	ESBL	yes	Alouache	2011	27	Microbes Environ
			yes	Blaak	2014c	171	Vet Microbiol
		MRSA	no	Kassem	2008	46	J Clin Microbiol
			yes	Enns	2012	46	Water Res
			yes	Goodwin	2009	43	Water Res
			yes	Goodwin	2012	46	Water Res
			yes	Hower	2013	65	Microb Ecol
			yes	Levin-Edens	2012	79	FEMS Microbiol Ecol

	AMR bacteria[a]	detected	first author	year	volume	journal
		yes	Plano	2011	11	BMC Microbiol
		yes	Plano	2013	65	Microb Ecol
		yes	Roberts	2013	4	Front Microbiol
		yes	Soge	2009	64	J Antimicrob Chemother
	VRE	yes	Dada	2013	185	Environ Monit Assess
		yes	Roberts	2009	107	J Appl Microbiol
		yes	Santiago-Rodriquez	2013	11	J Water Health
drinking water	ESBL	yes	Amaya	2012	18	Clin Microbiol Infect
		yes	De Boeck	2012a	18	Emerg Infect Dis
		yes	Talukdar	2013	8	PLoS ONE
	MRSA	-	-	-	-	-
	VRE	no	Schwartz	2003	43	FEMS Microbiol Ecol
		yes	Ateba	2013	11	J Water Health
urban water	ESBL	-	-	-	-	-
	MRSA	-	-	-	-	-
	VRE	-	-	-	-	-
irrigation water	ESBL	no	Blaak	2014a	168-169	Int J Food Microbiol
	MRSA	-	-	-	-	-
	VRE	-	-	-	-	-
shell(fish)	ESBL	-	-	-	-	-
	MRSA	-	-	-	-	-
	VRE	yes	Guardabassi	2004	70	Appl Environ Microbiol
		yes	Wilson	2002	79	Int J Food Microbiol
ambient air	ESBL	yes	Korzeniewska	2013a	91	Ecotox Environ Safe
		yes	Korzeniewska	2013c	128	J Environ Manage
	MRSA	yes	Friese	2013a	79	Appl Environ Microbiol
	VRE	-	-	-	-	-

	AMR bacteria[a]	detected	first author	year	volume	journal
fresh produce	ESBL	no	Blaak	2014a	168-169	Int J Food Microbiol
		no	Campos	2013	166	Int J Food Microbiol
		no	Kawamura	2014	11	Foodborne Pathog Dis
		yes	Egea	2011	30	Eur J Clin Microbiol
		yes	Hassan	2011	8	Foodborne Pathog Dis
		yes	Mesa	2006	58	J Antimicrob Chemother
		yes	Veldman	2014	177	Int J Food Microbiol
	MRSA	yes	Seo	2010	19	Food Sci Biotechnol
		yes	Sharafati-Chaleshtori	2010	26	Pak J Med Sci
	VRE	no	Boehme	2004	48	Mol Nutr Food Res
		no	Campos	2013	166	Int J Food Microbiol
		no	Koluman	2009	20	Food Control
		no	Kühn	2005	71	Appl Environ Microbiol
		no	Manero	2006	8	Environ Microbiol
		yes	Mohapi	2013	10	Life Sci J
		yes	Torre	2010	7	Ital J Public Health

[a] Abbreviations used: AMR bacteria, antimicrobial resistant bacteria; ESBL-Ent, extended-spectrum β-lactamase-producing Enterobacteriaceae; MRSA, methicillin-resistant *Staphylococcus aureus*; VRE, vancomycin-resistant *Enterococcus* spp.

Chapter 3

Prevalence of extended-spectrum β-lactamase-producing Enterobacteriaceae in humans living in municipalities with high and low broiler density

Patricia M.C. Huijbers
Marlieke de Kraker
Elisabeth A.M. Graat
Angela H.A.M. van Hoek
Marga G. van Santen
Mart C.M. de Jong
Engeline van Duijkeren
Sabine C. de Greeff

Clinical Microbiology and Infection (2013) 19:E256-E259.

Abstract

Prevalence of, and risk factors for, carriage of extended-spectrum β-lactamase (ESBL) -producing Enterobacteriaceae were determined for 1025 Dutch adults in municipalities with either high or low broiler densities. Overall prevalence of ESBL-carriage was 5.1%. The hypothesis that individuals in areas with high broiler densities are at greater risk for ESBL-carriage was rejected, as the risk was lower (OR=0.45; P=0.009) for these individuals. Owning a horse increased the risk (OR=4.69; P≤0.0001), but horse owners often owned multiple species of companion animals. Routes of transmission from animals to humans in the community, and the role of poultry in this process, remain to be elucidated.

Extended-spectrum β-lactamase (ESBL)-mediated resistance presents a threat for treatment of bacterial infections [1]. ESBL-producing Enterobacteriaceae have been detected in patients [2, 3], individuals from the community [4, 5], meat [3, 6], livestock [7, 8], and companion animals [9]. Transmission between humans and animals [10, 11] might occur through the food chain [2, 3], contact with livestock [12], or the environment [8]. An ESBL-prevalence of 4.9% has been found in Dutch hospitalised patients in the province with the highest number of broiler chickens [3], however little information is available about the prevalence of ESBL carriage in the Dutch community. ESBL-producing bacteria are present on all Dutch broiler farms, and 33% of farmers were ESBL carriers [12]. Individuals in areas with high broiler densities might therefore have an increased risk for ESBL carriage. The aim of this cross-sectional study was to determine prevalence of, and identify risk factors for, carriage of ESBL-producing Enterobacteriaceae in individuals living in municipalities with either high or low broiler densities.

A random sample of adults (≥ 18 years), stratified according to age and gender (Table 1), was taken from eight Dutch municipalities across four provinces. Per province, the municipality with the highest number of commercial broilers per km^2 and a municipality with a similar number of inhabitants, but with no commercial broilers, were selected. It was calculated that a sample size of 1800 individuals in both areas was required [13] based on an ESBL prevalence of 4.9% [3] in areas with high broiler densities, to show a difference of 3% (one-tailed, alpha-error=0.05, beta-error=0.20, assumed response=25%). In total 3949 individuals were asked to return a rectal swab, and a questionnaire on demographics, contact with animals, lifestyle, medical history, eating habits and travel. For each respondent, distance to the nearest broiler farm was obtained using geographic data. Exclusion criteria were living or working on a commercial broiler farm, and moving outside the study area. The study was approved by the Medical Ethics Committee of University Medical Centre Utrecht, the Netherlands (protocol number 11-277).

Rectal swabs were analysed within 3 days of collection, and not frozen before processing. Isolation of Enterobacteriaceae occurred by selective pre-enrichment with(out) 1 mg/L cefotaxime (Sigma, St. Louis, MO, USA), followed by screening on MacConkey agar no. 3 (Oxoid, Basingstoke, UK) supplemented with 1 mg/L cefotaxime. All oxidase-negative bacteria (BBL Dryslide Oxidase; Becton Dickinson, Franklin Lakes, NJ, USA) were tested phenotypically for ESBL-production by a combination disc-diffusion test using cefotaxime and ceftazidime, with and without clavulanic acid, according to CLSI guidelines [14]. A cefoxitin disc was used to detect combined ESBL/AmpC phenotypes. Individuals were classified as ESBL-positive when at least one isolate was identified phenotypically as an ESBL-producing gram-negative bacterium (BBL Crystal E/NF test; Becton Dickinson). Prevalences and their exact 95% CI were calculated based on the binomial distribution. Univariable and multivariable logistic regression analyses were performed for the variables in Table 2 to assess the relation between ESBL carriage and possible risk factors, according to the method of Hosmer and Lemeshow [15].

Table 1. Distribution of respondents over age and gender in areas with high and low broiler density

Age class	High broiler density			Low broiler density		
	No. of men (% of total)	No. of women (% of total)	Total	No. of men (% of total)	No. of women (% of total)	Total
18-40 years	36 (7)	72 (13)	108 (20)	32 (7)	68 (14)	100 (20)
41-65 years	122 (23)	187 (35)	309 (58)	114 (23)	155 (31)	269 (55)
≥66 years	65 (12)	51 (10)	116 (22)	66 (13)	57 (12)	123 (25)
Total	223 (42)	310 (58)	533 (100)	212 (43)	280 (57)	492 (100)

In total, 1033 (26.2%) individuals agreed to participate, of which 1025 individuals were eligible. Overall, prevalence of ESBL carriage was higher in areas with low broiler densities (6.7%; 33/492; exact 95% CI 4.7-9.3%) than in areas with high broiler densities (3.6%; 19/533; exact 95% CI 2.2-5.5%), and this difference was also present when combining test probabilities across provinces (P<0.01; χ^2=20.6 with 8 df). The hypothesis that individuals in municipalities with high broiler densities would be at greater risk for ESBL carriage was therefore rejected. Mean distance to the nearest broiler farm was smaller for individuals in municipalities with high broiler densities (2.2 km; SD 1.4, range 0.2-7.3), compared with low broiler densities (6.2 km; SD 2.9, range 0.9-11.8; P≤0.0001). It could be argued whether this is a distance of biological relevance. Moreover, the overall high broiler density in the Netherlands (1301 broilers/km^2), and mobility of individuals between municipalities and provinces, may have disturbed a possible relationship between broiler density and ESBL carriage, leading to the rejection of our hypothesis.

Sixteen variables could be included in the multivariable analysis (Table 2). Distance to closest broiler farm was also associated with probability of a person being ESBL-positive (P=0.04), but was not included in multivariable analysis as this variable was co-linear with broiler density. Risk factors for ESBL carriage in the community suggested in the literature, such as consumption of poultry meat, travelling abroad, recent hospitalisation and recent antibiotic use [2, 16, 17] were not confirmed in this study. The observed prevalence of ESBL carriage and the similarity in answers to the questionnaire, which resulted in categories with <10% of available data, make it difficult to draw statistically valid conclusions on these risk factors. Two variables remained in the final model showing no lack of fit (P=0.96): broiler density and owning/contact with a horse. Confounding was not present, and interaction was not significant (P=0.67). High broiler density decreased the risk for ESBL carriage (3.6% vs. 6.7%; OR=0.45; P=0.009). Owning/contact with a horse increased the risk (15.6% vs. 4.4%; OR=4.69; P≤0.0001). This might not be solely attributable to owning or having contact with a horse, as prevalence increased from 4% in individuals without companion animals to almost 12% in individuals who owned more than four different species of companion animals (Table 2).

After response analysis with respect to age, sex, province and broiler density (Table 1), it was assumed that a representative sample of Dutch adults was obtained. The observed overall

prevalence of 5.1% (52/1025; exact 95% CI 3.8-6.6%) therefore indicates the prevalence in the community, and is comparable with other countries [4, 5, 18, 19]. Contact with multiple species of companion animals might play a role in transmission, but further research on the mutual exchange of ESBL-producing Enterobacteriaceae between companion animals and their owners is needed. Other routes of transmission from animals to humans in the community, and the role of poultry in this process remain to be elucidated.

Table 2. Frequency (n and %), prevalence (%), and overall P-values in univariable logistic regression of possible risk factors (n=1025). Overall prevalence was 5.1%

Variable	Category	Frequency		Prev.	Overall P-value[a]
		n	%	%	
Research question					
Broiler density	Low	492	48.0	6.7	**0.02**
	High	533	52.0	3.6	
Distance to nearest broiler farm (per km; continuous)[b]	ESBL-negatives	4.1 ± 3.0 km			0.04
	ESBL-positives	5.0 ± 3.2 km			
General characteristics					
Province	Noord-Brabant	280	27.3	3.6	**0.05**
	Gelderland	219	21.4	3.7	
	Overijssel	268	26.2	4.5	
	Friesland	258	25.2	8.5	
Sex	Male	435	42.4	5.3	0.79
	Female	590	57.6	4.9	
Age (per year; continuous variable)[b]	ESBL-negatives	53.6 ± 15.6 years			0.58
	ESBL-positives	52.4 ± 14.1 years			
Country of birth	Abroad	27	2.7	0.0	0.27
	The Netherlands	991	97.3	5.3	
Contact with animals					
Owning/contact with companion animal	No	496	48.5	4.0	**0.14**
	Yes	527	51.5	6.1	
Cat	No	860	84.1	4.5	**0.08**
	Yes	163	15.9	8.0	
Dog	No	721	70.5	4.4	**0.16**
	Yes	302	29.5	6.6	
Rodent	No	879	85.9	4.9	0.50
	Yes	144	14.1	6.3	
Bird	No	940	91.9	5.1	0.91
	Yes	83	8.1	4.8	
Hobby chicken	No	928	90.7	4.7	**0.15**
	Yes	95	9.3	8.4	
Hobby sheep/goat	No	991	96.9	4.8	**0.10**
	Yes	32	3.1	12.5	
Horse	No	959	93.7	4.4	**0.001**
	Yes	64	6.3	15.6	

Table 2. Continued

Variable	Category	Frequency		Prev.	Overall P-value[a]
		n	%	%	
No. of companion animals species	0	496	48.5	4.0	0.31
	1	300	29.4	5.3	
	2	131	12.8	5.3	
	3	52	5.1	7.7	
	≥4	43	4.2	11.6	
Companion animal received antibiotics	No	945	93.5	4.6	**0.13**
	Yes	66	6.5	9.1	
Living on a livestock farm[d]	No	980	96.3	4.8	**0.16**
	Yes	38	3.7	10.5	
Working on a livestock farm[d]	No	945	93.2	4.9	0.74
	Yes	69	6.8	5.8	
Visiting a livestock farm[e]	No	719	70.5	5.3	0.67
	Yes	301	29.5	4.7	
Lifestyle					
No. of individuals in residence	≤2	630	61.5	4.3	0.15
	≥2	395	38.5	6.3	
Working in health care	No	853	83.4	5.5	0.14
	Yes	170	16.6	2.9	
Having a vegetable garden	No	851	83.4	5.2	0.81
	Yes	169	16.6	4.7	
Swimming in a river, lake of pond	No	940	92.1	5.0	0.65
	Yes	81	7.9	6.2	
Medical history (during last 6 months)					
Visiting general practitioner	No	456	44.9	6.6	**0.06**
	Yes	560	55.1	3.9	
Cases of urinary tract infection	No	954	93.6	5.0	0.70
	Yes	65	6.4	6.2	
Hospital admission	No	941	92.1	5.0	0.65
	Yes	81	7.9	6.2	
Visiting polyclinic	No	666	65.5	5.6	0.37
	Yes	351	34.5	4.3	
Eating habits					
Eating meat[c]	No	13	1.3	0.0	0.39
	Yes	1011	98.7	5.1	
Eating chicken meat	No	56	5.5	3.6	0.58
	Yes	967	94.5	5.2	
Eating meat purchased from farm	No	974	95.0	4.8	**0.16**
	Yes	51	5.0	9.8	
Eating vegetables from garden	No	811	79.1	5.6	**0.16**
	Yes	214	20.9	3.3	
Travel					
Travelling abroad	Inside Europe	995	93.4	4.8	**0.18**
	Outside Europe	68	6.7	8.8	

[a] Variables with P-value in bold (P<0.25) were included in the multivariable modelling procedure.
[b] Linearity of the logits was assessed before inclusion as continuous explanatory variables.
[c] Exact logistic regression.
[d] Living/working on livestock farms include farms with laying hens, veal calves, dairy cattle, breeding pigs, finishing pigs, sheep/goats; people living/working on broiler farms were excluded.
[e] Visiting livestock farms include broiler farms, in addition to the farms mentioned in footnote [d].

Acknowledgements

We thank our technicians P. Hengeveld and W.M. van Overbeek for their excellent work in the laboratory, B. van Benthem and A.W. van de Giessen for their valuable advice on the study design, and B. Bom for generating the geographic data. Preliminary results of this study were presented at the 3rd ASM conference on Antimicrobial Resistance in Zoonotic Bacteria and Foodborne Pathogens held in Aix-en-Provence from 26 to 29 July 2012.

References

1. Hawkey, P.M., Jones, A.M. 2009. The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy*. 64 Supplement 1:i3-i10.
2. Leverstein-van Hall, M.A., Dierikx, C.M., Cohen Stuart, J., *et al*. 2011. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clinical Microbiology and Infection.* 17:873-880.
3. Overdevest, I., Willemsen, I., Rijnsburger, M., *et al*. 2011. Extended-spectrum beta-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. *Emerging Infectious Diseases.* 17:1216-1222.
4. Vinué, L., Sáenz, Y., Martínez, S., *et al*. 2009. Prevalence and diversity of extended-spectrum β-lactamases in faecal *Escherichia coli* isolates from healthy humans in Spain. *Clinical Microbiology and Infection.* 15:954-956.
5. Geser, N., Stephan, R., Korczak, B.M., *et al*. 2012. Molecular identification of extended-spectrum-β-lactamase genes from *Enterobacteriaceae* isolated from healthy human carriers in Switzerland. *Antimicrobial Agents and Chemotherapy.* 56:1609-1612.
6. EFSA (European Food Safety Authority). 2011. Scientific opinion on the public health risks of bacterial strains producing extended-spectrum beta-lactamases and/or AmpC beta-lactamases in food and food-producing animals. *EFSA Journal*. 9:1-90.
7. Dierikx, C.M., van Essen-Zandbergen, A., Veldman, K., *et al*. 2010. Increased detection of extended-spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. *Veterinary Microbiology.* 145:273-278.
8. Hartmann, A., Locatelli, A., Amoureux, L., *et al*. 2012. Occurrence of CTX-M producing *Escherichia coli* in soils, cattle, and farm environment in France (Burgundy region). *Frontiers in Microbiology.* 3:article 83, pp 1-7.
9. Dierikx, C.M., van Duijkeren, E., Schoormans, A., *et al*. 2012. Occurrence and characteristics of extended-spectrum-β-lactamase-and AmpC-producing clinical isolates derived from companion animals and horses. *Journal of Antimicrobial Chemotherapy.* 67:1368-1374.
10. Smet, A., Martel, A., Persoons, D., *et al*. 2009. Comparative analysis of extended-spectrum beta-lactamase-carrying plasmids from different members of *Enterobacteriaceae* isolated from poultry, pigs and humans: evidence for a shared beta-lactam resistance gene pool? *Journal of Antimicrobial Chemotherapy.* 63:1286-1288.
11. Smet, A., Rasschaert, G., Martel, A., *et al.* 2011. *In situ* ESBL conjugation from avian to human *Escherichia coli* during cefotaxime administration. *Journal of Applied Microbiology.* 110:541-549.
12. Dierikx, C.M., Van der Goot, J., Fabri, T., *et al*. 2012 Extended-spectrum-beta-lactamase- and AmpC-beta-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers. *Journal of Antimicrobial Chemotherapy.* 68:60-67.
13. Thrusfield, M., Ortega, C., De Blas, I., *et al*. 2001. Win episcope 2.0: Improved epidemiological software for veterinary medicine. *Veterinary Record.* 148:567-572.
14. Clinical Laboratory Standards Institute. 2011. Performance standards for antimicrobial susceptibility testing. 21st Informational Supplement M100-21. Wayne, PA, USA.
15. Hosmer, D.W., Lemeshow, S. 2000. Applied logistic regression. 2nd edn. Hoboken, New Jersey: John Wiley & Sons, Inc.
16. Tängdén, T., Cars, O., Melhus, Å., *et al*. 2010. Foreign travel is a major risk factor for colonization with *Escherichia coli* producing CTX-M-type extended-spectrum beta-lactamases: a prospective study with Swedish volunteers. *Antimicrobial Agents and Chemotherapy.* 54:3564-3568.
17. Ben-Ami, R., Rodríguez-Baño, J., Arslan, H., *et al*. 2009. A multinational survey of risk factors for infection with extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in nonhospitalized patients. *Clinical Infectious Diseases.* 49:682-690.
18. Ben Sallem, R., Ben Slama, K., Estepa, V., *et al*. 2012. Prevalence and characterisation of extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* isolates in healthy volunteers in Tunisia. *European Journal of Clinical Microbiology and Infectious Diseases.* 31:1511-1516.
19. Luvsansharav, U.O., Hirai, I., Niki, M., *et al*. 2011. Prevalence of fecal carriage of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* among healthy adult people in Japan. *Journal of Infection and Chemotherapy.* 17:722-725.

Chapter 4

Extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* in broilers and people living and/or working on broiler farms: prevalence, risk factors and molecular characteristics

Patricia M.C. Huijbers
Elisabeth A.M. Graat
Anja P.J. Haenen
Marga G. van Santen
Alieda van Essen-Zandbergen
Dik J. Mevius
Engeline van Duijkeren
Angela H.A.M. van Hoek

Journal of Antimicrobial Chemotherapy (2014) 69:2669-2675.

Synopsis

Objectives: Estimate the prevalence of extended-spectrum β-lactamase (ESBL)- and AmpC β-lactamase-producing *Escherichia coli* carriage among broiler farmers, their family members and employees; identify and quantify risk factors for carriage, with an emphasis on contact with live broilers; and compare isolates from humans and broilers within farms with respect to molecular characteristics to gain insight into transmission routes.

Methods: A cross-sectional prevalence study was conducted on 50 randomly selected Dutch broiler farms. Cloacal swabs were taken from 20 randomly chosen broilers. Faecal swabs were returned by 141 individuals living and/or working on 47 farms. ESBL/AmpC-producing *E. coli* were isolated and, for selected isolates phylogenetic groups, plasmids and sequence types were determined. Questionnaires were used for risk factor analysis.

Results: All sampled farms were positive, with 96.4% positive pooled broiler samples. The human prevalence was 19.1%, with 14.3% and 27.1% among individuals having a low and high degree of contact with live broilers, respectively. Five pairs of human-broiler isolates had identical genes, plasmid families and *E. coli* sequence types, showing clonal transmission. Furthermore, similar ESBL/AmpC-genes on the same plasmid families in different *E. coli* sequence types in humans and broilers hinted at horizontal gene transfer.

Conclusions: Prevalence among people on broiler farms was higher than in previous studies involving patients and the general population. Furthermore, an increased risk of carriage was shown among individuals having a high degree of contact with live broilers. The (relative) contribution of transmission routes that might play a role in the dissemination of ESBL/AmpC-encoding resistance genes to humans on broiler farms should be pursued in future studies.

Introduction

Extended-spectrum β-lactamase (ESBL)- and AmpC β-lactamase-producing Enterobacteriaceae are frequently reported in broilers [1,2]. This raises public-health concerns as the intestinal microbiome of these animals might form a reservoir for ESBL/AmpC-encoding resistance genes, capable of being transmitted to humans [3,4]. Transmission via the food chain has been suggested [5,6], but transmission resulting from close contact between humans and animals on livestock farms is also plausible [2]. Contact with live broilers has already been identified as a risk factor for methicillin-resistant *Staphylococcus aureus* (MRSA) carriage among humans [7,8].

In the Netherlands, the prevalence of carriage of ESBL/AmpC-producing *Escherichia coli* (hereafter referred to ESBL/AmpC carriage) among broiler farmers was 33.3% (6/18), and β-lactamase genes located on identical plasmid families were detected in isolates both from farmers and their animals [2]. Furthermore, the prevalence among farmers was higher than that found among patients (4.9%) [5], and among humans not living on farms (5.1%) [9] in the Netherlands. This suggests that contact with broilers, and/or the farm environment, could be a risk factor for ESBL/AmpC carriage among humans.

Risk factors for ESBL/AmpC carriage among people living and/or working on broiler farms have not yet been reported. The objectives were therefore to estimate the prevalence among broiler farmers, their family members and employees, and to identify and quantify risk factors for carriage, with an emphasis on contact with live broilers. To gain an insight into transmission routes between broilers and humans, *E. coli* isolates from humans and broilers from the same farm were compared with respect to ESBL/AmpC genes, plasmid families and sequence types (STs).

Methods

From 5 July 2010 to 11 April 2011, a cross-sectional study of MRSA and ESBL/AmpC-producing bacteria was conducted on 50 Dutch broiler farms with 228 individuals living or working on these farms. Study populations were defined and sampled according to Geenen *et al.* [8]. Participating farms had an average of three broiler houses (range 1-6; broilers of one age and using an all-in-all-out system), with a median number of broilers per farm of 78000 (range 14400–200000). The age of broilers at sampling was 21-49 days, with an average of 31 days. In order to accurately estimate prevalence of ESBL/AmpC carriage among humans, given a prevalence of 33% among broiler farmers, a 95% confidence level and 10% accepted error, at least 85 individuals needed to be sampled.

Each farm was sampled by taking cloacal swabs from 20 broilers in total, the broilers being divided over all the broiler houses present on the farm. This sample size enables the detection of a positive farm at the 95% confidence level with a within-farm prevalence of at least 14%. The study was performed according to Dutch law on studies with animals. Farmers, family members and employees who voluntarily participated in the study returned an informed consent form, a faecal swab and a questionnaire on their lifestyle and health characteristics. For children aged <18 years parental consent was requested. Farmers also completed a questionnaire on the farm (management) characteristics. The median time between the sampling of broilers and the arrival of human samples was 1 day (range 0-28 days).

Broiler samples were pooled into 10 pools of 2 swabs each; human samples were examined individually. Bacteria were isolated by selective enrichment (Luria-Bertani broth with 1 mg/L cefotaxime) and cultured on selective plates for 18h at 37˚C (MacConkey agar no. 3 with 1 mg/L of cefotaxime). *E. coli*-like, indole-positive isolates (5 isolates per human sample and 10 isolates per broiler farm) were tested phenotypically for ESBL production by combination disc diffusion test according to CLSI guidelines [10]. A cefoxitin disc was used to detect the AmpC phenotypes.

E. coli phylogenetic groups were determined for a minimum of one (range 1-3) isolate per person testing positive as reported by Clermont *et al.* [11] and Escobar-Páramo *et al*. [12]. *E. coli* was confirmed in isolates that were negative for all three amplicons as described by Frahm and Obst [13]. Subsequently, phylogenetic groups were determined for a minimum of one (range 1-9) positive broiler isolate from each farm with human ESBL/AmpC carriage. More than one isolate per human or broiler was included if there were differences in the disc diffusion test, indicative of the presence of different ESBL/AmpC genes.

β-Lactamase genes were identified by PCR and sequencing in line with Dierikx *et al*. [14]. If broiler and human isolates showed similar *E. coli* phylogenetic groups and/or ESBL/AmpC genes within the same farm, multilocus sequence typing (MLST) of *E. coli* was performed as described by Wirth *et al*. [15]. Plasmid characterisation was performed for a selection of isolates representing the diversity of ESBL/AmpC genes in humans and broilers, and on isolate pairs with the same *E. coli* ST and ESBL/AmpC gene by transformation and PCR-based replicon typing as described by Hordijk *et al.* [16] and Carattoli *et al*. [17].

Prevalences and their exact 95% CIs were calculated based on the binomial probability function. Risk factor analysis could not be conducted at the farm level as all the farms were classified as positive (*i.e.* bacteria were phenotypically characterised as ESBL/AmpC-producing *E. coli* in at least one broiler sample). To assess the relationship between ESBL/AmpC carriage among humans and possible risk factors, univariable and multivariable logistic regression analyses were performed for the variables in Table 1 as reported by Hosmer and Lemeshow [18]. As observations on the same farm might not have been independent, a random effect of farms was included using an exchangeable covariance structure.

Results and discussion

This study investigates ESBL/AmpC carriage among not only farmers, but also all individuals living and/or working on broiler farms and is the first study reporting risk factors for carriage. Faecal swabs and informed consent forms were provided by 141/228 individuals from 47 farms (a response rate of 61.8%). An average of 3 individuals per farm (range 1-9) was included, with an average age of 36.6 years (SD 19.1, range 1-80). Similar to the results of Dierikx *et al*. [2], ESBL/AmpC-producing *E. coli* were present in broilers on all 50 farms, with a pooled sample prevalence of 96.4% (482/500; 95% CI 94.4-97.9%). In total, 27 humans originating from 21 farms tested positive (19.1%; 95% CI 13.0-26.6%). The prevalence in farmers (25.5%; exact 95% CI 13.9-40.4%) and employees (37.5%; exact 95% CI 8.5-75.5%) was similar to that of Dierikx *et al*. (33.3%) [2]. The prevalence in partners (11.4%; exact 95% CI 3.2-26.7%) and family members (15.7%; exact 95% CI 7.0-28.6%) was lower than in farmers and employees, but still higher in comparison with patients and the general population [5,9].

It was hypothesised that a high degree of contact with live broilers could be a risk factor for human ESBL/AmpC carriage on broiler farms. Information about the number of hours per week present in the broiler house and the number of hours per week having physical contact with live broilers was completed by about half of the participants (by 81 and 77 individuals, respectively). Analysis of variance of these variables showed that farmers (2.8 h and 1.8 h) and employees (1.8 h and 0.6 h) had a significantly higher degree of contact ($P<0.0001$) compared with partners (0.7 h and 0.5 h) and other family members (0.3 h and 0.1 h). In addition individuals reporting performance of activities in the broiler house (*i.e.* weighing, vaccination, blood sampling and/or health checks) were more often (χ^2 $P<0.0001$) farmers and employees (98.1%) than partners and family members (33.8%). Based on this information a new variable ('contact with broilers') was created, with farmers and employees in one category and partners and family members in the other. ESBL/AmpC carriage among humans related to possible risk factors are shown in Table 1. Sex, age, hours spent in the broiler house, performance of activities in the broiler house, and type of person correlated strongly (>0.5) with the 'contact with broilers' variable. To avoid multicollinearity only the latter variable was included in the multivariable model. Three out of the seven initially included variables (Table 1) remained in the final multivariable model, in which the random farm effect explained 8% of the non-explained variation. In this model, based on 118 individuals with a complete record, farmers and employees were at a higher risk of ESBL/AmpC carriage than partners and family members (27.1% versus 14.3%; OR=2.5; P=0.08). It seems that there is an increased risk of exposure to ESBL/AmpC-producing *E. coli* for humans on broiler farms and that this risk is larger for individuals in close contact with broilers. Two other factors associated with higher risk of ESBL/AmpC carriage in humans were having diabetes or a skin disease (41.2% versus 15.8%; OR=16.5; P=0.002) and sampling in July-December 2010

(33.3% versus 7.8% in January-May 2011; OR=13.0; P=0.002). Risk factors reported in the literature such as travel abroad [19] and antibiotic use [20] were not identified in the current study. The similarity of answers to the questionnaire, which resulted in categories with <10% of available data, makes it difficult, however, to draw statistically valid conclusions on these risk factors.

Table 1. Prevalence of ESBL/AmpC carriage (Prev) among people living and/or working on 47 Dutch broiler farms in relation to farm-related and individual characteristics (n=141),[a] the overall prevalence being 19.1%.

Variable	Category	Frequency[b]		Prev.	Overall P-value[c]
		n	%	%	
Research question					
Contact with broilers	Partners, family	86	61.0	14.0	**0.05**
	Farmers, employees	55	39.0	27.3	
General characteristics					
Sampling period	Jan-May 2011	73	51.8	9.6	**0.002**
	Jul-Dec 2010	68	48.2	29.4	
Region	South	48	34.0	22.9	0.84
	East	38	27.0	18.4	
	West	10	7.1	20.0	
	North	45	31.9	15.6	
Farm characteristics					
Presence in broiler house	No	22	17.5	13.6	0.40
	Yes	104	82.5	21.2	
Hours spent in broiler house	0	22	27.2	13.6	**0.02**
	≤ 2	32	39.5	9.4	
	> 2	27	33.3	37.0	
Physical contact with broilers	No	28	22.4	14.3	0.38
	Yes	97	77.6	19.7	
Hours in physical contact with broilers	0	28	36.4	14.3	0.79
	≤ 1	30	39.0	20.0	
	> 1	19	24.7	21.1	
Activities in the broiler house[d]	No activity	50	39.7	14.0	**0.17**
	≥ 1 activity	76	60.3	23.7	
Contact with livestock on other farms	No	98	77.2	22.5	**0.13**
	Yes	29	22.8	10.3	
Changing room in broiler house	No	14	9.9	14.3	0.61
	Yes	127	90.1	19.7	
Shower present in broiler house	No	78	55.3	24.4	**0.08**
	Yes	63	44.7	12.7	
Farm size	≤ 78000 broilers	63	46.7	15.9	0.46
	>78000 broilers	72	53.3	20.8	

Table 1. Continued

Variable	Category	Frequency[b]		Prev.	Overall P-value[c]
		n	%	%	
Individual characteristics					
Type of person	Partner	35	24.8	11.4	**0.21**
	Family member	51	36.2	15.7	
	Farmer	47	33.3	25.5	
	Employee	8	5.7	37.5	
Age	0-18 years	38	27.0	13.2	0.41
	19-65 years	97	68.8	20.6	
	≥ 65 years	6	4.3	33.3	
Sex	Male	72	56.3	27.8	**0.01**
	Female	56	43.8	8.9	
Family members in residence	≤ 2	25	18.5	16.0	0.57
	> 2	110	81.5	20.9	
Visit to hospital or polyclinic in past year	No	96	76.8	21.9	0.32
	Yes	29	23.2	13.8	
Antibiotic use during past 3 months	No	115	92.7	20.9	0.45
	Yes	9	7.3	11.1	
Having diabetes or skin disease(s)[e]	No	101	85.6	15.8	**0.02**
	Yes	17	14.4	41.2	
MRSA-positive in this study	No	133	94.3	17.3	**0.04**
	Yes	8	5.7	50.0	
Shared use of towels	No	48	39.7	14.6	**0.23**
	Yes	73	60.3	23.3	
Playing team sports	No	83	66.4	18.1	0.45
	Yes	42	33.6	23.8	
Travel abroad during past year	No	60	48.8	20.0	0.93
	Yes	63	51.2	20.6	

[a] In an 'intercept only' model (without explanatory variables) the random farm effect was not significant and explained only 1.9% of the non-explained variation.
[b] A number of questionnaires were not complete, resulting in variables with missing values.
[c] Variables with a P-value (based on the likelihood ratio test) in bold (P<0.25) were considered for multivariable modelling.
[d] Activities in the broiler house include weighing, vaccination, blood sampling and health checks.
[e] Skin diseases include psoriasis, eczema, impetigo, infected skin, infected wounds and boils.

The distribution of phylogenetic groups, ESBL/AmpC genes and plasmids for a selection of 43 human and 90 broiler *E. coli* isolates from 21 farms with humans testing positive is summarised in Table 2. The molecular characteristics of all the isolates are presented in Table S1. Phylogenetic groups A_0, A_1, $B1_1$, and D_2 predominated both in humans and in broilers. The most prevalent genes in isolates from humans as well as broilers were bla_{CMY-2}, $bla_{CTX-M-1}$ and bla_{SHV-12}, and the most recovered plasmid family was IncI1 (Table 2). The similarity of distribution of the phylogenetic groups, ESBL/AmpC genes and plasmid families in human

isolates compared with broiler isolates suggests an exposure to a local pool of resistance genes related to broilers and the farm environment. This is further supported by the fact that *bla*$_{CTX-M-15}$, which is one of the most prevalent genes found in humans in the Netherlands [5,21], was not found in the present study. In addition, *bla*$_{SHV-12}$ and *bla*$_{CMY-2}$ predominated but are only found sporadically in isolates from patients [5,6] and the community [21].

Table 2. Distribution of phylogenetic groups, ESBL/AmpC genes and plasmids among *E. coli* isolates from broilers and humans, representing the 21 farms with human carriage.

Molecular characteristic (PCR and sequencing)	**Broiler**		**Human**		**Total**	
	n	**%**	**n**	**%**	**n**	**%**
Phylogenetic groups						
D_2	29	32.2	8	18.6	37	27.8
A_0	19	21.1	10	23.2	29	21.8
$B1_1$	18	20.0	8	18.6	26	19.6
A_1	12	13.3	9	20.9	21	15.8
$B2_3$	8	8.9	2	4.7	10	7.5
D_1	4	4.5	4	9.3	8	6.0
$B2_2$	0	0.0	2	4.7	2	1.5
Total	**90**	**100**	**43**	**100**	**133**	**100**
ESBL/AmpC genes[a]						
bla$_{CMY-2}$	34	38.7	14	32.6	48	36.6
bla$_{CTX-M-1}$	25	28.4	12	27.9	37	28.2
bla$_{SHV-12}$	15	17.1	10	23.3	25	19.1
bla$_{TEM-52}$[b]	8	9.1	4	9.3	12	9.1
bla$_{CTX-M-2}$	2	2.3	1	2.3	3	2.3
AmpC promoter mutants[c]	0	0.0	2	4.6	2	1.5
bla$_{CTX-M-1}$, *bla*$_{CTX-M-9}$	1	1.1	0	0.0	1	0.8
bla$_{CTX-M-1}$, *bla*$_{SHV-12}$	1	1.1	0	0.0	1	0.8
bla$_{CTX-M-14}$	1	1.1	0	0.0	1	0.8
bla$_{CTX-M-32}$	1	1.1	0	0.0	1	0.8
Total[d]	**88**	**100**	**43**	**100**	**131**	**100**
Plasmid, ESBL/AmpC gene combinations						
IncI1, *bla*$_{CTX-M-1}$	16	37.2	8	30.8	24	34.8
IncK, *bla*$_{CMY-2}$	6	14.0	4	15.4	10	14.5
IncI1, *bla*$_{SHV-12}$	6	14.0	3	11.6	9	13.0
IncI1, *bla*$_{CMY-2}$	3	7.0	5	19.2	8	11.6

Table 2. Continued

Molecular characteristic (PCR and sequencing)	Broiler		Human		Total	
	n	%	n	%	n	%
IncX1, bla_{TEM-52}	2	4.7	2	7.7	4	5.8
IncX1, bla_{SHV-12}	2	4.7	0	0.0	2	2.9
IncN, bla_{SHV-12}	1	2.3	1	3.8	2	2.9
IncA/C, bla_{CMY-2}	1	2.3	0	0.0	1	1.4
IncB/O, bla_{SHV-12}	0	0.0	1	3.8	1	1.4
IncFIB, bla_{CMY-2}	1	2.3	0	0.0	1	1.4
IncFII, bla_{SHV-12}	1	2.3	0	0.0	1	1.4
IncHI2_IncP, $bla_{CTX-M-2}$	1	2.3	0	0.0	1	1.4
IncI1, $bla_{CTX-M-2}$	0	0.0	1	3.8	1	1.4
IncI1, $bla_{CTX-M-14}$	1	2.3	0	0.0	1	1.4
IncI1, bla_{TEM-52}	1	2.3	0	0.0	1	1.4
IncX1, $bla_{CTX-M-32}$	1	2.3	0	0.0	1	1.4
Untypable, bla_{SHV-12}	0	0.0	1	3.8	1	1.4
Total[e]	**43**	**100**	**26**	**100**	**69**	**100**

[a] A number of genes were found in combination with TEM-1b [CMY-2 (n=8), CTX-M-1 (n=10), CTX-M-2 (n=2), CTX-M-32 (n=1), SHV-12 (n=8), TEM-52 (n=5)]; see Table S1.
[b] One TEM-52 isolate was found with 1 mutation (C228T), and 11 TEM-52 isolates were found with 3 mutations (C18T, C228T and G396T) in this gene. All were synonymous mutations.
[c] One AmpC type 3 promoter mutant [mutations −42T−18A−1T(+23A+51T)+58T+81G] and one AmpC type 4 promoter mutant (mutations +22T+26G+27T+32A+70T+81G) were found.
[d] In 2 isolates from broilers, only bla_{TEM-1b} was found.
[e] Plasmids were not characterised for n=64 isolates.

MLST was performed for a selection of human and broiler isolates from 12 farms (Table 3). On the other 9 farms with humans testing positive (Table S1), ESBL/AmpC genes and/or *E. coli* phylogenetic groups were not similar between humans and broilers so MLST was not performed. In five cases, broiler-human isolate pairs showed the same ESBL/AmpC gene, plasmid family and *E. coli* ST (Table 3). Given the epidemiological relatedness of isolates collected in this study, a clonal transfer of ESBL/AmpC-producing *E. coli* between broilers and humans is likely. The ST diversity observed in the current study and by Dierikx *et al.* [2] further suggests that finding these broiler-human isolate pairs is not a coincidence. Clonal transfer is only the starting point for transmission as horizontal gene transfer [3,4] may occur within bacterial populations *e.g.* between *E. coli* with different STs and between *E. coli* and other bacterial species. Focussing on clonal transfer alone will therefore underestimate the frequency of transfer. In the current study identical ESBL/AmpC genes located on the same plasmid family were found in different *E. coli* STs (Table 3; farms 10, 12 and 16), suggesting a horizontal transfer of plasmids via conjugation between different *E. coli* strains.

Table 3. *E. coli* STs in isolates from 12 farms where humans and broilers had similar ESBL/AmpC genes and phylogenetic groups within the same farm.

Farm	Isolate	Source	Phylogenetic group	*E. coli* ST	Clonal complex	β-Lactamase genes (PCR and sequencing) [a,b]	Replicon type of plasmid with ESBL/ AmpC gene
04	HR1_1	Farmer	D_2	648		*bla*$_{SHV-12}$	incB/O
	C09_1	Broiler	D_2	648		*bla*$_{SHV-12}$	incI1
07	HR1_1	Farmer	D_2	117		*bla*$_{SHV-12}$	incI1
	C02_1	Broiler	D_2	117		*bla*$_{SHV-12}$	incFII
08	**GR1_3**	**Family**	**$B1_1$**	**351**		***bla*$_{CMY-2}$; *bla*$_{TEM-1b}$**	**incI1**
	C02_1	**Broiler**	**$B1_1$**	**351**		***bla*$_{CMY-2}$**	**incI1**
10	HR1_3	Farmer	A_0	93	ST168	*bla*$_{SHV-12}$; *bla*$_{TEM-1b}$	incN
	GR2_1	Family	A_0	399	ST399	*bla*$_{CTX-M-1}$	incI1
	C02_1	Broiler	A_0	189	ST165	*bla*$_{CTX-M-1}$; *bla*$_{TEM-1b}$	incI1
12	HR1_1	Farmer	$B2_3$	131		*bla*$_{CMY-2}$	incI1
	HR1_3	Farmer	$B1_1$	641	ST86	*bla*$_{CMY-2}$	
	HR1_5	Farmer	D_2	NEW[c]		*bla*$_{CTX-M-1}$	incI1
	C01_2	Broiler	D_2	1640		*bla*$_{CTX-M-1}$; *bla*$_{TEM-1b}$	incI1
	C08_1	Broiler	$B1_1$	1146		*bla*$_{CMY-2}$	
	C09_2	Broiler	D_2	1775		*bla*$_{CMY-2}$; *bla*$_{TEM-1b}$	incI1
	C10_1	Broiler	$B2_3$	355		*bla*$_{CMY-2}$	
15	**HR1_3**	**Farmer**	**D_2**	**117**		***bla*$_{CMY-2}$**	**incI1**
	GR1_4	**Family**	**D_2**	**117**		***bla*$_{CMY-2}$**	**incI1**
	C03_1	Broiler	D_2	57	ST35	*bla*$_{CMY-2}$	incK
16	HR1_3	Farmer	A_1	48	ST10	*bla*$_{CTX-M-1}$; *bla*$_{TEM-1b}$	incI1
	C03_1	Broiler	A_1	10	ST10	*bla*$_{CTX-M-1}$	incI1
20	**GR2_1**	**Family**	**A_1**	**10**	**ST10**	***bla*$_{CTX-M-1}$**	**incI1**
	C04_1	**Broiler**	**A_1**	**10**	**ST10**	***bla*$_{CTX-M-1}$**	**incI1**
25	**GR2_4**	**Family**	**A_0**	**1818**		***bla*$_{TEM-52}$**[d]	**incX1**
	C01_1	**Broiler**	**A_0**	**1818**		***bla*$_{TEM-52}$**[d]	**incX1**
	GR2_1	**Family**	**A_0**	**373**	**ST168**	***bla*$_{CMY-2}$**	**incK**
	C03_1	**Broiler**	**A_0**	**373**	**ST168**	***bla*$_{CMY-2}$**	**incK**
	C05_1	Broiler	D_2	38	ST38	*bla*$_{CMY-2}$; *bla*$_{TEM-1b}$	incK
27	**HR1_1**	**Farmer**	**A_1**	**88**	**ST23**	***bla*$_{CTX-M-1}$**	**incI1**
	HR1_5	**Farmer**	**A_1**	**88**	**ST23**	***bla*$_{CTX-M-1}$**	**incI1**
	C06_1	**Broiler**	**A_1**	**88**	**ST23**	***bla*$_{CTX-M-1}$**	**incI1**
	C08_1	Broiler	A_0	2223		*bla*$_{CTX-M-1}$	incI1

Table 3. Continued

Farm	Isolate	Source	Phylogenetic group	*E. coli* ST	Clonal complex	β-Lactamase genes (PCR and sequencing) [a,b]	Replicon type of plasmid with ESBL/AmpC gene
28	HR1_1	Farmer	A_0	1324		$bla_{CTX-M-1}$	incI1
	C09_1	Broiler	A_0	641	ST86	$bla_{CTX-M-1}$	
37	GR2_1	Family	A_1	23	ST23	bla_{SHV-12}; bla_{TEM-1b}	incI1
	GR3_2	Family	D_2	1163		bla_{CMY-2}; bla_{TEM-1b}	incI1
	C05_1	Broiler	A_0	2509		bla_{TEM-52} [d]; bla_{TEM-1b}	
	C07_1	Broiler	D_2	57	ST350	bla_{CMY-2}; bla_{TEM-1b}	incK

[a] Bold text indicates human-broiler or human-human isolate pairs from the same farm with the same ESBL/AmpC gene, plasmid family and *E. coli* ST.
[b] With the primers used in this study no distinction was made between TEM-1b and TEM-198; however for readability of the table, TEM-1b was inserted.
[c] MLST results: adk35, fumC3, gyrB234, icd342, mdh45, purA5, recA95.
[d] Isolates were found with 3 mutations (C18T, C228T and G396T). All were synonymous mutations.

Transmission between humans and broilers has been shown but knowledge about the (relative) contribution of transmission routes that might play a role in the dissemination of ESBL/AmpC-encoding resistance genes is lacking. Direct contact between humans and live broilers seems to play a major role, given the highest prevalence in farmers and employees. It is important, however, also to consider transmission via the farm environment and between humans within the household, given the relatively high prevalence in family members. *E. coli* have a high survival rate in the environment, which might lead to the accumulation of these bacteria [22], both inside and outside the broiler house, and indirect transmission to individuals living on farms. Environmental samples were not collected in the current study. A high rate of intestinal colonisation with ESBL-producing organisms was shown for the household members of patients suffering from community acquired urinary tract infections, and up to 66.6% of isolates from case patients and their corresponding household members had indistinguishable pulsed-field gel electrophoresis patterns [23]. In the current study two isolates were found, one from a farmer and one from a family member, from the same farm with bla_{CMY-2}, located on a plasmid from the incI1 family, in an *E. coli* with ST117, which might indicate human-to-human transmission. The family member reported no contact with live broilers, and rarely entered the broiler house.

This study contributes to the growing body of evidence supporting the risk for humans working and/or living on broiler farms and the transmission of ESBL/AmpC-producing Enterobacteriaceae between broilers and humans. To further elucidate the role of broilers in ESBL/AmpC carriage among humans on broiler farms, future studies should attempt to quantify the transmission between broilers, and between humans and broilers, taking into account indirect (via the environment) and direct transmission routes, as well as clonal spread and horizontal gene transfer.

Acknowledgements

Preliminary results were presented at the Spring Meeting of the Dutch Society for Medical Microbiology (NVMM) and the Royal Netherlands Society for Microbiology (KVvM), Papendal, the Netherlands, 2013 (abstract O120) and at the Annual Conference of the Netherlands Epidemiology Society (WEON) on Epidemiology in Global Health, Utrecht, the Netherlands, 2013 (abstract O24). We would like to thank all farmers, their family members and employees for participating in this study. We are grateful to P.L. Geenen, P. Doornenbal, W.M. van Overbeek, B.V. Lambrechts, C.C. Kappert and G.A.C. Lammers for their valuable assistance.

Funding

This work is supported by: the Netherlands Food and Consumer Product Authority (NVWA); the Dutch Product Board for Poultry and Eggs (PPE); and the Dutch Ministry of Health, Welfare and Sport.

References

1. Smet, A., Martel, A., Persoons, D., *et al*. 2013. Diversity of extended-spectrum β-lactamases and class C β-lactamases among cloacal *Escherichia coli* isolates in Belgian broiler farms. *Antimicrobial Agents and Chemotherapy*. 52:1238-1243.
2. Dierikx, C.M., van der Goot, J., Fabri, T., *et al*. 2013. Extended-spectrum-β-lactamase-and AmpC-β-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers. *Journal of Antimicrobial Chemotherapy.* 68:60-67.
3. Smet, A., Martel, A., Persoons, D., *et al*. 2009. Comparative analysis of extended-spectrum-β-lactamase-carrying plasmids from different members of Enterobacteriaceae isolated from poultry, pigs and humans: evidence for a shared β-lactam resistance gene pool? *Journal of Antimicrobial Chemotherapy.* 63:1286-1288.
4. Smet, A., Rasschaert, G., Martel A., *et al*. 2011. *In situ* ESBL conjugation from avian to human *Escherichia coli* during cefotaxime administration. *Journal of Applied Microbiology.* 110:541-549.
5. Overdevest, I., Willemsen, I., Rijnsburger, M., *et al*. 2011. Extended-spectrum β-lactamase genes of *Escherichia coli* in chicken meat and humans, The Netherlands. *Emerging Infectious Diseases.* 17:1216-1222.
6. Voets, G.M., Fluit, A.C., Scharringa, J., *et al*. 2013. Identical plasmid AmpC beta-lactamase genes and plasmid types in *E. coli* isolates from patients and poultry meat in the Netherlands. *International Journal of Food Microbiology.* 167:359-362.
7. Mulders, M.N., Haenen, A.P.J., Geenen, P.L., *et al*. 2010. Prevalence of livestock-associated MRSA in broiler flocks and risk factors for slaughterhouse personnel in The Netherlands. *Epidemiology and Infection.* 138:743-755.
8. Geenen, P.L., Graat, E.A.M., Haenen, A.P.J., *et al*. 2013. Prevalence of livestock-associated MRSA on Dutch broiler farms and in people living and/or working on these farms. *Epidemiology and Infection.* 141:1099-1108.
9. Huijbers, P.M.C., de Kraker, M., Graat, E.A.M., *et al*. 2013. Prevalence of extended-spectrum β-lactamase-producing Enterobacteriaceae in humans living in municipalities with high and low broiler density. *Clinical Microbiology and Infection.* 19:E256-E259.
10. Clinical and Laboratory Standards Institute. 2011. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-first Informational Supplement M100-21*. CLSI, Wayne, PA, USA.
11. Clermont, O., Bonacorsi, S., Bingen, E. 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Applied Environmental Microbiology.* 66:4555-4558.
12. Escobar-Páramo, P., Le Menac'h, A., Le Gall, T., *et al.* 2006. Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. *Environmental Microbiology.* 8:1975-1984.
13. Frahm, E., Obst, U. 2003. Application of fluorogenic probe technique (TaqMan PCR) to the detection of *Enterococcus* spp. and *Escherichia coli* in water samples. *Journal of Microbiological Methods.* 52:123-131.
14. Dierikx, C.M., van Duijkeren, E., Schoormans, A.H.W., *et al*. 2012. Occurrence and characteristics of extended-spectrum-β-lactamase and AmpC-producing clinical isolates derived from companion animals and horses. *Journal of Antimicrobial Chemotherapy.* 67:1368-1374.
15. Wirth, T., Falush, D., Lan, R., *et al.* 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Molecular Microbiology.* 60:1136-1151.
16. Hordijk, J., Wagenaar, J.A., Kant, A., *et al*. 2013. Cross-sectional study on prevalence and molecular characteristics of plasmid mediated ESBL/AmpC-producing *Escherichia coli* isolated from veal calves at slaughter. *Plos One*. 8:e65681.
17. Carattoli, A., Bertini, A., Villa, L., *et al.* 2005. Identification of plasmids by PCR-based replicon typing. *Journal of Microbiological Methods.* 63:219-228.
18. Hosmer, D.W., Lemeshow, S. 2000. *Applied Logistic Regression, Second edition*. New Jersey: John Wiley & Sons, Inc.
19. Paltansing, S., Vlot, J.A., Kraakman, M.E.M., *et al.* 2013. Extended-spectrum β-lactamase-producing Enterobacteriaceae among travellers from the Netherlands. *Emerging Infectious Diseases.* 19:1206-1213.
20. Ben-Ami, R., Rodríguez-Baño, J., Arslan, H., *et al*. 2009. A multinational survey of risk factors for infection with extended-spectrum β-lactamase-producing Enterobacteriaceae in nonhospitalized patients. *Clinical Infectious Diseases.* 49:682-690.
21. Reuland, A.E., Overdevest, I.T.M.A., al Naiemi, N., *et al.* 2012. High prevalence of ESBL-producing Enterobacteriaceae carriage in Dutch community patients with gastrointestinal complaints. *Clinical Microbiology and Infection.* 19:542-549.

22. Van Bunnik, B.A.D., Ssematimba, A., Hagenaars, T.J., *et al*. 2014. Small distances can keep bacteria at bay for days. *PNAS.* 111:3556-3560.
23. Valverde, A., Grill, F., Coque, T.M., *et al*. 2008. High rate of intestinal colonization with extended-spectrum-β-lactamase-producing organisms in household contacts of infected community patients. *Journal of Clinical Microbiology.* 46:2796-2799.

Supplementary Table. Characteristics of β-lactamase genes and plasmids present in *E. coli* isolates from broilers, farmers, family members and employees.

Farm	Isolate	Source	Phylogenetic group	*E. coli* ST	Clonal complex	β-lactamase genes (PCR and sequencing) [a,b]	Replicon type of plasmid with ESBL/AmpC gene
04	HR1_1	Farmer	D_2	648		*bla*$_{SHV-12}$	incB/O
	C02_1	Broiler	A_1			*bla*$_{TEM-52}$	incX1
	C05_3	Broiler	D_2			*bla*$_{CTX-M-1}$; *bla*$_{TEM-1b}$	incI1
	C06_1	Broiler	D_2			*bla*$_{SHV-12}$	incI1
	C09_1	Broiler	D_2	648		*bla*$_{SHV-12}$	incI1
	C10_1	Broiler	D_2			*bla*$_{SHV-12}$	incI1
05	HR1_1	Farmer	$B1_1$			*bla*$_{TEM-52}$	incX1
	HR1_3	Farmer	$B1_1$			*bla*$_{TEM-52}$	
	HR1_5	Farmer	$B1_1$			*bla*$_{TEM-52}$	
	C01_1	Broiler	$B1_1$			*bla*$_{TEM-1b}$	
	C04_2	Broiler	A_0			*bla*$_{TEM-52}$	
	C05_1	Broiler	A_0			*bla*$_{CMY-2}$	incFIB
	C06_1	Broiler	$B1_1$			*bla*$_{TEM-52}$; *bla*$_{TEM-1b}$	
	C07_1	Broiler	A_1			*bla*$_{SHV-12}$	incI1
	C10_2	Broiler	A_0			*bla*$_{CTX-M-1}$	incI1
07	HR1_1	Farmer	D_2	117		*bla*$_{SHV-12}$	incI1
	C02_1	Broiler	D_2	117		*bla*$_{SHV-12}$	incFII
	C08_1	Broiler	D_1			*bla*$_{TEM-52}$; *bla*$_{TEM-1b}$	incI1
	C08_2	Broiler	A_0			*bla*$_{CTX-M-1}$	incI1
08	HR1_1	Farmer	A_1			*bla*$_{CMY-2}$	incK
	HR1_5	Farmer	$B2_3$			*bla*$_{CTX-M-1}$; *bla*$_{TEM-1b}$	incI1
	GR1_3	Family	$B1_1$	351		*bla*$_{CMY-2}$; *bla*$_{TEM-1b}$	incI1
	C02_1	Broiler	$B1_1$	351		*bla*$_{CMY-2}$	incI1
	C04_1	Broiler	$B1_1$			*bla*$_{CTX-M-1}$	
	C05_1	Broiler	A_0			*bla*$_{CMY-2}$	incA/C
	C06_1	Broiler	$B1_1$			*bla*$_{CTX-M-1}$; *bla*$_{TEM-1b}$	incI1
	C07_1	Broiler	$B1_1$			*bla*$_{CTX-M-1}$	
	C09_1	Broiler	D_2			*bla*$_{SHV-12}$	incI1
09	MR1_3	Employee	$B2_2$			*bla*$_{CMY-2}$	incK
	C02_1	Broiler	D_2			*bla*$_{SHV-12}$	
	C04_1	Broiler	$B1_1$			*bla*$_{CMY-2}$	
	C06_1	Broiler	$B2_3$			*bla*$_{CTX-M-1}$	

Supplementary Table. Continued

Farm	Isolate	Source	Phylogenetic group	*E. coli* ST	Clonal complex	β-lactamase genes (PCR and sequencing) [a,b]	Replicon type of plasmid with ESBL/ AmpC gene
10	HR1_3	Farmer	A_0	93	ST168	bla_{SHV-12}; bla_{TEM-1b}	incN
	HR1_5	Farmer	A_0			bla_{SHV-12}; bla_{TEM-1b}	
	GR2_1	Family	A_0	399	ST399	$bla_{CTX-M-1}$	incI1
	GR2_5	Family	A_1			$bla_{CTX-M-1}$	
	C02_1	Broiler	A_0	189	ST165	$bla_{CTX-M-1}$; bla_{TEM-1b}	incI1
	C03_1	Broiler	D_1			bla_{TEM-52}; bla_{TEM-1b}	
	C06_1	Broiler	D_2			$bla_{CTX-M-1}$; bla_{SHV-12}	
	C07_1	Broiler	$B1_1$			$bla_{CTX-M-14}$	incI1
	C08_1	Broiler	D_2			bla_{SHV-12}; bla_{TEM-1b}	
	C08_2	Broiler	$B1_1$			bla_{SHV-12}	
11	MR1_4	Employee	$B1_1$			$bla_{CTX-M-2}$; bla_{TEM-1b}	incI1
	C01_1	Broiler	A_1			$bla_{CTX-M-1}$	
	C03_1	Broiler	D_2			bla_{SHV-12}; bla_{TEM-1b}	incI1
	C03_2	Broiler	A_0			$bla_{CTX-M-1}$	
	C08_1	Broiler	A_1			$bla_{CTX-M-1}$	incI1
	C10_1	Broiler	A_1			$bla_{CTX-M-1}$	
12	HR1_1	Farmer	$B2_3$	131		bla_{CMY-2}	incI1
	HR1_3	Farmer	$B1_1$	641	ST86	bla_{CMY-2}	
	HR1_5	Farmer	D_2	NEW[c]		$bla_{CTX-M-1}$	incI1
	C01_2	Broiler	D_2	1640		$bla_{CTX-M-1}$; bla_{TEM-1b}	incI1
	C02_1	Broiler	A_0			bla_{CMY-2}	incI1
	C04_1	Broiler	A_0			bla_{TEM-1b}	
	C06_1	Broiler	$B1_1$			bla_{CMY-2}	
	C08_1	Broiler	$B1_1$	1146		bla_{CMY-2}	
	C09_2	Broiler	D_2	1775		bla_{CMY-2}; bla_{TEM-1b}	incI1
	C09_3	Broiler	A_0			$bla_{CTX-M-1}$	
	C10_1	Broiler	$B2_3$	355		bla_{CMY-2}	
15	HR1_3	Farmer	D_2	117		bla_{CMY-2}	incI1
	GR1_4	Family	D_2	117		bla_{CMY-2}	incI1
	C02_2	Broiler	D_2			bla_{CMY-2}	
	C03_1	Broiler	D_2	57	ST35	bla_{CMY-2}	incK
	C08_1	Broiler	A_0			bla_{CMY-2}	
16	HR1_1	Farmer	A_1			$bla_{CTX-M-1}$; bla_{TEM-1b}	
	HR1_3	Farmer	A_1	48	ST10	$bla_{CTX-M-1}$; bla_{TEM-1b}	incI1
	HR1_5	Farmer	A_1			$bla_{CTX-M-1}$; bla_{TEM-1b}	
	C02_1	Broiler	$B1_1$			$bla_{CTX-M-1}$; bla_{TEM-1b}	incI1

Supplementary Table. Continued

Farm	Isolate	Source	Phylogenetic group	*E. coli* ST	Clonal complex	β-lactamase genes (PCR and sequencing) [a,b]	Replicon type of plasmid with ESBL/ AmpC gene
	C03_1	Broiler	A_1	10	ST10	*bla*$_{CTX-M-1}$	incI1
	C04_1	Broiler	$B1_1$			*bla*$_{CTX-M-1}$	
	C05_1	Broiler	A_0			*bla*$_{CMY-2}$	incK
	C06_1	Broiler	A_1			*bla*$_{CTX-M-2}$; *bla*$_{TEM-1b}$	
	C07_1	Broiler	A_1			*bla*$_{CTX-M-2}$	incHI2_incP
	C07_2	Broiler	$B1_1$			*bla*$_{CTX-M-1}$; *bla*$_{CTX-M-9}$	
	C08_1	Broiler	A_0			*bla*$_{CMY-2}$	
17	GR2_1	Family	A_0			*bla*$_{CMY-2}$; *bla*$_{TEM-1b}$	
	GR3_1	Family	D_1			*bla*$_{SHV-12}$	incI1
	GR3_3	Family	D_1			*bla*$_{SHV-12}$	
	C02_1	Broiler	D_2			*bla*$_{CTX-M-32}$; *bla*$_{TEM-1b}$	incX1
	C03_1	Broiler	D_2			*bla*$_{SHV-12}$	
	C05_1	Broiler	D_2			*bla*$_{SHV-12}$	incX1
	C06_1	Broiler	D_2			*bla*$_{SHV-12}$	incX1
	C07_1	Broiler	D_1			*bla*$_{CMY-2}$	
	C08_1	Broiler	D_2			*bla*$_{CMY-2}$	
	C09_1	Broiler	D_2			*bla*$_{SHV-12}$	
	C10_1	Broiler	$B1_1$			*bla*$_{CTX-M-1}$	incI1
18	GR1_1	Family	D_1			*bla*$_{CMY-2}$	incK
	C03_1	Broiler	D_2			*bla*$_{CMY-2}$	
	C04_1	Broiler	A_1			*bla*$_{CMY-2}$	
	C06_1	Broiler	D_2			*bla*$_{CMY-2}$	incK
	C10_1	Broiler	D_2			*bla*$_{CMY-2}$	
20	GR2_1	Family	A_1	10	ST10	*bla*$_{CTX-M-1}$	incI1
	C04_1	Broiler	A_1	10	ST10	*bla*$_{CTX-M-1}$	incI1
	C09_1	Broiler	A_1			*bla*$_{SHV-12}$	incN
	C10_1	Broiler	$B1_1$			*bla*$_{CTX-M-1}$	incI1
22	GR1_1	Family	$B1_1$			AmpC type 3 promotor mutant[d]	
	C08_1	Broiler	D_1			*bla*$_{CMY-2}$	
25	GR2_1	Family	A_0	373	ST168	*bla*$_{CMY-2}$	incK
	GR2_4	Family	A_0	1818		*bla*$_{TEM-52}$	incX1
	GR2_5	Family	D_2			*bla*$_{CMY-2}$	
	C01_1	Broiler	A_0	1818		*bla*$_{TEM-52}$	incX1
	C03_1	Broiler	A_0	373	ST168	*bla*$_{CMY-2}$	incK
	C05_1	Broiler	D_2	38	ST38	*bla*$_{CMY-2}$; *bla*$_{TEM-1b}$	incK
	C06_1	Broiler	$B2_3$			*bla*$_{CMY-2}$	

Supplementary Table. Continued

Farm	Isolate	Source	Phylogenetic group	*E. coli* ST	Clonal complex	β-lactamase genes (PCR and sequencing) [a,b]	Replicon type of plasmid with ESBL/ AmpC gene
27	HR1_1	Farmer	A_1	88	ST23	*bla*$_{CTX-M-1}$	incI1
	HR1_5	Farmer	A_1	88	ST23	*bla*$_{CTX-M-1}$	incI1
	C01_1	Broiler	$B1_1$			*bla*$_{TEM-52}$; *bla*$_{TEM-1b}$	
	C06_1	Broiler	A_1	88	ST23	*bla*$_{CTX-M-1}$	incI1
	C08_1	Broiler	A_0	2223		*bla*$_{CTX-M-1}$	incI1
28	HR1_1	Farmer	A_0	1324		*bla*$_{CTX-M-1}$	incI1
	HR1_4	Farmer	A_0			*bla*$_{CTX-M-1}$	
	C03_1	Broiler	$B1_1$			*bla*$_{CTX-M-1}$; *bla*$_{TEM-1b}$	incI1
	C09_1	Broiler	A_0	641	ST86	*bla*$_{CTX-M-1}$	
31	GR4_5	Family	D_1			*bla*$_{CMY-2}$; *bla*$_{TEM-1b}$	
	C03_1	Broiler	$B2_3$			*bla*$_{CMY-2}$	
	C05_1	Broiler	D_2			*bla*$_{CMY-2}$	
	C06_1	Broiler	D_2			*bla*$_{CMY-2}$	
	C07_1	Broiler	$B2_3$			*bla*$_{CMY-2}$	
37	HR1_1	Farmer	A_0			*bla*$_{SHV-12}$; *bla*$_{TEM-1b}$	non-typable
	HR1_5	Farmer	A_0			*bla*$_{SHV-12}$; *bla*$_{TEM-1b}$	
	GR2_1	Family	A_1	23	ST23	*bla*$_{SHV-12}$; *bla*$_{TEM-1b}$	incI1
	GR2_3	Family	$B2_2$			*bla*$_{SHV-12}$; *bla*$_{TEM-1b}$	
	GR3_2	Family	D_2	1163		*bla*$_{CMY-2}$; *bla*$_{TEM-1b}$	incI1
	C03_1	Broiler	A_0			*bla*$_{CTX-M-1}$	incI1
	C05_1	Broiler	A_0	2509		*bla*$_{TEM-52}$; *bla*$_{TEM-1b}$	
	C07_1	Broiler	D_2	57	ST350	*bla*$_{CMY-2}$; *bla*$_{TEM-1b}$	incK
40	GR4_2	Family	$B1_1$			*bla*$_{CMY-2}$; *bla*$_{TEM-1b}$	
	C04_1	Broiler	$B2_3$			*bla*$_{CMY-2}$	
	C06_1	Broiler	$B2_3$			*bla*$_{CMY-2}$	
	C09_1	Broiler	$B2_3$			*bla*$_{CMY-2}$	
46	HR1_3	Farmer	D_2			AmpC type 4 promotor mutant[e]; *bla*$_{TEM-1b}$	
	C05_1	Broiler	D_2			*bla*$_{CMY-2}$	
	C08_1	Broiler	D_2			*bla*$_{CMY-2}$	

[a] With the primers used in this study no distinction was made between TEM-1b and TEM-198, however for readability of the Table, TEM-1b was inserted.
[b] One TEM-52 isolate was found with 1 mutation (C228T) and 11 TEM-52 isolates were found with 3 mutations in this gene (C18T, C228T, G396T). All were synonymous mutations.
[c] MLST results: adk35, fumC3, gyrB234, icd342 mdh45, purA5, recA95.
[d] Positions of mutations in AmpC promoter region: −42T−18A−1T(+23A+51T)+58T+81G.
[e] Positions of mutations in AmpC promoter region: +22T+26G+27T+32A+70T+81G.

Chapter 5

Methicillin-resistant *Staphylococcus aureus* and extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* in broilers and in people living and/or working on organic broiler farms

Patricia M.C. Huijbers
Angela H.A.M. van Hoek
Elisabeth A.M. Graat
Anja P.J. Haenen
Alice Florijn
Paul D. Hengeveld
Engeline van Duijkeren

Veterinary Microbiology (2014) 176:120-125.

Abstract

The aim of this study was to estimate the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) and extended-spectrum and AmpC β-lactamase (ESBL/AmpC)-producing *Escherichia coli* among broilers, and humans living and/or working on organic broiler farms; further characterise isolates; and compare these results with those from conventional farms.

In the Netherlands, only 9 certified organic broiler farms were present. On 8 of these farms, 60 throat swabs and 20 cloacal swabs were taken per farm for MRSA and ESBL/AmpC-*E. coli* detection, respectively, at an average age of both 34 (T1) and 68 (T2) days. Faecal swabs and questionnaires were returned by 27 out of 36 humans. For selected ESBL/AmpC-producing *E. coli* isolates, phylogenetic groups, β-lactamase genes, plasmid families and sequence types were determined.

MRSA was not detected in broiler and human samples. ESBL/AmpC-producing *E. coli* were isolated from broilers on 7/8 farms at T1 and on all farms at T2. Furthermore, 3 farmers at T1, and 2 farmers and 1 family member at T2 were positive. Genes found in broilers and humans were almost exclusively $bla_{\text{CTX-M-1}}$ and $bla_{\text{CMY-2}}$. Given the high overall human ESBL/AmpC-prevalence (18.5%), which is similar to conventional farms, contact with live broilers is assumed a risk factor for carriage. Farm and sample-level prevalence at T1 are consistent with those from conventional farms. At T2, just before slaughter, sample-level prevalence of ESBL/AmpC-*E.coli* appears to have decreased (94.3 vs. 80%), which could have important consequences for contamination of retail meat.

Introduction

Human infections with methicillin-resistant *Staphylococcus aureus* (MRSA), and extended-spectrum and AmpC β-lactamase (ESBL/AmpC)-producing *Escherichia coli* have both been associated with reservoirs in livestock [1,2]. This raises a public health concern as transmission could occur between animals and humans. Livestock-associated (LA)-MRSA and ESBL/AmpC-producing *E. coli* have been found in broilers, and in individuals working and/or living on conventional Dutch broiler farms [3,4,5]. The prevalences of MRSA and ESBL/AmpC-producing *E. coli* are higher in humans on these farms than in the community [4,5,6,7], and contact with live broilers has been identified as a risk factor for human carriage [5,8].

In comparison to conventional farms, organic broiler farms have lower flock densities, different breeds, restrictions in antimicrobial use, availability of outdoor areas and later slaughter age. These factors could lead to a lower prevalence of MRSA and ESBL/AmpC-producing *E. coli* among broilers on organic compared to conventional farms and might also affect carriage of humans. There are currently no studies investigating carriage of MRSA and ESBL/AmpC-producing *E. coli* among humans and broilers on organic farms. The aim of this study was to estimate the prevalence of MRSA and ESBL/AmpC-producing *E. coli* among broilers and humans living and/or working on organic broiler farms; further characterise isolates from humans and broilers; and compare these results with those from conventional farms.

Material and methods

Study population

From October 2011 to April 2012 a prevalence study for MRSA and ESBL/AmpC-producing *E. coli* was conducted on 8 out of 9 certified organic broiler farms in the Netherlands. Certified organic broiler farms are those that comply with legal regulations laid down by the European Commission. Chapter 2 of Commission Regulation No 889/2008 describes production rules for different livestock species, including broilers [9]. Participating farms had a median of 1.5 broiler houses (range 1-22) and the median number of broilers per farm was 4500 (range 4000-6900). The human study population consisted of broiler farmers, their family members working and/or living on the farm and employees working on the farm.

Sample collection and questionnaires

Broiler samples were collected by one employee of the Animal Health Service according to Geenen *et al.* [4] for MRSA and according to Huijbers *et al.* [5] for ESBL/AmpC-producing *E. coli* at two different times (T1 and T2) during the production cycle. Broiler age was on average

34 (range 24-48) days at T1 and 68 (range 62-69) days at T2. T1 was chosen so that results could be compared to the studies on conventional farms where broilers were sampled at an average age of 31 days [4,5]. As organic broilers have a later slaughter age, T2 was also selected to assess the situation just before slaughter. A randomly chosen flock was sampled for MRSA by taking throat swabs from 60 broilers (pooled to five samples), and for ESBL/AmpC-producing *E. coli* by taking cloacal swabs from 20 broilers (pooled to 10 samples). The study was performed according to Dutch law on studies with animals. In addition, for MRSA-detection, five environmental wipes were taken from all broiler houses on the farm, and five environmental wipes were taken from the farm residence (*i.e.,* favourite armchair, TV remote control, inside and outside door handles and the favourite pet).

Human samples were also collected at T1 and T2. People who voluntarily participated in the study took a nose swab for MRSA-detection and a faecal swab for detection of ESBL/AmpC-producing *E. coli*, as described by Geenen *et al.* [4] and Huijbers *et al*. [5], respectively. All participants signed a written informed consent and for children aged <18 years parental consent was requested. In addition, a questionnaire about farm management was completed by farmers, and a separate questionnaire on lifestyle and health characteristics was completed by farmers, family members and employees.

Microbiological examination

MRSA was isolated by incubation in Mueller-Hinton enrichment broth with 6.5% NaCl, followed by selective enrichment (broiler and environmental samples) or plating on ChromID MRSA plates (human samples), and plating on Columbia agar with 5% sheep blood and Brilliance MRSA agar [4]. A farm was classified as MRSA-positive if at least one broiler sample or environmental wipe tested positive for MRSA at T1 or T2. ESBL/AmpC-producing Enterobacteriaceae were isolated by selective enrichment and culture on selective plates. ESBL/AmpC *E. coli*-like isolates were confirmed phenotypically by combination disc diffusion test, including a cefoxitin disc for the detection of AmpC phenotypes [5]. Further molecular characteristics, *i.e.*, phylogenetic group and ESBL/AmpC-gene were determined for one or two human isolates and a minimum of four (range 4-9) broiler isolates per farm. On farms with positive humans, multilocus sequence typing (MLST) and PCR-based replicon typing were performed on a minimum of one human isolate and at least one broiler isolate if they showed the same phylogenetic group and ESBL/AmpC-gene [5]. MLST was also performed on isolates from broilers and humans showing the $B2_3$ or D phylogenetic groups because virulent extra-intestinal strains of *E. coli* mainly belong to these groups [10]. A farm was classified as ESBL/AmpC-positive if at least one broiler sample tested positive for ESBL/AmpC-producing *E. coli* at T1 or T2.

Statistical analysis

Prevalences and their exact 95% confidence intervals (95% CI) were calculated based on the binomial distribution. In order to compare organic and conventional farms, data from Geenen *et al.* [4] and Huijbers *et al.* [5] with respect to the presence of MRSA or ESBL/AmpC-producing *E. coli* on farms, among broiler samples and humans were merged with data from the current study. These studies were done by the same institute and were comparable in design. Exact logistic regression was used to investigate the association between type of farm (*i.e.*, organic vs. conventional farms) and the presence of MRSA or ESBL/AmpC-producing *E. coli*. Multiple observations on the same farm might not be independent, so a random farm effect was included using an exchangeable covariance structure, and explained 36.5% and 2.0% of all non-explained variation in the models for broilers and humans, respectively.

Results and discussion

Descriptive statistics

In total, 27 out of 36 individuals agreed to participate. These participants included 9 farmers, 16 family members and two employees. Per farm, this ranged from two to seven individuals, with an average age of 40.6 years (range 4-76 years).

MRSA-carriage among broilers and humans

MRSA was not detected in all 80 pooled throat swabs and 120 environmental wipes, so all farms were classified MRSA-negative. The prevalence of positive organic farms (0/8) was not statistically different (P=1.0) from conventional farms (4/50; [4]). On-farm antimicrobial treatment has been associated with antimicrobial resistance [11]. Since antimicrobial consumption is lower on organic farms, it was hypothesised that both prevalence of positive organic farms as well as within-flock prevalence would be lower. Due to the small number of organic broiler farms in the Netherlands this cannot be determined at the national level, however. MRSA was also not detected in 27 samples from humans (0%; 95% CI 0.0-12.8), and in 75 environmental wipes taken from the farm residence (0%; 95% CI 0.0-4.8). Since prevalences were not different from those from conventional farms, risk factors for carriage of MRSA among humans living and/or working on organic and conventional farms are assumed to be comparable.

ESBL/AmpC-carriage among broilers and humans

All farms were ESBL/AmpC-positive, although one farm only tested ESBL/AmpC-positive at T2 (Farm 8). Possibly, the number of ESBL/AmpC-producing *E. coli* was below detection limit, or the longer vacancy between production cycles compared to other farms (25 vs.

median 10 days) might have resulted in a delay in farm contamination. The prevalence of positive farms at T1 (7/8) was not significantly different (P=0.28) from conventional farms (50/50; [5]). This was not expected considering the lower consumption of antimicrobials on organic broiler farms. On positive farms, the prevalence of ESBL/AmpC-producing *E. coli* at the sample-level (66/70=94.3%) at T1 was not different from conventional farms (96.4%; P=0.57). To the authors' knowledge there are no other studies comparing farm- and sample-level prevalences of ESBL/AmpC-producing *E. coli* between organic and conventional broiler farms. Schwaiger *et al.* [12] showed lower resistance rates for several β-lactam antibiotics, including second generation cephalosporins, in *E. coli* isolates from organically reared laying hens compared to conventionally reared laying hens. It should be noted however, that all *E. coli* isolates were sensitive to cefepime, cefotaxime and ceftazidime and ceftiofur, and they were not tested for ESBL/AmpC-production. On positive organic farms the prevalence of ESBL/AmpC-producing *E. coli* at the sample-level decreases from 94.3% to 80.0% (64/80; 95% CI 69.6-88.1) at T2 (P=0.08). It can be speculated that fewer positive animals enter the slaughterhouse, potentially leading to retail meat that is less contaminated. This is supported by Cohen-Stuart *et al.* [13] who found that the prevalence of ESBL-producing bacteria was lower among organic (84%) compared to conventional (100%) retail meat samples in the Netherlands, and that the median bacterial load on organic samples was lower than on conventional ones. The decrease might also hold true for broilers on conventional farms if they would have a longer production cycle, but at their usual slaughter age of 6 weeks, a very high percentage of positive broilers enter the slaughterhouse.

ESBL/AmpC-genes and phylogenetic groups were determined in 49 isolates from broilers (Table 1). The genes found in broilers were almost exclusively $bla_{\text{CTX-M-1}}$ (n=31) and $bla_{\text{CMY-2}}$ (n=17). In addition, an isolate harbouring $bla_{\text{TEM-52}}$ (n=1) was found. The genes $bla_{\text{CTX-M-1}}$ and $bla_{\text{CMY-2}}$ were also highly prevalent on conventional farms in the Netherlands [3,5]. Interestingly $bla_{\text{SHV-12}}$, which was frequently identified in broilers on conventional farms, was not found in the present study [3,5]. This might be explained by the small number of organic farms, but could also be due to the different broiler breeds or hatcheries associated with organic compared to conventional broiler production. The latter two factors have previously been shown to influence the occurrence of antimicrobial resistance on conventional broiler farms [11]. The predominant phylogenetic group was $B1_1$ (n=15), but $B2_3$ (n=9), D_2 (n=10), A_0 (n=7), D_1 (n=4) and A_1 (n=4) were also found. On conventional farms there was a similar distribution of phylogenetic groups [5]. Plasmid families were determined for 3 broiler isolates, which showed $bla_{\text{CMY-2}}$ located on IncI1, $bla_{\text{CMY-2}}$ on IncK, and $bla_{\text{CTX-M-1}}$ on IncI1. MLST of isolates from phylogenetic groups $B2_3$ yielded two different sequence types: ST131 (n=5) and ST429 (n=2). Group D_1 isolates showed ST69 (n=2) and unknown sequence types (n=2), and group D_2 showed ST93 (n=2), ST117 (n=2), ST648 (n=2), ST155 (n=1) and ST569 (n=1). *E. coli* with ST131, commonly harbouring $bla_{\text{CTX-M-15}}$, has been recognised as a pandemic clone responsible

for extra-intestinal infections in humans [14]. In the current study, isolates with ST131 were found in broilers, but these harboured $bla_{CTX\text{-}M\text{-}1}$ and $bla_{CMY\text{-}2}$ instead of $bla_{CTX\text{-}M\text{-}15}$. Reports of *E. coli* ST131 harbouring ESBL/AmpC-genes in animals are few [14], however two other studies have shown a single ST131 isolate harbouring $bla_{CTX\text{-}M\text{-}1}$ in a wild seagull and a domestic pig [15,16]. This is a potentially worrisome development given the global success of ST131 [14].

This is the first study investigating carriage of ESBL/AmpC-producing *E. coli* among humans on organic broiler farms. Overall, 18.5% (95% CI 6.3-38.1) of humans living and/or working on four broiler farms were ESBL/AmpC-positive, which is higher than in the general Dutch population (5.1%; [7]). At T1, positive humans were present on 37.5% of farms, which is similar to the percentage on conventional farms (42.0%, P=1.0). The prevalence of positive humans at T1 (three humans from three different farms) on organic broiler farms (11.1%; 95% CI 2.4-29.2) was also comparable (P=0.22) to conventional broiler farms (19.1%, [5]). Given the similarities in prevalence, risk factors are assumed to be comparable to those found for conventional farms. There, an increased risk of carriage was shown among individuals having a high degree of contact with live broilers [5].

ESBL/AmpC-genes identified in humans were $bla_{CMY\text{-}2}$ (n=3), $bla_{CTX\text{-}M\text{-}1}$ (n=1), and promotor mutants (n=1). Similar to studies on conventional farms, $bla_{CTX\text{-}M\text{-}15,}$ the most predominant gene found in humans in the Netherlands [12], was not found in humans on organic farms. It appears that the presence of ESBL/AmpC-producing *E. coli* in humans can be both persistent and intermittent, as only one farmer was positive at both T1 and T2. This individual had the same gene ($bla_{CMY\text{-}2}$), located on the same plasmid family (IncI1), in an *E. coli* with the same ST (ST131) at both sampling times. Persistent carriage has been shown before in a longitudinal study among travellers who were positive for ESBL-producing Enterobacteriaceae up to 6 months post-travel [18]. In contrast to Huijbers *et al.* [5], clonal transfer of ESBL/AmpC-producing *E. coli* between humans and broilers was not shown. This might be related to the low number of organic broiler farms existing in the Netherlands, resulting in fewer isolates from broilers and humans and a decreased chance of observing clonal transfer. Dierikx *et al.* [3] also did not find evidence of clonal transfer on 26 conventional farms, however. Horizontal transfer was suspected as similar ESBL/AmpC-genes and plasmid families were found among broiler and human isolates in both the current study and the studies by Dierikx *et al*. [3] and Huijbers *et al*. [5]. On one farm, a farmer and a family member showed the same *E. coli* ST (ST95) with $bla_{CMY\text{-}2}$ on an IncI1 plasmid at different sampling times. The family member reported no contact with live broilers and rarely entered the broiler house, therefore it can be speculated that transmission occurred between family members. However, transmission via the farm environment, which has been previously shown to be contaminated with ESBL/AmpC-producing *E. coli* [19], should also be considered. MLST of isolates from phylogenetic groups $B2_3$ yielded two different sequence types: ST131 (n=2) and ST95 (n=2). The isolates with ST131 harboured $bla_{CMY\text{-}2}$ as shown in broilers, but these were not animals from the

same farm. Group D_2 isolates showed ST117 (n=1). *E. coli* ST117, found both in broilers and humans in the current study, has been previously associated with hospitalised patients and retail chicken meat [17]. Unfortunately the authors do not specify whether this is meat originating from chickens raised on organic or conventional farms. Zoonotic potential has been suggested for *E. coli* with ST95, ST117 and ST131 as both avian pathogenic (APEC) and human extra-intestinal (ExPEC) *E. coli* strains have been shown to belong to these clonal groups [20,21]. Furthermore, similarity in virulence traits, and the ability of certain APEC and human ExPEC strains to cause disease in a murine and avian models, respectively, lend support to this hypothesis [21].

Table 1. Characteristics of β-lactamase genes and plasmid families present in *E. coli* isolates from broilers, farmers, family members and employees at sampling times T1 and T2.

Farm_ time	Source	Phylogenetic group	*E. coli* ST	Clonal complex	β-lactamase genes (PCR and sequencing)	Replicon type of plasmid with ESBL/ AmpC-gene[f]
1_T1	**Farmer**	A_1			Promotor mutant[a]	
	Farmer	$B2_2$			Promotor mutant[a]	
	Broiler	$B1_1$			bla_{CMY-2}	
	Broiler	$B2_3$	131		$bla_{CTX-M-1}$	
	Broiler	$B1_1$			$bla_{CTX-M-1}$	
	Broiler	$B1_1$			bla_{CMY-2}	
1_T2	Broiler	$B1_1$			$bla_{CTX-M-1}$	
	Broiler	$B1_1$			$bla_{CTX-M-1}$	
	Broiler	$B1_1$			bla_{CMY-2}	
2_T1	Broiler	D_2	117		bla_{CMY-2}	
	Broiler	$B2_3$	131		$bla_{CTX-M-1}$	
	Broiler	$B1_1$			$bla_{CTX-M-1}$	
2_T2	Broiler	$B1_1$			$bla_{CTX-M-1}$	
	Broiler	D_2	117		bla_{CMY-2}	
	Broiler	$B2_3$	131		$bla_{CTX-M-1}$	
3_T1[b]	Broiler	D_2			$bla_{CTX-M-1}$	
	Broiler	A_0	93	ST168	$bla_{CTX-M-1}$	
	Broiler	D_2	93	ST168	$bla_{CTX-M-1}$	
	Broiler	D_2	648		$bla_{CTX-M-1}$	
	Broiler	D_2			$bla_{CTX-M-1}$	
3_T2	**Farmer**	D_2	117		$bla_{CTX-M-1}$	NT
	Farmer	A_0	1818		$bla_{CTX-M-1}$	
	Broiler	A_1			$bla_{CTX-M-1}$	
	Broiler	D_2	93	ST168	$bla_{CTX-M-1}$	
	Broiler	D_2	648		$bla_{CTX-M-1}$	incI1

Table 1. Continued

Farm_time	Source	Phylogenetic group	*E. coli* ST	Clonal complex	β-lactamase genes (PCR and sequencing)	Replicon type of plasmid with ESBL/AmpC-gene[f]
4_T1	**Farmer**	$B2_3$	131		bla_{CMY-2}	incI1
	Broiler	$B1_1$			$bla_{CTX-M-1}$	
	Broiler	A_0			bla_{CMY-2}	incI1
4_T2	**Farmer**	$B2_3$	131		bla_{CMY-2}	incI1
	Broiler	A_0			bla_{CMY-2}[d]	
	Broiler	A_0			bla_{CMY-2}	NT
	Broiler	A_0			bla_{CMY-2}	
	Broiler	D_1	Unknown[c1]		bla_{CMY-2}	NT
5_T1	Broiler	$B1_1$			$bla_{CTX-M-1}$	
	Broiler	$B1_1$			$bla_{CTX-M-1}$	
	Broiler	$B2_3$	429		bla_{CMY-2}	
5_T2	Broiler	$B1_1$			$bla_{CTX-M-1}$	
	Broiler	$B2_3$	429		bla_{CMY-2}	
6_T1	**Farmer**	$B2_3$	95	ST95	bla_{CMY-2}	incK
	Broiler	A_1			$bla_{CTX-M-1}$; bla_{TEM-1b}[e]	
	Broiler	$B1_1$			$bla_{CTX-M-1}$	
	Broiler	A_1			$bla_{CTX-M-1}$	
6_T2	**Family**	$B2_3$	95	ST95	bla_{CMY-2}	incK
	Broiler	$B2_3$	131		bla_{CMY-2}	
	Broiler	$B2_3$			$bla_{CTX-M-1}$	
	Broiler	A_1			$bla_{CTX-M-1}$	
	Broiler	$B2_3$	131		bla_{CMY-2}	incK
	Broiler	A_0			$bla_{CTX-M-1}$	
7_T1	Broiler	$B1_1$			$bla_{CTX-M-1}$	
	Broiler	A_0			$bla_{CTX-M-1}$; bla_{TEM-1b}[e]	
	Broiler	$B1_1$			$bla_{CTX-M-1}$	
7_T2	Broiler	D_1	Unknown[c2]		$bla_{CTX-M-1}$[d] 1mm	
	Broiler	D_2	155		$bla_{CTX-M-1}$	
8_T2	Broiler	$B2_3$			bla_{TEM-52}[d] 1mm	
	Broiler	D_2	569		bla_{CMY-2}	
	Broiler	D_1	69		bla_{CMY-2}	
	Broiler	D_1	69		bla_{CMY-2}	

[a] Positions of mutations in AmpC promoter region: −42T−18A−1T+58T+81G.
[b] Farmer reported treatment of an unspecified bacterial infection with antibiotics during the production cycle.
[c1] MLST results: adk6, fumC23, gyrB44, icd11, mdh361_1mm, purA239, recA7 (mm = mismatches). [c2] MLST results: adk337_2mm, fumC100_5mm, gyrB266_2mm, icd158, mdh118, purA16_1mm, recA107.
[d] One CMY-2 isolate has a non-synonymous mutation; A736T (S246C). In addition, one CTX-M-1 isolate was found with a mutation (C741T) and one TEM-52 isolate had a mutation (C228T). Both are synonymous mutations.
[e] With the primers used in this study no distinction was made between TEM-1b and TEM-198, however for readability of the Table, TEM-1b was inserted.
[f] NT = Plasmid typing was unsuccessful, no transformants were obtained.

Conclusion

MRSA was not detected in broilers or humans on Dutch organic broiler farms. In contrast, ESBL/AmpC-producing *E. coli* were detected in broilers on all farms, with a prevalence of ≥80% at the sample-level and in 18.5% of humans. Given the higher prevalence of ESBL/AmpC-producing *E. coli* among humans living and/or working on organic farms compared to the general Dutch population and similarity to the prevalence in humans on conventional farms, contact with live broilers could also be a risk factor for carriage on organic farms. Farm- and sample-level prevalence of MRSA and ESBL/AmpC-producing *E. coli* in broilers at T1 are consistent with those from conventional farms. Sample-level prevalence of ESBL/AmpC-producing *E. coli* in broilers appears to have decreased at T2, however. Fewer positive animals entering the slaughterhouse might lead to retail meat that is less contaminated.

Acknowledgements

This work was financially supported by the Netherlands Food and Consumer Product Authority (NVWA, grant number V330091). We would like to thank all farmers, their family members and employees for participating in this study.

References

1. van Loo, I., Huijsdens, X.W., Tiemersma, E., *et al*. 2007. Emergence of methicillin-resistant *Staphylococcus aureus* of animal origin in humans. *Emerging Infectious Diseases*. 13:1834-1839.
2. Carattoli, A. 2008. Animal reservoirs for extended spectrum β-lactamase producers. *Clinical Microbiology and Infection*. 14:117-123.
3. Dierikx, C., van der Goot, J., Fabri, T., *et al*. 2013. Extended-spectrum-β-lactamase-and AmpC-β-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers. *Journal of Antimicrobial Chemotherapy*. 68:60-67.
4. Geenen, P.L., Graat, E.A.M., Haenen, A., *et al*. 2013. Prevalence of livestock-associated MRSA on Dutch broiler farms and in people living and/or working on these farms. *Epidemiology and Infection*. 141:1099-1108.
5. Huijbers, P.M.C., Graat, E.A.M., Haenen, A.P.J., *et al*. 2014. Extended-spectrum and AmpC beta-lactamase-producing *Escherichia coli* in broilers and people living and/or working on broiler farms: prevalence, risk factors and molecular characteristics. *Journal of Antimicrobial Chemotherapy*. 69:2669-2675.
6. Bode, L.G.M., Wertheim, H.F.L., Kluytmans, J.A.J.W., *et al.* 2011. Sustained low prevalence of methicillin-resistant *Staphylococcus aureus* upon admission to hospital in The Netherlands. *Journal of Hospital Infection*. 79:198-201.
7. Huijbers, P.M.C., de Kraker, M., Graat, E.A.M., *et al*. 2013. Prevalence of extended-spectrum β-lactamase-producing Enterobacteriaceae in humans living in municipalities with high and low broiler density. *Clinical Microbiology and Infection*. 19:E256-E259.
8. Mulders, M.N., Haenen, A.P.J., Geenen, P.L., *et al*. 2010. Prevalence of livestock-associated MRSA in broiler flocks and risk factors for slaughterhouse personnel in The Netherlands. *Epidemiology and Infection*. 138:743-755.
9. European Commission, 2008. Commission Regulation (EC) No 889/2008 of 5 September 2008 laying down detailed rules for the implementation of Council Regulation (EC) No 834/2007 on organic production and labelling of organic products with regard to organic production, labelling and control. *Official Journal of the European Union*. L 250:1-84.
10. Picard, B., Garcia, J.S., Gouriou, S., *et al*. 1999. The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. *Infection and Immunity*. 67:546-553.
11. Persoons, D., Haesebrouck, F., Smet, A., *et al*. 2011. Risk factors for ceftiofur resistance in *Escherichia coli* from Belgian broilers. *Epidemiology and Infection*. 139:765-771.
12. Schwaiger, K., Schmied, E.M.V., Bauer, J. 2008. Comparative analysis of antibiotic resistance characteristics of Gram-negative bacteria isolated from laying hens and eggs in conventional and organic keeping systems in Bavaria, Germany. *Zoonoses and Public Health*. 55:331-341.
13. Cohen-Stuart, J., van den Munckhof, T., Voets, G., *et al*. 2012. Comparison of ESBL contamination in organic and conventional retail chicken meat. *International Journal of Food Microbiology*. 154:212-214.
14. Nicolas-Chanoine, M.H., Bertrand, X., Madec, J.Y. 2014. *Escherichia coli* ST131, an intriguing clonal group. *Clinical Microbiology Reviews*. 27:543-574.
15. Simões, R.R., Poirel, L., Da Costa, P.M., *et al*. 2010. Seagulls and beaches as reservoirs for multidrug-resistant *Escherichia coli*. *Emerging Infectious Diseases*. 16:110-112.
16. Schink, A.K., Kadlec, K., Kaspar, H., Mankertz, J., *et al*. 2013. Analysis of extended-spectrum-β-lactamase-producing *Escherichia coli* isolates collected in the GERM-Vet. Monitoring programme. *Journal of Antimicrobial Chemotherapy*. 68:1741-1749.
17. Overdevest, I., Willemsen, I., Rijnsburger, M., *et al*. 2011. Extended-spectrum β-lactamase genes of *Escherichia coli* in chicken meat and humans, the Netherlands. *Emerging Infectious Diseases*. 17:1216-1222.
18. Tängdén, T., Cars, O., Melhus, Å., *et al*. 2010. Foreign travel is a major risk factor for colonization with *Escherichia coli* producing CTX-M-type extended-spectrum β-lactamases: a prospective study with Swedish volunteers. *Antimicrobial Agents and Chemotherapy*. 54:3564-3568.
19. Laube, H., Friese, A., von Salviati, C., *et al*. 2014. Transmission of ESBL/AmpC-producing *Escherichia coli* from broiler chicken farms to surrounding areas. *Veterinary Microbiology*. 172:519-527.
20. Maluta, R.P., Logue, C.M., Casas, M.R.T., *et al*. 2014. Overlapped sequence types (STs) and serogroups of avian pathogenic (APEC) and human extra-intestinal pathogenic (ExPEC) *Escherichia coli* isolated in Brazil. *PLoS One*. 9:e105016.
21. Tivendale, K.A., Logue, C.M., Kariyawasam, S., *et al*. 2010. Avian-pathogenic *Escherichia coli* strains are similar to neonatal meningitis *E. coli* strains and are able to cause meningitis in the rat model of human disease. *Infection and Immunity*. 78:3412-3419.

Chapter 6

Transmission dynamics of extended-spectrum β-lactamase and AmpC β-lactamase-producing *Escherichia coli* in a broiler flock without antibiotic use

Patricia M.C. Huijbers
Elisabeth A.M. Graat
Angela H.A.M. van Hoek
Christiaan Veenman
Mart C.M. de Jong
Engeline van Duijkeren

Submitted.

Abstract

Extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* (ESBL/AmpC-EC) are found throughout the broiler production chain. Transmission of resistance between broilers and humans could occur at any point, representing a potential public health issue. Insight in farm transmission dynamics could provide a basis for control, leading to fewer contaminated broilers. The aim was to quantify transmission rates and routes of ESBL/AmpC-EC, and specific phylogenetic groups, in an organic broiler flock without antibiotic use. In each of two consecutive production rounds, 80 randomly chosen broilers were followed individually. Cloacal swabs from these, 20 other randomly chosen broilers, and 11 environmental samples were taken at several moments from arrival till slaughter age. ESBL/AmpC-EC were isolated by selective pre-enrichment, and ESBL/AmpC-genes, *E. coli* phylogenetic groups and sequence types were determined. Transmission parameters (β) were estimated using a Generalised Linear Model with a susceptible-infectious-susceptible model. Effect of direct broiler contact as compared to contact through the environment and previous infection status (yes/ no) were included as explanatory variables. The reproduction ratio (R) was calculated by multiplying β with the length of the infectious period. On day 1, prevalence was 28.8% (95%CI 19.2-40.0%) and 0.0% (95%CI 0.0-4.5%) among individually followed broilers, in round 1 and 2 respectively. In round 2, the environment was positive before arrival of day-old chicks. After 3 days, almost 100% of broilers and environmental samples were positive in both rounds. Most samples were positive for CTX-M-1 group genes, and A1 and B1 were the predominant phylogenetic groups. From day 3 there was a shift towards more phylogenetic groups and sequence types. R was 1.70 (95%CI 0.55-5.25) for total ESBL/AmpC-EC. Risk for an individual to become infected was lower if it had been infected previously ($\beta_{\text{previously infected}}$ =0.02 vs. $\beta_{\text{not previously infected}}$ =3.41; P<0.0001). For phylogenetic groups separately, R was 0.88 (95%CI 0.38-2.07), 0.51 (95%CI 0.27-0.98), 0.99 (95%CI 0.65-1.51) for A1, B1 and rest (i.e. A0, B2, D1, D2) groups, respectively. When broilers were not previously infected, the environment was relatively more important for transmission of the A1 group, while this was direct contact between broilers for the B1 group. Positive day-old chicks and the environment both play a role in introduction and transmission of ESBL/AmpC-EC in flocks. These results suggest that, even without selective pressure from antibiotics, total ESBL/AmpC-EC persistence, and resulting endemic situation, seem to be caused by shifts in infections by different phylogenetic groups. It implies that contaminated broilers enter the slaughterhouse.

Introduction

High prevalences of extended-spectrum β-lactamase and AmpC β-lactamase-producing *Escherichia coli* (ESBL/AmpC-EC) are reported in broiler (great) grandparent stock [1-5], on broiler farms [1,6-8], at the slaughterhouse [9], and in retail meat [2,10]. It is hypothesised that particular ESBL/AmpC-EC clones, or plasmids carrying β-lactamase genes, are introduced through breeding stock, and transmit through the broiler production chain, also in the absence of known selective antibiotic pressure [2,3]. This could present a threat for public health, as transmission of ESBL/AmpC-EC might occur between broilers and humans at any point in the production chain. ESBL/AmpC-mediated resistance transmitted in that way could complicate treatment of human bacterial infections [11]. Quantification of transmission may be a first step in further understanding the apparent persistence of ESBL/AmpC-EC in the broiler production chain, even in absence of antibiotic use.

Transmission can be expressed quantitatively by the basic reproduction ratio (R_0), which is defined as the expected number of secondary cases caused by one typical infected individual during its entire infectious period in a completely susceptible population [12]. If R_0 is less than one, major outbreaks or persistence of the infectious agent in the population is never possible, whereas when R_0 is larger than one major outbreaks may occur, possibly leading to persistence [13]. Longitudinal field studies have been used before to quantify transmission of antibiotic resistance, for example of methicillin-resistant *Staphylococcus aureus* (MRSA) in pigs [14]. Longitudinal studies have been conducted measuring ESBL/AmpC-EC prevalence [1,15], however no such study has been conducted on an organic broiler farm, and no attempt has been made to quantify transmission. ESBL/AmpC-EC have been isolated from broilers and the broiler house environment on conventional farms [1,15], suggesting that both direct and indirect routes might play a role in transmission. The aim of the current study was, therefore, to quantify transmission rates for different routes of ESBL/AmpC-EC transmission. Broilers, tagged to allow individual tracking, were followed from arrival till slaughter age, on an organic broiler farm. Positive tested samples were further characterised with respect to ESBL/AmpC-genes and *E. coli* genotypes for subsequent calculation of transmission rates for total ESBL/AmpC-EC, and specific phylogenetic groups separately.

Material and methods

Farm selection and sampling

From 11 June – 11 November 2013 a longitudinal study was conducted during two consecutive production rounds on an organic broiler farm, where a production round was defined as the period from arrival of day-old chicks until slaughter. The farm was selected out of a total of 9

organic broiler farms present in The Netherlands in 2013, according to the following criteria: more than one year organic production, no other livestock species present on the farm, and convenience of geographic location. On this farm there were two separate broiler houses with approximately 4800 individuals each. There were two age groups, but per house only broilers of the same age were present. No medication was used during either production round. From day 42 of age until slaughter age (approximately 70 days), broilers were allowed access to the outdoor pasture. Between rounds the broiler house was left empty for 7 days, during which time litter and faeces were removed mechanically, and it was cleaned with a high pressure water hose.

Upon arrival in the broiler house (day 1) in each production round, 80 broilers were randomly selected and labelled for individual tracking by applying a tag to the neck skin with a textile gun and nylon thread (QuickTag Fine Gauge Mk3 Long Needle Gun, Roxan Developments Ltd., United Kingdom). The number of broilers used was based on an expected prevalence of 5.0% in day-old chicks, an estimate obtained by sampling day-old chicks from the same farm prior to starting the current study, 95% confidence, and 5% absolute precision. The sample size was corrected for an estimated 10% loss to follow-up. Cloacal swabs from broilers with tag, randomly selected broilers without tag, environmental wipes from feeding, drinking water, and heating systems, and air samples from the broiler house were taken between arrival on the farm (day 1) and slaughter age (day 70): 7 times in round 1 (day 1, 3, 4, 7, 10, 42, and 70), and 4 times in round 2 (day 1, 2, 3, 70). The exception was day 70 in round 2 where a random selection of 100 broilers was taken because tags were no longer visible for identification. An overview of the types and numbers of all collected samples is given in Tables 1 and 2. Due to the large difference in prevalence on day 1 in round 1 between the 80 broilers with tag and 20 randomly chosen broilers without tag (28.8% vs. 5.0%), it was decided to investigate the effect of tagging in round 2. Cloacal swabs were taken from 80 randomly selected broilers before and after tagging. The same was done for 20 extra randomly chosen broilers using the textile gun without nylon threads. Due to the high prevalence of ESBL/AmpC-EC on day 3 in round 1, it was decided to sample day 2 in round 2 (Table 2).

The current study was conducted in accordance with the Dutch Law on Animal Health and Welfare, and approved by the Animal Welfare Committee of Wageningen University (registration code 2012110.b).

Microbiological examination

Bacteria were isolated from cloacal swabs, human faecal swabs, and environmental wipes by selective enrichment (Luria-Bertani broth with 1 mg/L cefotaxime), and culture on selective plates for 18h at 37˚C (MacConkey agar no. 3 with 1 mg/L cefotaxime). For broiler faeces, the paper lining of the transport box, soil, feed and litter samples initial suspensions were made by making a 10^{-1} dilution in Luria-Bertani broth, and homogenising using a pulsifier for 1 minute.

Subsequently, 100µl of the suspension was streaked onto selective plates (MacConkey agar no. 3 with 1 mg/L cefotaxime), and cultured for 18h at 37˚C. Oxidase and indole tests were performed on isolates with an *E. coli*-like morphology. Isolates were suspected to be *E. coli* when the oxidase test was negative and the indole test was positive. After day 1 of round 1 it appeared that indole negative isolates were also *E. coli*, therefore only oxidase positive isolates were excluded from that point onwards. *E. coli*-like isolates (range 1-4 isolates per sample) were then tested phenotypically for ESBL-production by combination disc-diffusion test according to CLSI guidelines [16]. A cefoxitin disc was used to detect AmpC phenotypes.

Subsequently, *E. coli* phylogenetic groups were determined for all isolates with an ESBL/AmpC-phenotype according to Clermont *et al.* [17] and Escobar-Páramo *et al*. [18]. *E. coli* were confirmed in isolates that were negative for all three amplicons using matrix-assisted laser desorption ionisation-time of flight. β-Lactamase-genes were identified in all isolates with an ESBL/AmpC-phenotype by using group specific primers for the detection of TEM, SHV and OXA-1-like genes (Multiplex I); CTX-M group 1, group 2 and group 9 genes (Multiplex II); and CIT genes as specified by Dallenne *et al*. [19]. A selection of CTX-M group 1 positive isolates (n=29) was sequenced and $bla_{\text{CTX-M-1}}$ was identified in these isolates. When isolates with an AmpC-phenotype were negative in the CIT and both multiplex PCRs these were excluded from analysis. Multilocus sequence typing (MLST) of *E. coli* was performed on a selection of isolates from round 1 and 2, according to Wirth *et al*. [20]. In round 1, MLST was performed on all day 1 isolates that did not originate from broiler cloacal swabs i.e. isolates from air inside the broiler house, broiler faeces, transport box paper lining, delivery van, and the sample collector (n=31), on a random selection of day 1 isolates from broilers with and without tag (45/72), and on a random selection of isolates (142/206) from 20 randomly selected broilers with tag. In round 2, MLST was performed on all day 1 isolates that did not originate from broiler cloacal swabs i.e. isolates from environmental wipes inside the broiler house, transport box paper lining, delivery van, and the sample collector (n=21), and on an isolate from the only positive broiler (without tag) on day 1 (n=1).

Quantification of transmission

A susceptible-infectious-susceptible (SIS-)model [14,21] was used to quantify transmission of total ESBL/AmpC-EC and for separate phylogenetic groups within the study population. In this model, the transmission rate parameter, β, is the average number of cases caused by a typical infected individual in a totally susceptible population per unit of time.

For each sampling moment, a broiler was classified infected (I) if at least one isolate recovered from a cloacal swab was positive for *E. coli* and an ESBL/AmpC-gene, and susceptible (S) if the cloacal swab tested negative. Broilers were also classified as infected or susceptible for A1, B1 and rest (i.e. A0, $B2_3$, D1, D2) phylogenetic groups separately. Missing values were animals that died or could not be traced due to invisible or missing neck tags. Based on this, three variables were determined for each time interval (Δt): the number broilers that

were classified as infected (I) (and the total number sampled) at the start of the interval, the number of tagged susceptible animals (S) at the start of the interval, and the number of new cases (C) among the tagged broilers, i.e. those that are susceptible at the beginning of the interval and are positive at the end of the interval. This was determined separately for individuals previously and not previously infected. Underlying assumptions were: (1) all broilers were in random contact, (2) all individuals were equally susceptible when not infected and (3) infected individuals were equally infectious over time.

The number of new cases can only be determined for the tagged animals but their infection can be caused by any of the animals in the flock or any part of the environment of the broiler house. Therefore, based on the method of Broens *et al*. [14], total infection pressure (IP_{total}) was introduced, and calculated as the sum of the proportion of infected (tagged plus random) broilers ($IP_{broiler}$), and the proportion of all environmental samples that were positive (i.e. air samples and environmental wipes from feed, water and heating systems; $IP_{environment}$). The number of new cases (C) per time interval (Δt) depends on $\beta_{environment}$ and $\beta_{broiler}$, the number of susceptible individuals (S), and total infection pressure (IP_{total}). Infected (I) individuals become susceptible again at recovery rate, α (Figure 1).

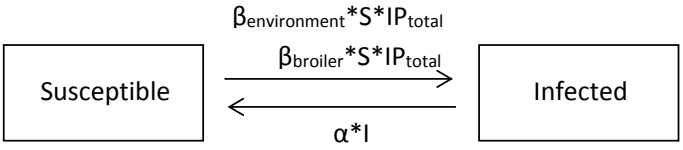

Figure 1. SIS-model used to quantify ESBL/AmpC-EC transmission rates. β, transmission parameter; S, susceptible individuals; IP_{total}, total infection pressure; I, infected individuals; α, recovery rate.

Similar to Broens *et al*. [14], the probability for each broiler to become infected during a time period Δt depends on $\beta_{environment}$, $\beta_{broiler}$, IP_{total}, and is equal to: $1 - e^{-\beta*IPtotal*\Delta t}$. From this probability, it can be shown that the number of new cases (C) in a period Δt follows a binomial distribution with parameter $1 - e^{-\beta*IPtotal*\Delta t}$ and binomial total S, the number of tagged susceptible individuals at the start of each time period (Δt). Consequently, the relation between the expected number of cases per unit of time (E(C)) and the transmission rate β, IP_{total}, and the number of susceptibles is as follows [14,22]:

$$E(C) = S * (1 - e^{-\beta*IPtotal*\Delta t}) \quad (1)$$

The data were analysed with SAS 9.2 [23] using a Generalised Linear Model with a complementary log-log link function, the term log (IP_{total} * Δt) as offset variable, C as the number of new cases, and S as the number of trials in the binomial process. The relation between the expected (E) value of a number of new cases out of a number of susceptibles during a time period Δt is presented in the following basic statistical model:

$$\text{cloglog}(E\ C/S) = \log(\beta) + \log(IP_{total} * \Delta t) \quad (2)$$

Exponentiation of the estimated parameter log(β), gives the transmission rate parameter β. The reproduction ratio, R, can then be calculated by multiplying β with the average length of the infectious period (T), which was estimated from the data. For round 2, R could not be calculated due to the small number of available data points.

To quantify the relative effect of transmission through direct contact with broilers compared to total transmission through direct contact and via the environment, a continuous explanatory variable was included in the model. This variable (pIP) was calculated as $IP_{broiler}$ divided by IP_{total}. Another explanatory variable ('previnf') was added describing whether or not broilers were infected (yes or no) during a previous time period. The full model is as follows:

$$\text{cloglog}(E\ C/S) = C0 + C1(pIP) + C2(previnf) + C3(pIP * previnf) + \log(IP_{total} * \Delta t) \quad (3)$$

where C0 is the intercept, and C1 and C2 are the regression coefficients for the two explanatory variables, and C3 for their interaction. It follows that the transmission rate parameters for the two routes (broiler or environment) and previous infection status (yes or no) can be calculated as follows:

$$\begin{aligned}
\beta_{environment,not\ previously\ infected} &= e^{C0} \\
\beta_{broiler,not\ previously\ infected} &= e^{C0+C1} \\
\beta_{environment,previously\ infected} &= e^{C0+C2} \\
\beta_{broiler,previously\ infected} &= e^{C0+C1+C2+C3}
\end{aligned} \quad (4)$$

First, the analysis was done without explanatory variables (intercept only model) to estimate the reproduction ratio for ESBL/AmpC-EC total, and for the A1, B1 and rest phylogenetic groups separately. Secondly, the analysis including explanatory variables was done. Variables with $P>0.05$ were removed from the model, and in case both variables remained in the model the interaction term was tested and considered significant at $P<0.05$.

Results

ESBL/AmpC-EC prevalence and ESBL/AmpC-genes

In round 1, the prevalence of ESBL/AmpC-EC was 28.8% (95% CI 19.2-40.0%) in broilers with tag, and 5.0% (95% CI 0.1-24.9%) in broilers without tag within two hours after arrival at the broiler farm (Table 1). At day 3 these percentages were 98.8% (95% CI 93.2-100.0%), and 100% (95% CI 83.2-100.0%) respectively. Prevalence at slaughter (day 70) was 39.4%

(95% CI 27.6-52.2%) in broilers with tag, and 25.0% (95% CI 8.7-49.1%) in broilers without tag. Except for one broiler with tag that was found positive at every sampling in round 1, broilers showed intermittent carriage. Upon arrival at the farm in round 2, ESBL/AmpC-EC was not detected in any broiler with tag (95% CI 0.0-4.5%), and in 5.0% (95% CI 0.1-24.9%) of broilers without tag (Table 2). Before tagging the prevalence was higher (5.0%) than after tagging (0.0%), but not significantly different (P=0.06), so the hypothesis that tagging results in higher prevalence of ESBL/AmpC-EC was rejected. On day 2, prevalences were 74.7 (95% CI 63.6-83.8%) in broilers with tag, and 65.0 (95% CI 40.8-84.6%) in broilers without tag. On day 3 these were 9l.1% (95% CI 82.6-96.4%) and 90.0% (95% CI 68.3-98.8%), respectively. At slaughter, the prevalence was 81.0% (95% CI 71.9-88.2%).

Table 1. Types and numbers of collected samples, and prevalence of ESBL/AmpC-EC in production round 1.

Day	Sample description[a]	Total collected	Positive	Prevalence (%)
1	Broiler without tag	20	1	5.0
	Broiler with tag	80	23	28.8
	Air broiler house	6	5	83.3
	Feed, water, heating system broiler house	5	0	0.0
	Broiler faeces	1	1	100.0
	Transport box	3	3	100.0
	Delivery van	2	2	100.0
	Sample collector	3	1	33.3
	Residence farmer	4	0	0.0
3	Broiler without tag	20	20	100.0
	Broiler with tag	80	79	98.8
	Air broiler house	6	6	100.0
	Feed, water, heating system broiler house	5	5	100.0
	Broiler faeces	2	2	100.0
4	Broiler without tag	20	20	100.0
	Broiler with tag	78	76	97.4
	Air broiler house	6	4	66.7
	Feed, water, heating system broiler house	5	5	100.0
	Broiler faeces	1	1	100.0
7	Broiler without tag	20	18	90.0
	Broiler with tag	75	69	92.0
	Air broiler house	6	4	66.7
	Feed, water, heating broiler house	5	5	100.0
	Broiler faeces	1	1	100.0
10	Broiler without tag	20	15	75.0
	Broiler with tag	73	61	83.6
	Air broiler house	6	4	66.7
	Feed, water, heating system broiler house	5	5	100.0

Table 1. Continued

Day	Sample description[a]	Total collected	Positive	Prevalence (%)
	Broiler faeces	2	2	100.0
	Sample collector	3	1	33.3
	Residence farmer	4	0	0.0
42	Broiler without tag	20	3	15.0
	Broiler with tag	68	13	19.1
	Air broiler house	6	0	0.0
	Feed, water, heating system broiler house	5	5	100.0
	Broiler faeces	2	2	100.0
	Soil free-range pasture	3	0	0.0
	Sample collector	3	1	33.3
	Residence farmer	4	0	0.0
70	Broiler without tag	20	5	25.0
	Broiler with tag	66	26	39.4
	Air broiler house	6	0	0.0
	Feed, water, heating system broiler house	5	3	60.0
	Broiler faeces	2	2	100.0
	Soil free-range pasture	3	1	33.3
	Sample collector	3	1	33.3

[a] Air: selective plates containing cefotaxime spread out over three locations, and left open for one hour in broiler house. Feed, water, heating system: environmental wipes taken from feeding system (front and back, three feeders per wipe), drinking water system (front and back, ten nipples per wipe), and heating system (top) in broiler house. Faeces: fresh, collected from floor of broiler house. Delivery van: environmental wipes from cargo area. Transport box: faeces from paper lining at bottom of box. Soil: free-range pasture in front of broiler house, halfway and back (9 cores each location). Sample collector: faecal swab from individuals taking samples. Residence farmer: environmental wipes from dog, TV remote control, favourite armchair, and door handle inside and outside of residence.

The number of positive environmental wipes from feeding, drinking water, and heating systems and air samples from the broiler house varied between rounds and sampling times, from no positive samples to all samples positive. Prevalence of ESBL/AmpC-EC in broiler faeces, the delivery van, transport boxes, and soil from the outdoor pasture also varied between rounds and between sampling times (Tables 1 and 2). In round 2, samples collected from the heating system, and the floor of both the broiler house, and hygiene barrier were already positive before arrival of the broilers. ESBL/AmpC-EC were not detected at the farm residence in round 1. One sample collector was positive throughout round 1, and at the beginning of round 2.

Isolates that were positive for CTX-M-1 group genes were obtained from most broiler samples and samples from other sources in both round 1 and 2. In round 1, isolates from one broiler were all negative in the CIT and both multiplex PCR's, and considered ESBL/AmpC-positive, because the *E. coli* isolates expressed an ESBL-phenotype. In round 2, one broiler had CIT genes only, and isolates from two broilers and two broiler house samples

were all negative in the CIT and both multiplex PCR's. Isolates obtained from the latter PCR negative samples showed an AmpC-phenotype. These were excluded from analysis because chromosomal mutations might be involved, and the focus was on plasmid-located genes.

Table 2. Types and numbers of collected samples, and prevalence of ESBL/AmpC-EC in production round 2.

Day	Sample description[a]	Total collected	Positive	Prevalence (%)
1	Broiler without tag (before)[b]	20	1	5.0
	Broiler without tag (after)[b]	20	0	0.0
	Broiler with tag (before)[c]	80	0	0.0
	Broiler with tag (after)[c]	80	0	0.0
	Air broiler house (before)[d]	6	0	0.0
	Air broiler house (after)[d]	6	0	0.0
	Environmental wipes broiler house[e]	11	4	36.4
	Feed[f]	1	0	0.0
	Litter[f]	1	0	0.0
	Transport box	2	1	50.0
	Delivery van	2	2	100.0
	Sample collector	3	1	33.3
2	Broiler without tag	20	13	65.0
	Broiler with tag	79	59	74.7
	Air broiler house	6	2	33.3
	Feed, water, heating system broiler house	5	5	100.0
	Broiler faeces	1	1	100.0
3	Broiler without tag	20	18	90.0
	Broiler with tag	79	72	91.1
	Air broiler house	6	4	66.7
	Feed, water, heating system broiler house	5	5	100.0
	Broiler faeces	1	1	100.0
70	Broiler without tag	100	81	81.0
	Air broiler house	6	1	16.7
	Feed, water, heating system broiler house	5	5	100.0
	Broiler faeces	2	2	100.0
	Soil free-range pasture	3	1	33.3

[a] Environmental wipes, transport box, delivery van, sample collector, soil and faeces samples taken as in round 1.
[b] Cloacal swabs taken before and after use of textile gun without nylon threads.
[c] Cloacal swabs taken before and after use of textile gun for application of neck tags.
[d] Air samples taken before and after arrival of day-old chicks i.e. first set of selective plates containing cefotaxime opened for half an hour before arrival of chicks, and second set of selective plates opened for half an hour after arrival of chicks in broiler house.
[e] All day 1 environmental wipes taken in broiler house before arrival of chicks at following locations: feeding system, drinking water system, and heating system. Additional wipes taken from broiler house (i.e. floor, wall, separation curtain, inside of heating system), and from hygiene barrier attached to broiler house (i.e. floor, farmer's boots).
[f] Feed and litter were taken from inside the broiler house before arrival of the chicks.

E. coli phylogenetic groups and sequence types

The predominant *E. coli* phylogenetic groups in broilers with and without tag, environmental wipes from feed, water and heating systems, and air samples taken inside the broiler house throughout round 1 and 2 were A1 and B1 (Table 3). The predominant *E. coli* sequence types in isolates further characterised by MLST were ST10 and ST88, belonging to the A1 phylogenetic group, and ST58 and ST155, belonging to the B1 phylogenetic group (Tables 4 and 5). *E. coli* with different phylogenetic groups could be isolated from the same sample on the same day. From day 3, there was a shift towards more different groups (Table 3). Furthermore, none of the 20 randomly selected broilers out of 80 with tag from round 1 were positive for the same *E. coli* sequence type at all sampling times (Table 5).

Table 3. *E. coli p*hylogenetic groups per day in broiler and broiler house samples in round 1 and 2.

Round	Day	Sample[a]	n	Prevalence (%)						
				Total[c]	A1	A0	B1	$B2_3$	D1	D2
1	1	Broiler	100[b]	24.0	24.0	0.0	2.0	1.0	0.0	0.0
		House	11	45.5	45.5	0.0	0.0	0.0	0.0	0.0
	3	Broiler	98	99.0	89.8	0.0	29.6	1.0	12.2	2.0
		House	11	100.0	90.9	0.0	45.5	0.0	27.3	0.0
	4	Broiler	95	97.9	84.2	0.0	34.7	0.0	9.5	2.1
		House	11	81.8	63.6	0.0	27.3	0.0	18.2	0.0
	7	Broiler	93	92.5	84.9	0.0	17.2	0.0	8.6	2.2
		House	11	81.8	63.6	0.0	27.3	0.0	27.3	0.0
	10	Broiler	87	81.6	63.2	0.0	13.8	0.0	6.9	8.0
		House	11	81.8	81.8	0.0	9.1	0.0	0.0	0.0
	42	Broiler	86	18.6	12.8	0.0	5.8	0.0	0.0	0.0
		House	11	45.5	36.4	9.1	18.2	0.0	0.0	0.0
	70	Broiler	86	36.0	10.5	0.0	5.8	0.0	0.0	23.3
		House	11	27.3	9.1	0.0	27.3	0.0	0.0	0.0
2	1	Broiler	100	1.0	1.0	0.0	0.0	0.0	0.0	0.0
		House	23	17.4	17.4	0.0	0.0	0.0	0.0	4.3
	2	Broiler	99	71.7	69.7	1.0	14.1	2.0	1.0	1.0
		House	11[b]	54.5	45.5	0.0	9.1	0.0	0.0	0.0
	3	Broiler	99	89.9	79.8	1.0	35.4	1.0	0.0	3.0
		House	11	72.7	72.7	0.0	54.5	0.0	0.0	0.0
	70	Broiler	100[b]	81.0	75.0	3.0	18.0	1.0	0.0	0.0
		House	13	61.5	53.8	0.0	15.4	7.7	0.0	0.0

[a] Broiler refers to cloacal swabs from broilers with and without tag. House refers to environmental wipes, and air samples taken inside the broiler house.

[b] One isolate with unknown phylogenetic group.

[c] Prevalences for individual phylogenetic groups do not add up to total because *E. coli* with different phylogenetic groups could be isolated from the same sample on the same day.

In round 1, all tested samples from day 1 harboured at least one *E. coli* isolate that was identified as ST88 (Tables 4 and 5). The only exception was the sample collector, where only *E. coli* isolates with ST636 ($B2_3$ phylogenetic group) were detected. In addition to being positive for ST88, broiler faeces, the paper lining at the bottom of the transport box, and environmental wipes from the delivery van were positive for ST155 (B1), ST106 (D2), and ST10 (A1) and ST58 (B1), respectively. While ST58 and ST155 were detected in broilers with and without tag on day 1, ST10 and ST106 were not detected in broilers with tag until day 3. In round 2, all tested samples from day 1 also harboured at least one isolate with ST88. Environmental wipes from the heating system (new ST, A1; ST117, D2), and the floor of the hygiene barrier (ST43, A1) harboured sequence types that were not detected in round 1, however.

Table 4. *E. coli* phylogenetic groups and sequence types in samples collected on day 1 in round 1 and 2.

Round	Sample description	Phylogenetic group(s)	Sequence type(s)
1	Broiler without and with tag	A1; B1; $B2_3$	88, 4358; 58, 155; 140
	Air broiler house	A1	88
	Feed, water, heating system broiler house	-	-
	Broiler faeces	A1; B1	88; 155[c]
	Transport box	A1; D2	88; 106
	Delivery van	A1; B1	10, 88; 58
	Sample collector	$B2_3$	636
	Residence farmer	-	-
2[a]	Broiler without tag	A1	88
	Air broiler house	-	-
	Environmental wipes broiler house[b]	A1; D2	43, 88, new; 117
	Transport box	A1	88
	Delivery van	A1	88, nt
	Sample collector	$B2_3$	636

-, sample was ESBL/AmpC-negative; new, new sequence type; nt, non-typable.
[a] Due to the absence of fresh broiler faeces, none were collected.
[b] Heating system (ST88, ST117, new), floor broiler house (ST88), floor hygiene barrier (ST43).
[c] Housekeeping gene adk 6 with one mutation, closest match is ST155.

Table 5. *E. coli* sequence types per day from 20 broilers randomly selected from tagged broilers in round 1.

Broiler ID	Day						
	1	**3**	**4**	**7**	**10**	**42**	**70**
K103[a]	88	10	10	10	10	-	155
K104[a]	88	10	106	10; 106	10; 155[b]	-	-
K105	88	10	10; 58	10; 106	10; 106	-	155
K106	-	155	nt	10	10	-	-
K109	-	10; 3258	3258	10	3258	-	1551
K117	-	10	10	88	2485	-	88
K123	-	106	-	10	88	10	-
K128[a]	-	10	10; 58	10; 155[b]	-	-	10
K133[a]	88; 4358	10; 58	10; 58	10	3258	-	-
K140[a]	-	10; 155	155	10; 155	155	-	1551
K142	-	10	10; 2001	10	3258	10	-
K147	88	10	10	10	-	-	-
K150	-	10; 155	88	10	10	-	-
K155	-	10	88	-	-	-	-
K157	-	-	155	88; 155	155	155	155; 1551
K163	88	10; 58	58; 2001	88	58; 88	88	1551
K164	-	10; 58	10; 58	10	10	-	-
K169	88	10; 155	10; 58	10	10	-	1551
K173	88; 155	10; 155	10	106	10	88	-
K179	-	88	10	-	10	-	-

nt, non-typable;-, sample was ESBL/AmpC-negative.

[a] Randomly selected broilers where all collected isolates were typed to explore variation in ST within samples.

[b] Housekeeping gene adk 6 with one mutation, closest match is ST155.

Transmission quantification

Table 6 shows the model input summarised per interval for ESBL/AmpC-EC total, and for the A1, B1, and rest (i.e. A0, B2, D1, D2) phylogenetic groups separately, which was used for transmission quantification. The reproduction ratio for total ESBL/AmpC-EC, A1 group, B1 group and rest group, respectively, was 1.70 (95% CI 0.55-5.25), 0.88 (95% CI 0.38-2.07), 0.51 (95% CI 0.27-0.98), and 0.99 (95% CI 0.65-1.51) (Table 7).

Table 6. Summarised model input used for estimation of transmission parameters for total ESBL/AmpC-EC, and A1, B1 and rest phylogenetic groups.

ESBL/AmpC	Interval (days)	Previously infected	Susceptible (S)	Cases (C)[a]	Δt (days)	$IP_{broiler}$	$IP_{environment}$
Total	1-3	No	57	56.5	2	0.24	0.45
		Yes	0	0			
	3-4	No	1	1	1	0.99	1.00
		Yes	0	0			
	4-7	No	0	0	3	0.98	0.82
		Yes	2	2			
	7-10	No	0	0	3	0.92	0.82
		Yes	5	3.5			
	10-42	No	0	0	32	0.82	0.82
		Yes	11	1.5			
	42-70	No	0	0	28	0.19	0.45
		Yes	53	19.5			
A1	1-3	No	57	52.5	2	0.24	0.45
		Yes	0	0			
	3-4	No	5	2.5	1	0.90	0.91
		Yes	1	1			
	4-7	No	3	3	3	0.84	0.64
		Yes	10	9.5			
	7-10	No	0	0	3	0.85	0.64
		Yes	11	6.5			
	10-42	No	0	0	32	0.63	0.82
		Yes	20	2.5			
	42-70	No	0	0	28	0.13	0.36
		Yes	57	4.5			
B1	1-3	No	78	24.5	2	0.02	0.0
		Yes	0	0			
	3-4	No	52	14.5	1	0.30	0.45
		Yes	1	1			
	4-7	No	38	3.5	3	0.35	0.27
		Yes	9	1.5			
	7-10	No	34	2.5	3	0.17	0.27
		Yes	26	4.5			
	10-42	No	30	3.5	32	0.14	0.09
		Yes	28	0.5			
	42-70	No	26	1.5	28	0.06	0.18
		Yes	36	2.5			
Rest (A0, B2, D1 D2)	1-3	No	79	10.5	2	0.01	0.0
		Yes	0	0			

Table 6. Continued

ESBL/AmpC	Interval (days)	Previously infected	Susceptible (S)	Cases (C)[a]	Δt (days)	$IP_{broiler}$	$IP_{environment}$
	3-4	No	67	7.5	1	0.15	0.27
		Yes	1	0.5			
	4-7	No	57	5.5	3	0.12	0.18
		Yes	9	1.5			
	7-10	No	50	5.5	3	0.11	0.27
		Yes	15	2.5			
	10-42	No	41	0.5	32	0.15	0.0
		Yes	17	0.5			
	42-70	No	40	8.5	28	0.006	0.09
		Yes	26	9.5			

Δt, time interval between samplings; $IP_{broiler}$, proportion of infected broilers; $IP_{environment}$, proportion of positive environmental samples.

[a] When C/S<1, 0.5 was added to the number of cases, and when I=0, 0.5 was added to the number of infected (I) animals.

Table 7. Transmission rate parameters (β) and reproduction ratios (R) with 95% confidence interval for total ESBL/AmpC-EC and A1, B1 and rest phylogenetic groups in round 1 from the 'intercept only' model.

ESBL/AmpC	Infectious period (days)	β (day^{-1})	R	95% CI
Total	26.84	0.06	1.70	0.55-5.25
A1	18.44	0.05	0.88	0.38-2.07
B1	8.63	0.06	0.51	0.27-0.98
Rest (A0, B2, D1 and D2)	10.93	0.09	0.99	0.65-1.51

Effects of the relative effect of transmission through direct contact with broilers compared to total transmission through direct contact and via the environment (pIP), and previous infection status are shown in Table 8. For total ESBL/AmpC-EC, the effect of pIP was not significant (P=0.21), and the risk for an individual to become infected was lower if it was infected previously ($\beta_{previously\ infected}$=0.02 vs. $\beta_{not\ previously\ infected}$=3.41; P<0.0001). For the A1 phylogenetic group there was significant interaction (P<0.0001) between pIP and previous infection status. The environmental route played a greater role when individuals were not previously infected ($\beta_{environment,\ not\ previously\ infected}$=18.39 vs. $\beta_{broiler,\ not\ previously\ infected}$=0.02), while the animal route played a relatively greater role when individuals were previously infected ($\beta_{broiler,\ previously\ infected}$=982.50 vs. $\beta_{environment,\ previously\ infected}$=2.73x$10^{-6}$). For the B1 phylogenetic group there was also significant interaction (P<0.0001). In this case, however, the animal route played a relatively greater role when animals were not previously infected ($\beta_{broiler,\ not\ previously\ infected}$=6.02 vs. $\beta_{environment,\ not\ previously\ infected}$=0.0004), and there was no effect of infection pressure from

broilers when animals were previously infected (P=0.65). For the rest phylogenetic groups (i.e. A0, B2, D1, D2), the effect of previous infection was not significant (P=0.91). The relative proportion of infection pressure from broilers played a relatively smaller role than that from the environment ($\beta_{broiler}$=0.05 vs. $\beta_{environment}$=0.16; P=0.001).

Table 8. Estimates of transmission parameters and their 95% confidence for total ESBL/AmpC-EC and A1, B1 and rest phylogenetic groups in round 1.

ESBL/AmpC	Transmission parameter	Estimate (day^{-1})	95% CI	P-values
Total	$\beta_{not\ previously\ infected}$	3.41	1.91-6.11	<0.0001
	$\beta_{previously\ infected}$	0.02	0.01-0.03	<0.0001
A1	$\beta_{environment,\ not\ previously\ infected}$	18.39	2.41-140.34	0.005
	$\beta_{broiler,\ not\ previously\ infected}$	0.02	0.0007-0.69	0.015
	$\beta_{environment,\ previously\ infected}$	$2.73x10^{-6}$	$1.63x10^{-7}$-$4.56x10^{-5}$	<0.0001
	$\beta_{broiler,\ previously\ infected}$	982.50	53.67-17985.64	<0.0001
B1	$\beta_{environment,\ not\ previously\ infected}$	0.0004	0.0001-0.001	<0.0001
	$\beta_{broiler,\ not\ previously\ infected}$	6.02	3.71-9.77	<0.0001
	$\beta_{environment,\ previously\ infected}$	0.03	0.006-0.14	<0.0001
	$\beta_{broiler,\ previously\ infected}$	0.01	0.001-0.13	0.648
Rest	$\beta_{environment}$	0.16	0.11-0.23	<0.0001
(A0, B2, D1, D2)	$\beta_{broiler}$	0.05	0.03-0.08	0.001

Discussion

This is the first study to quantify transmission of ESBL/AmpC-EC in a broiler flock, and explore the effect of direct broiler contact relative to the environment on transmission. It is also the first longitudinal study on an organic broiler farm. A reproduction ratio of 1.70 for total ESBL/AmpC-EC indicates that it transmits and persists in the broiler flock. Interestingly, this does not hold true for individual phylogenetic groups as indicated by reproduction ratios less than one for groups separately. This might mean that total ESBL/AmpC-EC persistence is determined by repeated shifts from one phylogenetic group to another during the period between arrival at the farm and slaughter age.

Although the prevalence in tagged broilers on day 1 in round 1 and 2 was considerably different (28.8% vs. 0.0%), the same general pattern in prevalence was seen in both production rounds, with a peak occurring around day 3, and a decrease towards slaughter age. Furthermore, the risk for an individual to become infected with ESBL/AmpC-EC was lower if it had been infected previously. An explanation for these results might be the acquisition of immunity to particular *E. coli* genotypes. Age dependent-susceptibility to *E.*

coli O157:H7 has been shown previously for broilers [24], but whether this also holds true for commensal *E. coli* populations has not been investigated to the authors' knowledge. The genotypic data from the current study showed that the A1 phylogenetic group, or specifically ST88, predominated in broiler samples on day 1, and genotypes became more diverse from day 3 onwards. It can be speculated that this is a result of changes in the availability of niches for particular populations of *E. coli*, as the first weeks post-hatch are marked by changes in relative abundances of different bacterial species, and varies between compartments of the intestinal tract (i.e. duodenum, ileum, caecum; van der [25]). Horizontal transfer of CTX-M-1 group genes between *E. coli* of different phylogenetic groups and sequence types should also be considered as a potential explanation of the diversification of ESBL/AmpC-EC genotypes after day 3, as *in situ* experiments have shown that conjugation of plasmids still appeared without selective pressure from an antimicrobial agent [26]. The plasmids harbouring CTX-M-1 group genes were not investigated in the current study, but taking into account within-host dynamics of the bacterial population seems important in accurately describing the transmission dynamics of antibiotic resistant bacteria. Models combining this information with an epidemiological model representing the spread of resistance within the population could be a next step in understanding the apparent persistence of ESBL/AmpC-EC in the broiler production chain [27,28].

E. coli with ST88 were found at the end of production round 1 in broilers, and also in the broiler house at the beginning of round 2 before arrival of day-old chicks. This suggests that these particular *E. coli* can survive for prolonged periods of time outside their host, and might infect a new flock. In a survival experiment where bedding material and faeces from broilers were examined daily for concentrations of *E. coli*, it was shown that *E. coli* could survive for a minimum of 35 days ($4.97x10^7$ Colony Forming Units) under broiler house conditions and without addition of new faecal material [29], lending support to this idea. The MLST results are complemented by the results obtained from the model with explanatory variables, which show that the environment was relatively more important in the transmission of the A1 phylogenetic group, to which ST88 belongs, and direct contact between broilers was relatively more important for the transmission of the B1 phylogenetic group, for those broilers that were not previously infected. ST58, belonging to the B1 phylogenetic group, was found in day-old chicks and the delivery van, but not in the broiler house environment on day 1 of round 1. Direct contact between broilers was relatively more important for transmission of the A1 phylogenetic group for broilers that were previously infected. This is also suggested by the MLST results which indicate a switch from ST88 on day 1 to ST10 on day 3. Due to the labour intensive nature of MLST only a small selection of isolates were typed, but using *E. coli* sequence types instead of phylogenetic groups will provide even more details on transmission dynamics. Together, these results suggest that the contaminated environment before the start of the production round and positive day-old chicks both play a role in the introduction

and transmission of ESBL/AmpC-EC in the new flock. Intervention strategies should focus on preventing the introduction of ESBL/AmpC-EC into the flock. Cleaning and disinfection of the broiler house and hygiene barrier between rounds reduces these bacteria in the environment [30], but in order to prevent their introduction by positive day-old chicks it is necessary to consider all previous steps in the broiler production chain, that is (great)grandparent and parent stock, and hatcheries. It is suggested that ESBL/AmpC-*E. coli* are introduced through breeding stock and that there is transmission through the production chain [3].

Following individual broilers in the current study provides a unique insight in ESBL/AmpC-EC dynamics. Carriage of ESBL/AmpC-EC was intermittent in all broilers in the sense that no broiler was positive for ESBL/AmpC-EC with the same phylogenetic group or sequence type during the entire production round, which indicates how complex transmission of ESBL/AmpC-EC actually is. Several factors should be taken into account when interpreting the results. Firstly, the length of the last two intervals in which measurements were taken, were 32 and 28 days. This is long in comparison to the infectious period for total ESBL/AmpC-EC, and even more so in comparison to the infectious period for the individual phylogenetic groups, and perhaps also individual sequence types. Some cases (C), but also infected (I) individuals might have been missed during these intervals as individuals might have switched multiple times between susceptible and infected states. In a study modelling the epidemiology of Verocytotoxin-producing *E. coli* (VTEC) serogroups in young calves, it was shown that there is a difference in the rate at which different serogroups are lost from calves, with serogroups being divided into those with high and low recovery rate [31]. A higher recovery rate can reduce the total number of infections observed, and can have an impact on how often consecutive infections are observed [31]. Secondly, it should be taken into account that *E. coli* phylogenetic groups and sequence types were determined for a limited number of isolates (range 1-3) per positive sample. Analysis of multiple isolates from one tagged broiler showed two different sequence types (ST88 and ST4358) from the A1 phylogenetic group on the same day. This signifies that selection of a limited number of isolates might result in missing less dominant strains of ESBL/AmpC-EC, but perhaps also underestimating the number of 'mixed' infections. This was also observed in a study looking at VTEC in calves [31]. Lastly, from day 3 onwards all oxidase negative isolates were included, instead of only those oxidase negative isolates that were also indole positive. It might be expected that this explains the increase in number of different phylogenetic groups from day 3, but the indole negative isolates belonged primarily to the A1 phylogenetic group (data not shown).

In conclusion, positive day-old chicks and the environment both play a role in the introduction and transmission of ESBL/AmpC-EC into the new flock. Furthermore, these resistant bacteria are able to transmit and persist in a broiler flock, even without selective pressure from antibiotics. The resulting endemic situation implies that contaminated broilers enter the slaughterhouse.

Acknowledgements

We would especially like to thank the farmer and his family for allowing us conduct this study on their farm. We would also like to thank our technicians W.M. van Overbeek and P.D. Hengeveld for their excellent work in the laboratory. Financial support for this project was provided by the Netherlands Food and Consumer Product Authority (NVWA).

References

1. Dierikx, C.M., van der Goot, J.A., Smith, H.E., *et al*. 2013. Presence of ESBL/AmpC-producing *Escherichia coli* in the broiler production pyramid: a descriptive study. *PLoS One*. 8:e79005.
2. Agersø, Y., Jensen, J.D., Hasman, H., *et al*. 2014. Spread of extended spectrum cephalosporinase-producing *Escherichia coli* clones and plasmids from parent animals to broilers and to broiler meat in a production without use of cephalosporins. *Foodborne Pathogens and Disease*. 11:740-746.
3. Nilsson, O., Börgesson, S., Landén, A., *et al*. 2014. Vertical transmission of *Escherichia coli* carrying plasmid-mediated AmpC (pAmpC) through the broiler production pyramid. *Journal of Antimicrobial Chemotherapy*. 69:1497-1500.
4. Zurfluh, K., Wang, J., Klumpp, J., *et al*. 2014. Vertical transmission of highly similar $bla_{CTX\text{-}M\text{-}1}$-harboring IncI1 plasmids in *Escherichia coli* with different MLST types in the poultry production pyramid. *Frontiers in Microbiology*. 5:519.
5. Mo, S.S., Norström, M., Slettemeås, J.S., *et al*. 2014. Emergence of AmpC-producing *Escherichia coli* in the broiler production chain in a country with a low antimicrobial usage profile. *Veterinary Microbiology*. 171:315-320.
6. Smet, A., Martel, A., Persoons, D., *et al*. 2008. Diversity of extended-spectrum β-lactamases and class C β-lactamases among cloacal *Escherichia coli* isolates in Belgian broiler farms. *Journal of Antimicrobial Chemotherapy*. 52:1238-1243.
7. Huijbers, P.M.C., Graat, E.A.M., Haenen, A.P.J., *et al*. 2014a. Extended-spectrum and AmpC beta-lactamase-producing *Escherichia coli* in broilers and people living and/or working on broiler farms: prevalence, risk factors and molecular characteristics. *Journal of Antimicrobial Chemotherapy*. 69:2669-2675.
8. Huijbers, P.M.C., van Hoek, A.H.A.M., Graat, E.A.M., *et al*. 2014b. Methicillin-resistant *Staphylococcus aureus* and extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* in broilers and people living and/or working on organic broiler farms. *Veterinary Microbiology*. 176:120-125.
9. Reich, F., Atanassova, V., Klein, G. 2013. Extended-spectrum β-lactamase-and AmpC-producing Enterobacteria in healthy broiler chickens, Germany. *Emerging Infectious Diseases*. 19:1253-1259.
10. Cohen-Stuart, J., van den Munckhof, T., Voets, G., *et al*. 2012. Comparison of ESBL contamination in organic and conventional retail chicken meat. *International Journal of Food Microbiology*. 154:212-214.
11. de Kraker, M.E.A., Davey, P.G., Grundmann, H. 2011. Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteraemia: Estimating the burden of antimicrobial resistance in Europe. *PLoS Medicine*. 8:e1001104.
12. Diekmann, O., Heesterbeek, J.A.P., Metz, J.A.J. 1990. On the definition and the computation of the basic reproduction ration R_0 in models for infectious diseases in heterogeneous populations. *Journal of Mathematical Biology*. 28:365-382.
13. Diekmann, O., Heesterbeek, J.A.P., Britton, T. 2012. Mathematical tools for understanding infectious disease dynamics. Princeton University Press, New Jersey, USA.
14. Broens, E.M., Espinosa-Gongora, C., Graat, E.A.M., *et al*. 2012. Longitudinal study on transmission of MRSA CC398 within pig herds. *BMC Veterinary Research*. 8:58.
15. Laube, H., Friese, A., von Salviati, C., *et al*. 2013. Longitudinal monitoring of ESBL/AmpC-producing *Escherichia coli* in German broiler chicken fattening farms. *Applied Environmental Microbiology*. 79:4815-4820.
16. Clinical and Laboratory Standards Institute (CLSI)., 2011. *Performance Standards for Antimicrobial Susceptibility Testing: Twenty-first Informational Supplement M100-21*. Wayne, Pennsylvania, USA.
17. Clermont, O., Bonacorsi, S., Bingen, E. 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. *Applied Environmental Microbiology*. 66:4555-4558.
18. Escobar-Páramo, P., Le Menac'h, A., Le Gall, T., *et al*. 2006. Identification of forces shaping the commensal *Escherichia coli* genetic structure by comparing animal and human isolates. *Environmental Microbiology.* 8:1975-1984.
19. Dallenne, C., Da Costa, A., Decre, D., *et al*. 2010. Development of a set of multiplex PCR assays for the detection of genes encoding important β-lactamases in Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy*. 65:490-495.
20. Wirth, T., Falush, D., Lan, R., *et al*. 2006. Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Molecular Microbiology*. 60:1136-1151.
21. Velthuis, A.G.J., Bouma, A., Katsma, W.E.A., *et al*. 2007. Design and analysis of small-

scale transmission experiments with animals. *Epidemiology and Infection*. 135:202-217.
22. Velthuis, A.G.J., de Jong, M.C.M., Kamp, E.M., *et al*. 2003. Design and analysis of an *Actinobacillus pleuropneumoniae* transmission experiment. *Preventive Veterinary Medicine*. 60:53-68.
23. SAS Institute Inc., 2008. SAS/STAT® 9.2 User's Guide. SAS Institute, Cary, North Carolina, USA.
24. Stavric, S., Buchanan, B., Gleeson, T.M. 1993. Intestinal colonisation of young chicks with *Escherichia coli* O157:H7 and other verotoxin-producing serotypes. *Journal of Applied Bacteriology*. 74:557-563.
25. van der Wielen, P.W.J.J., Keuzenkamp, D.A., Lipman, L.J.A., *et al*. 2002. Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. *Microbial Ecology*. 44:286-293.
26. Smet, A., Rasschaert, G., Martel, A., *et al*. 2011. *In situ* ESBL conjugation from avian to human *Escherichia coli* during cefotaxime administration. *Journal of Applied Microbiology*. 110:541-549.
27. Lanzas, C., Lu, Z., Gröhn, Y.T. 2010. Mathematical modelling of the transmission and control of foodborne pathogens and antimicrobial resistance at preharvest. *Foodborne Pathogens and Disease*. 8:1-10.
28. Andraud, M., Rose, N., Laurentie, M., *et al*. 2011. Estimation of transmission parameters of a fluoroquinolone-resistant *Escherichia coli* strain between pigs in experimental conditions. *BMC Veterinary Research*. 42:44.
29. van Bunnik, B.A.D., Ssematimba, A., Hagenaars, T.J., *et al*. 2014. Small distances can keep bacteria at bay for days. *Proceedings of National Academy of Sciences of the United States of America*. 111:3556-3560.
30. Luyckx, K.Y., van Weyenberg, S., Dewulf, J., *et al*. 2015. On-farm comparisons of different cleaning protocols in broiler houses. *Poultry Science*. 94:1986-1993.
31. Liu, W.C., Jenkins, C., Shaw, D.J., *et al*. 2005. Modelling the epidemiology of Verocytotoxin-producing *Escherichia coli* serogroups in young calves. *Epidemiology and Infection*. 133:449-458.

Chapter 7

General Discussion

Introduction

The aim of this thesis was to investigate the transmission of antibiotic resistant bacteria between animals and humans, and more specifically to investigate the transmission of ESBL-producing *Escherichia coli* between broilers, and between broilers and humans in varying degrees of contact with broilers. This could help to elucidate the role of broilers in the transmission of antibiotic resistance to humans.

In this chapter the conclusions of the thesis are used to discuss the contribution of poultry to human carriage of ESBL-producing Enterobacteriaceae. Thereafter, transmission of ESBL-producing Enterobacteriaceae between animals and humans, and between humans in the general population through other sources and pathways is examined. Finally, implications of the results, directions for future research, and the main conclusions of this thesis are given.

Contribution of poultry to human carriage of ESBL-producing Enterobacteriaceae

Clinically relevant antibiotic resistant (AMR) bacteria, that is ESBL-producing Enterobacteriaceae, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant *Enterococcus* spp., are present in the natural environment, more specifically in soil, water, air and wildlife ([1]; *Chapter 2*). Examination of the vicinity of broiler farms showed that air 50 m downwind and 100 m upwind of the broiler house, and soil surfaces 50 to 500 m from the broiler house are contaminated with ESBL-producing Enterobacteriaceae. Isolates originating from inside and outside the broiler house on the same farm showed 100% similarity by pulsed-field gel electrophoresis (PFGE; [2]). Furthermore, soil and flies on broiler farms, and surface water in the neighbourhood of these farms frequently contained ESBL-producing *E. coli* which were similar according to ESBL-gene, plasmid and sequence type to isolates obtained from broiler faeces on the same farm [3]. Individuals living close to broiler farms might, therefore, be exposed to ESBL-producing Enterobacteriaceae through the surrounding environment. Living in a municipality with high broiler density, however, did not increase the risk for carriage among humans in the general population. The overall high broiler density in the Netherlands (1301 broilers/km^2) and mobility of individuals between municipalities and provinces may have disturbed a possible relationship between broiler density and ESBL-carriage ([4]; *Chapter 3*). On the other hand, it is not known whether bacteria from poultry present in air, water, soil or wildlife can really infect humans.

All investigated Dutch broiler farms were positive for ESBL-producing *E. coli*, and these bacteria were highly prevalent on both conventional (85%, [5]; 100%, [6]; 96.4%, [7]; *Chapter 4*) and organic (94.3%, [8]; *Chapter 5*) farms at 5 weeks of age, which is just before slaughter on

conventional farms. On organic farms, the sample level prevalence decreased to 39.4-81.0% at slaughter, which takes place at approximately 70 days of age ([8]; *Chapter 5; Chapter 6*). It should be noted that this decrease might also hold true for broilers on conventional farms if they would have a longer production round. Not only transmission via the environment, but close physical contact with broilers might, therefore, lead to increased risk for carriage of ESBL/AmpC-producing *E. coli* among humans. Prevalence of carriage among humans living or working on both conventional (33%, [5]; 19.1%, [7]; *Chapter 4*) and organic (18.5%; [8]; *Chapter 5*) broiler farms was higher compared to humans in the general population (5.1%; [4]; *Chapter 3*) and Dutch patients (4.9%; [9]). It was also shown that farmers and employees who spent more hours in the stable, had more physical contact with broilers, and performed more activities in the broiler house (e.g. weighing, vaccination, health checks) than their partners or family members, were more often positive (27.1% vs. 14.3%; OR=2.5; P=0.08). Furthermore, evidence for clonal transmission of ESBL-producing *E. coli* between humans and broilers on farms was found ([7]; *Chapter 4*). This suggests that there is an increased risk of exposure for humans on broiler farms, and that this is more pronounced for individuals in close contact with broilers. ESBL-producing *E. coli* are able to transmit and persist in a broiler flock, even without selection pressure from antibiotics, as indicated by a reproduction ratio of 1.70 for total ESBL/AmpC-producing *E. coli* on an organic broiler farm (*Chapter 6*). This suggests that broilers form a reservoir of antibiotic resistance genes for other flocks and humans living or working on these farms.

The results from this thesis show that direct contact between humans, live broilers and the broiler farm environment, as experienced by people living or working on these farms, contributes to human carriage. To place this in perspective, however, there were 576 broiler farms in the Netherlands in 2014. Assuming that the average Dutch household size of 2.2 individuals also applies to the farmers' households, this means that 1267 individuals are living on broiler farms, which is only a very small percentage (0.009%; 1267/14875853) of the total Dutch population [10]. Therefore, the role of live broilers in the transmission of ESBL-producing *E. coli* to the general population is negligible.

Transmission of ESBL-producing Enterobacteriaceae in the Dutch general population

Among the 99.99% of the Dutch general population without continuous exposure to broilers, 5.1% are carriers of ESBL-producing Enterobacteriaceae (*Chapter 3*). The question is what other sources and pathways might play a role in the transmission of these resistant bacteria in the general population. Transmission between humans and other animal species, between humans, and to humans through travel abroad and consumption of food products will be discussed.

Transmission between humans and other animal species

Both occupational and domestic exposure to animals might play a role in transmission between humans and other animal species and will be discussed below.

Occupational exposure to animals

As with broilers, it can be speculated that transmission between and among humans, pigs, or cattle on farms might occur through direct contact or via the (farm) environment. Three studies have investigated carriage of ESBL-producing Enterobacteriaceae in humans living or working on pig farms. On two Danish pig farms, samples from three out of five people living or working on these farms were ESBL-positive. Human, animal, and environmental *E. coli* strains harboured indistinguishable or closely related IncN plasmids carrying $bla_{\text{CTX-M-1}}$ [11]. On 39 Danish pig farms, ESBL-producing *E. coli* were detected among 10% (19/195) of farmers, employees and residents on these farms. Overall, 70% (136/195) of individuals reported contact with pigs, and 18 out of 19 ESBL-positive individuals reported contact with pigs. For individuals having contact with pigs, the risk of being ESBL-positive was higher when ESBL-producing *E. coli* were detected in the pigsties, compared to when they were not detected (15/72; 3/64; 21% vs. 5%; P=0.0056). ESBL-producing *E. coli* isolates with the same $bla_{\text{CTX-M}}$, phylogenetic group, PFGE type and sequence type were detected in pigs and in humans in contact with pigs [12]. On 40 Dutch pig multiplier farms, overall prevalence of human ESBL-carriage was 6% (8/142). Living or working on a farm with ESBL-positive pigs (13.5%; 7/52) compared to a farm with ESBL-negative pigs (1.1%; 1/90) was associated with an increased risk of carriage (OR=12.5; P=0.02). Human carriage increased with increasing number of working hours on the farm per week (OR=1.04 per working hour; P=0.0008). On one farm, two pig *E. coli* isolates and two human isolates were identical with respect to ESBL-gene, plasmid type and sequence type, and on another farm one pig *E. coli* isolate and one human isolate were identical. It is not clear whether the two isolates that came from the same farm were from separate individuals [13]. These studies suggest that, as seen for individuals living on broiler farms, contact with positive pigs is associated with an increased risk of carriage. A similar situation might be observed in individuals living or working on veal calf farms. It was shown that human carriage of methicillin-resistant *Staphylococcus aureus* (MRSA) on veal calf farms was associated with intensity of animal contact and number of positive animals on the farm [14], but this has not been investigated for EBSL-producing Enterobacteriaceae.

Slaughterhouse employees or veterinarians might also be exposed to ESBL-positive animals, but there are no studies investigating transmission of ESBL-producing Enterobacteriaceae between humans and animals in these situations. For MRSA, however, contact with live pigs and broilers for individuals working at slaughterhouses has been shown a risk factor for carriage [15,16].

Domestic exposure to animals

While living and working on a farm, working at a slaughterhouse or working as a veterinarian represents occupational exposure to animals, there are also opportunities where humans might come into contact with animals in a domestic situation such as at farm open house events, petting zoos or by owning companion animals.

In the Netherlands, there are approximately 530 petting zoos (affiliated with the Dutch 'Vereniging Samenwerkende Kinderboerderijen Nederland') which receive an estimated 29.3 million visitors per year. Types of animals most commonly held are horse, donkey, cow, pig, sheep, goat, chicken, rabbit and Guinee pig [17]. There are reports of outbreaks of *E. coli* O157 infections associated with visits to farms and petting zoos [18,19], but there are no studies investigating prevalence of ESBL-producing Enterobacteriaceae or their transmission between humans and animals in these situations.

It has been estimated that 18% of Dutch households owned at least one dog, and 24% of households owned at least one cat. The estimated numbers of cats and dogs in the Netherlands in 2012 were, 2.7 and 1.6 million respectively [20]. Close contact between companion animals and people within the same household offers favourable conditions for transmission by direct contact or indirectly through contamination of domestic environments. No studies exist, however, that investigate transmission of ESBL-producing Enterobacteriaceae between and among dogs, cats and humans within the household. With respect to horses, a study at a veterinary clinic in the Czech Republic showed that *E. coli* isolated from a horse, a drinking trough, and a human had the same PFGE type, implying clonal transmission between humans and horses. Furthermore, similar plasmids harbouring $bla_{CTX\text{-}M\text{-}1}$ were found in isolates from horses, flies, and drinking troughs, suggesting horizontal gene transfer [21]. It was also shown that owning or having contact with a horse increased the risk for ESBL-carriage (15.6% vs. 4.4%; OR=4.69) among individuals in the general Dutch population. This might not be solely attributable to owning or having contact with a horse, as prevalence increased from 4% in individuals without companion animals to 12% in individuals who owned more than four different species of companion animals ([4]; *Chapter 3*). Owning a pet was also associated with an increased risk for ESBL-carriage (OR=6.7) among volunteers recruited at an infection control conference (nurses and physicians from hospitals in Germany and Austria; [22]).

There are studies available showing *E. coli* strain sharing between and among dogs, cats and humans in the same household, suggesting that this might also hold true for ESBL-producing Enterobacteriaceae. In a cross-sectional study that included 228 adults, children and pets from 63 households, strain sharing (i.e. highly related PFGE profile in isolates from two individuals) was more common among pet-pet pairs (22/38) than among human-human pairs (47/154) in the same household, and was more common among human-human pairs than among pet-human pairs (31/179; [23]). In a longitudinal study on 18 humans and 13 dogs living in 8 households, long-term carriage or intermittent shedding was demonstrated in most

humans and dogs; 10/18 humans studied were shown to share a clone least once with another person or with a dog in their household [24]. Although strain sharing does not necessarily indicate transmission, it is a likely explanation for these observations because of close physical contact between humans, and with their dogs or cats.

Transmission of ESBL-producing Enterobacteriaceae between humans and animals within the household is plausible, but might also occur outside the home (petting zoos and farm visits). What has not been considered so far, but is important to mention, is that households on livestock farms may also own one or more companion animals, and family members might visit petting zoos or other farms. It can be speculated that transmission between humans (within and between different households), livestock and companion animals also occurs and might be important in understanding the persistence of ESBL-producing Enterobacteriaceae.

Transmission between humans

So far studies on transmission between humans and animals resulting from professional or domestic exposure have been discussed, but in order to understand transmission in the general population human-to-human transmission should also be considered. Duration of carriage in humans will also be dealt with here because this has consequences for within and between household transmission, that is long-term carriers might form a reservoir of resistance genes for other household members leading to persistence of ESBL-producing Enterobacteriaceae within the household.

Transmission within and between households

Investigation of human-to-human transmission in the general population seems to focus mainly on the household, although there are other places such as child day-care centres [25] where human-human transmission of ESBL-producing Enterobacteriaceae might be important. Human-to-human transmission is also relevant for medical personnel in contact with patients who are carriers, but this is beyond the scope of this discussion. Available studies in the general population mainly concern household contacts of index patients with an infection caused by ESBL-producing Enterobacteriaceae. The prevalence of faecal carriage was significantly higher in household members, and non-household relatives of patients diagnosed with community-acquired urinary tract infections caused by ESBL-producing *E. coli* (23.8%; 25/105) than in unrelated individuals, not living in the same household (7.4%; 4/54). Out of 19 families (household members and non-household relatives) with a least two carriers, eight families included carriers of isolates with the same PFGE pattern and ESBL-gene [26]. In another Spanish study, faecal carriage was 70% (28/40) among intensive care patients with community acquired infections with ESBL-producing bacteria, and was 16.7% (9/54) in household contacts of these patients. This is higher than the prevalence in the general Spanish population measured in the same geographic area (3.7%; [27]). Furthermore, up

to 66.7% (6/9) of intensive care patients and their corresponding household members had indistinguishable PFGE patterns [28]. In Norway, 20% (12/60) of household contacts of infants who became carriers of two unrelated *bla*$_{CTX-M-15}$ carrying *Klebsiella pneumoniae* clones during a neonatal intensive care unit outbreak, were carriers of the same strain as the infants [29]. In Switzerland, ESBL-producing *E. coli* was found in 35.2% (31/88) of household contacts of patients presenting with carriage or infection with ESBL-producing Enterobacteriaceae, and based on *E. coli* phylogenetic group, sequence type and PFGE pattern, transmission was plausible for 22.7% (20/88) of contacts. Furthermore, evidence for transmission of ESBL-producing *K. pneumoniae* was found in 25.0% (2/8) contacts [30].

Strain sharing among household members seems to occur regularly, especially if one of the members was infected with ESBL-producing Enterobacteriaceae. This suggests that patients returning home might be an important source of ESBL-producing Enterobacteriaceae for their household contacts, including both humans and pets. Furthermore, if patients are returning to their home on a livestock farm this might also have implications for transmission between household members, their pets and livestock. It can also be speculated that patients and individuals living or working on farms form a source of ESBL-producing Enterobacteriaceae for humans outside their own household [31].

Duration of carriage

Information about the duration of carriage of ESBL-producing Enterobacteriaceae in humans in the general population is lacking, however some information is available for specific sub-groups such as patients and travellers. In infants who became carriers of *bla*$_{CTX-M-15}$ carrying *Klebsiella pneumoniae* clones during a neonatal intensive care unit outbreak (n=51) and colonised parents (n=11) there was a significant difference in median length of carriage: for infants this was 12.5 (IQR (interquartile range) 9.0-17.5) months, and for parents 2.5 (IQR 1.0-5.0) months [29]. In adult Swedish patients who were diagnosed with an infection caused by ESBL-producing Enterobacteriaceae, carriage was observed in 84% (51/61) of patients after one month, in 66% of patients (36/61) after 3 months, in 55% (31/61) after 6 months, and in 43% (26/61) after 12 months. In 28% (17/61) of patients, ESBL-production was found in a different bacterial strain or species during follow up than at the beginning of the study [32]. In Sweden, 24 out of 100 travellers who were initially ESBL-negative became positive after travel, and five out of 21 participants who completed the follow-up after 6 months were still colonised, but it was not reported whether this was with the same strain [33]. Of 113 Dutch travellers who had initially negative pre-travel samples and positive samples immediately after return, 16.8% (19/113) were still colonised after 6 months, and 7/19 participants had samples that were positive for *E. coli* with the same sequence type and CTX-M-group gene [34].

These studies show that carriage in humans can be persistent and intermittent, but it is difficult to give an overall estimate of duration of carriage due to, for example, differences in definition of persistence and clearance, lack of molecular information, and the fact that information is limited to specific groups of individuals (not a random sample).

Travel abroad

In 2014, 12.5 million Dutch people went on vacations: 4.6 million went abroad only, 3.1 went on vacations in the Netherlands only, and 4.8 went on vacations abroad as well as in the Netherlands [10]. Prevalences of ESBL-producing Enterobacteriaceae among humans in the general population, as well as distributions of ESBL-genes, vary across geographic regions [35]. Individuals might face an increased level of exposure when travelling to countries where prevalences are higher than in the Netherlands. Multiple studies have identified travel abroad as a risk factor for carriage of ESBL-producing Enterobacteriaceae in humans in the general population, with increased rates of colonisation after travel. In Canadian patients with community-onset infections caused by ESBL-producing *E. coli* 43.6% (71/163) reported overseas travel, and this was associated with increased risk of infection (RR=5.7; P=<0.0001; [36]). Of 100 ESBL-negative Swedish travellers planning a trip outside of Northern Europe, 24 had acquired ESBL-producing Enterobacteriaceae during travel. Travellers visiting India showed the highest rate of acquisition of ESBL-producing Enterobacteriaceae with 88% (7/8) colonised upon return, compared to 0% (0/1), 0% (0/2), 0% (0/6), 4% (1/25), 13% (2/16), 29% (4/14), and 32% (10/31) colonised for travellers to South America, North America, Central America, Africa, Southern Europe, Middle East and Asia (excluding India), respectively (three participants visited more than one continent; [33]). In Dutch travellers (n=370), 8.6% were colonised with ESBL-producing Enterobacteriaceae before travel, and 30.5% acquired these bacteria during travel. Travel to South and East Asia were associated with an increased risk, while travel to Southeast Asia, Central Asia, Africa, the Middle East, South America, and Central America and the Caribbean were not [34].

These studies indicate that travellers returning to their home country might be an important source of ESBL-producing Enterobacteriaceae for the general population, also in light of the evidence given for within and between household transmission.

Food products

ESBL- and AmpC-producing Enterobacteriaceae have been isolated from various types of retail meat, including chicken, turkey, pork, and beef, and in vegetables meant for raw consumption, such as celery, carrots and lettuce.

Resistance to cefotaxime, an indicator for ESBL, in randomly selected *E. coli* isolates recovered from raw chicken, pork and beef was 9.1%, 3.6%, and 1.2%, respectively, in Europe [37]. In the Netherlands, this was 2.3%, 1.9%, 1.9%, and 0.9% from turkey, chicken, beef

and pork, respectively. No positive isolates were found in veal and lamb [38]. With selective enrichment, prevalences in raw fresh meat were 67.0% in chicken, 50.9% in turkey, 3.1% in veal, 2.7% in pork and 2.2% in beef. Again no positive isolates were found in lamb [38]. It should be noted that an unknown portion of samples originated from imported meat, therefore this does not necessarily correspond with what is present in faecal samples from animals in the Netherlands. Concentrations of ESBL-producing bacteria on meat are not included in the European and Dutch monitoring programmes, however some data for chicken meat is available. Median loads of ESBL-producing microorganisms were 80 CFU/25g (range <20-1360) in conventional poultry meat samples versus <20 CFU/25g (range 0-260) in organic samples [39], but these are not available for other types of meat.

Vegetables are also not included in the European and Dutch monitoring programmes, but two Dutch studies investigated samples from for example blanched celery, bunched carrots, butterhead lettuce, endive, iceberg lettuce, bean sprouts, spring onion, radish and parsnip. A 5% (63/1216) prevalence of third-generation cephalosporin resistant Enterobacteriaceae in vegetables after selective enrichment was shown, but only 0.8% of all samples were ESBL-producing faecal Enterobacteriaceae [40]. Another study showed a 6% (7/119) prevalence of ESBL-producing faecal Enterobacteriaceae in vegetables after selective enrichment [41].

Thorough cooking destroys bacteria, but there may be cross contamination in the kitchen between meat and vegetables meant for raw consumption through handling of the food or via implements [42]. ESBL-producing *E. coli* were detected on cutting boards after poultry meat preparation and before cleaning in both the hospital kitchen and private homes. ESBL-producing *E. coli* were not detected on cutting boards after preparation of beef, pork, lamb, game and vegetables. The meat and vegetables themselves were not sampled in this study, however [43]. Undercooking of meat preparations such as minced meat, burger or sausage might also lead to ingestion of ESBL-producing Enterobacteriaceae as these bacteria might be found in the meat, and not just on the surface [42]. Vegetables can become contaminated with ESBL-producing Enterobacteriaceae by growth in contaminated soil, and irrigation with contaminated water, but also during processing [44]. When consumed raw, ESBL-producing Enterobacteriaceae may be transferred to humans.

In Chapter 3 of this thesis consumption of meat, chicken meat and vegetables from the garden were investigated, but not found to be a risk factor for carriage among humans in the general Dutch population ([4]; *Chapter 3*). Other studies investigating meat and/or vegetable consumption, reached a similar conclusion [26,45]. Circumstantial evidence for transmission of ESBL-producing Enterobacteriaceae to humans is available from other studies, however. For example, a foodborne outbreak of ESBL-producing Enterobacteriaceae has been reported, namely the German outbreak of *E. coli* O104:H4 harbouring $bla_{\text{CTX-M-15}}$ associated with fenugreek sprouts [46]. Case-control and cohort studies implicated a certain producer of sprouts as the source of the outbreak, but these bacteria were not detected in the sprouts

themselves or in environmental samples [47]. Similar genes, plasmids and *E. coli* sequence types (based on multi-locus sequence typing; MLST) have been found in isolates from meat and humans, suggesting transfer of ESBL-producing *E. coli* to humans [48]. Whole-genome sequencing (WGS) of isolates from the aforementioned study collected from humans and broiler meat showed that there was a difference of 1263 Single-Nucleotide Polymorphisms (SNPs) per Mbp core genome between them, while for spatiotemporally-related isolates from human and pigs that were expected to be clonally related this was only 1.8 SNPs per Mbp core genome [49]. Drawing conclusions about transmission based on gene, plasmid and sequence type can therefore be highly speculative in isolates that are not spatiotemporally related.

It is clear that humans are exposed to ESBL-producing Enterobacteriaceae via food products, but present knowledge is insufficient to determine the consequences of ingestion for carriage. Factors that need to be considered are the ability of bacteria of livestock origin to survive and multiply in the human gastrointestinal tract, and the role of horizontal gene transfer between bacteria of livestock and human origin. It has been shown that inoculation of mice with ESBL-producing *E. coli* of avian origin leads to short-term colonisation, and conjugation of plasmids was not observed [50]. However, an *in situ* continuous flow culture system simulating the human caecum and ascending colon showed that conjugation of plasmids from avian to human *E. coli* is possible, even without selective pressure from antibiotics [51]. It can be speculated that the role of chicken meat in human carriage is limited, but data are lacking to be able to suggest the same for other types of meat and vegetables.

Implications of results and directions for future research

A number of sources and pathways that might be relevant for the transmission of ESBL-producing Enterobacteriaceae between humans, and between animals and humans have been given in the sections above. The relative contribution of each of these to carriage among humans remains to be elucidated, however. Attribution is the process of determining how much of a given infection is due to particular sources and pathways [52]. Attribution is also a useful tool to prioritise intervention strategies, but in order to determine the impact of these measures it is necessary to understand transmission dynamics and ultimately quantify transmission in human and animal populations. In this section, approaches that might be used to attribute ESBL/AmpC-producing Enterobacteriaceae to different sources and routes, and factors that might hamper the development of these approaches are given. This is followed by a discussion of the importance of understanding dynamics of transmission in livestock and other animal populations, and potential strategies for control. Finally, the main conclusions of this thesis are given.

Relative contribution of different sources and routes to carriage among humans in the general population

Two different approaches might be used to attribute ESBL/AmpC-producing Enterobacteriaceae to different sources and routes. First, to estimate the relative exposure to resistance determinants through various transmission routes (e.g. direct contact, via the environment, travel abroad, consumption of food) a comparative exposure assessment might be used. To estimate the exposure dose for each transmission route, information on the prevalence and dose of the resistance determinant in the source, the changes in prevalence and quantity of the resistance determinant throughout the transmission chain, and the frequency at which humans are exposed by that route is required. The exposure doses are compared, and the human disease burden caused by particular resistance determinants is partitioned to each of the various transmission routes, proportionally to the size of the exposure dose [52]. Second, to estimate the relative importance of different sources (e.g. livestock, companion animals, environment) for exposure to the resistance determinant in humans, a source attribution model based on microbial subtyping data might be used. The principle behind this method is to compare bacterial genetic profiles found in the various sources with the ones found in humans based on for example, DNA sequence data. Information that would be required for a source attribution model based on microbial subtyping includes a collection of isolates from humans and different sources, subtyping methods that can provide detailed knowledge of all types in relevant sources, and a model to infer sources of human infection based on the subtype distributions obtained within each investigated source [53].

Models have been developed that attribute foodborne infections to their sources and pathways using microbial subtyping [54,55] and comparative exposure assessment [56]. Perhaps these models could be adapted for ESBL-producing Enterobacteriaceae. Several factors can be identified that might hamper the adaptation of these models for ESBL-producing Enterobacteriaceae, and indicate directions for future research. First, selection of typing methods that are discriminatory enough to enable recognition of correlation between human isolates and their sources, but not too discriminatory so that there is a risk that correlated isolates may not be recognised as related [53]. It has been shown that similarity between isolates cannot be based on sequence type, plasmid family and ESBL-gene alone in case isolates are not spatiotemporally related [49]. Whole-genome sequencing might provide a suitable alternative, but then the question still remains whether it is more accurate to look at virulence and resistance gene content, the genetic backbone of the host strain or a combination of these. Furthermore, transfer of resistance genes between chromosome and plasmid, between plasmids, and between bacteria makes it difficult to establish correlations between isolates from humans and those from possible sources. Second, detailed molecular typing data is not available from all putative sources. Isolates from patients, livestock and meat have been systematically collected in the Dutch and European monitoring programmes,

but this is not the case for companion animals, humans in the general population, and the natural environment for example. Studies quantifying ESBL-producing Enterobacteriaceae in putative sources and changes in concentration along various transmission pathways to sites for human exposure are available for broilers, but is lacking for other sources. For example, quantitative data is available on ESBL-producing *E. coli* shed by broilers [57], on reduction through processing in the slaughterhouse [58], and on concentration in retail meat [39]. Approaches to determine the relative contribution of different sources to carriage of ESBL-producing Enterobacteriaceae are considered here but it is important to keep in mind that the relationship between exposure, infection, carriage and disease is unclear, making it difficult to determine the implications of different sources and pathways for human health.

Dynamics of transmission in livestock and other animal populations

Equally important to future research into sources and pathways that contribute to human carriage, is the continued elucidation of transmission dynamics in livestock, but also other animal populations and more specifically companion animals as these are in close contact to humans.

In order to understand transmission dynamics on livestock farms, longitudinal studies have been conducted examining the prevalence of ESBL-producing Enterobacteriaceae in different livestock species ([6,59,60]; *Chapter 6*). On two pig farms all investigated age groups were positive for CTX-M positive coliforms: sows in the last week of gestation (35-71%), piglets during the first week after birth (56-91%) and just before weaning (71-97%), weaners 6 weeks after weaning (21-56%) and finishers just before slaughter (16-17%). Prevalence of carriage increased until weaning, while a decrease was observed in weaning and finishing sections, with the lowest prevalence being found before slaughter. Mean faecal counts of CTX-M positive coliforms decreased from piglets (10^7 CFU/g) to weaners (10^5 CFU/g) and from weaners to finishers (10^3 CFU/g). On a third farm, which applied all-in/all-out management in the farrowing and finishing sections and continuous production in the weaning section compared to continuous production in all sections on the other two farms, only weaners tested positive (10%). On one farm $bla_{\text{CTX-M-9}}$ were found while on the other two farms this was $bla_{\text{CTX-M-1}}$, and no shift in genotypes was reported. Data on individual animals were unfortunately not presented and environmental samples of the different sections were not taken [59]. On three veal calf farms, prevalence was comparable upon arrival of the animals at the farm (18-26%) and a similar distribution of primarily $bla_{\text{CTX-M-1}}$ genes was shown. In the following ten weeks of the study, each farm showed a different pattern of prevalence: on one farm ESBL/AmpC-producing *E. coli* were not detected after the first sampling, on another farm prevalence increased to 37% at week 3 and then decreased below detection level at week 10, and on the third farm prevalence decreased to 1.4% at week 10. Between arrival and the next sampling moment at week 3 a shift in the predominant gene group occurred

from $bla_{CTX\text{-}M\text{-}1}$ to $bla_{CTX\text{-}M\text{-}9}$, which according to the authors might be explained by presence below detection level at arrival, presence and acquisition through the stable environment or as a result of antibiotic treatment. Although calves were housed individually until week 6, no data on individual animals was reported and no samples of the farm environment were taken [60]. On three conventional broiler farms, environmental samples from the broiler house were positive for EBSL/AmpC-producing *E. coli* before arrival of chicks, and in day-old chicks the prevalence ranged from 0-20% upon arrival at the farm. After one week the prevalence on these farms was 96-100% and it was 100% on all farms until the end of the study at 5 weeks of age. ESBL-genes, plasmids and sequence types were not determined so shifts in genotypes during the study period were not observed [6]. In a longitudinal study on an organic farm, broilers were followed individually from arrival till slaughter age (*Chapter 6*). This is the first study to quantify transmission of ESBL/AmpC-producing *E. coli* in an animal population and explore the effect of direct contact, relative to the environment in transmission. Total ESBL/AmpC-producing *E. coli* are able to transmit and persist in an organic broiler flock, but this was not the case for individual phylogenetic groups as indicated by reproduction ratios less than one for groups separately. Persistence of total ESBL/AmpC-producing *E. coli* might therefore be determined by repeated shifts from one phylogenetic group to another between arrival on the farm and slaughter age. Furthermore, the risk for an individual to become infected with ESBL/AmpC-producing *E. coli* was lower if it had been infected previously, which might be explained by the acquisition of immunity to particular *E. coli* genotypes. The environment was relatively more important in the transmission of the A1 phylogenetic group, and direct contact between broilers was relatively more important for the transmission of the B1 phylogenetic group, for those broilers that were not previously infected. These results suggest that the contaminated environment before the start of the production round and positive day-old chicks both play a role in the introduction and transmission of ESBL/AmpC-EC in the new flock.

A decrease in prevalence of ESBL-producing Enterobacteriaceae was seen on all types of farms towards slaughter except on conventional broiler farms ([6,59,60]; *Chapter 6*), but as mentioned previously this might be related to the length of production round. Furthermore, there appears to be a shift in genotypes on both veal calf and organic broiler farms, but unfortunately data is lacking to determine if this is also the case on pig and conventional broiler farms. It would be useful to apply the methods from Chapter 6 on other livestock farms to estimate duration of carriage, quantify transmission parameters, and elucidate the role of direct contact, relative to the environment in transmission. Also, it is important to confirm present findings for organic broiler farms and compare this to conventional broiler farms. Information on transmission dynamics will provide starting points for intervention on livestock farms, which will lead to fewer contaminated animals. Fewer contaminated animals both on farms and entering the slaughterhouse could affect carriage in humans exposed to these animals.

For companion animals only one longitudinal study has been published, which included 38 dogs from 24 owners that were sampled once per month for a period of 6 months. Dogs showed a monthly, and in some cases even a weekly, shift in shedding of ESBL-producing Enterobacteriaceae. It was also shown that when dogs were positive this was not with the same ESBL-gene throughout the study period. Unfortunately, *E. coli* genotypes and plasmids were not determined [61]. In Chapter 6 it was speculated that persistence of ESBL/AmpC-producing *E. coli* during a production round was determined by shifts in genotypes. This might also be an explanation for persistence in dogs, but the factors driving these shifts remain to be elucidated.

Control strategies on livestock farms

ESBL-producing Enterobacteriaceae from livestock might represent a reservoir of resistance genes, and reasons for maintenance of this reservoir are needed to inform control strategies. In the Netherlands, the total use of antimicrobials in farm animals was reduced by 56% between 2007 and 2012, and the aim is to accomplish a 70% reduction in 2015 [62]. Cefotaxime-resistant *E. coli* decreased in farm animals from 2008-2013, but has levelled off in 2014 [38]. Prudent use of antimicrobials and efforts to reduce antibiotic use should be continued, however it is has been suggested that while reducing antibiotic use will have an impact on resistance, it will not eliminate it. Resistance is often associated with reduced bacterial fitness, and it is proposed that a reduction in antibiotic use would benefit the fitter susceptible bacteria, allowing them to outcompete resistant strains over time. Compensatory evolution and co-selection of resistance to more than one antibiotic limit the reversibility of resistance, however [63]. In an *in vitro* experiment fitness cost resulting in a lower bacterial growth rate or lower maximum density due to presence of the plasmid IncI1 carrying the $bla_{\text{CTX-M-1}}$ gene, frequently identified in humans and animals, were not observed [64], lending support to this idea. This suggests that reduction of antibiotic use alone will not be enough to eliminate the reservoir of resistance genes in livestock.

Maintenance of resistance genes in livestock might also be explained by the presence of other potential reservoirs of resistance genes (e.g. on the same farm), that is humans, companion animals, and vermin (i.e. flies, rats). Although it has been indicated that reducing antibiotic use will not reverse resistance, also prudent use of antimicrobials in humans and companion animals should be pursued. The Netherlands had an antibiotic consumption rate of 10.4 defined daily doses (DDD) per 1000 inhabitants per day in humans in 2013, which is the lowest in Europe. In comparison, the highest consumption rate was shown in Greece and was 32.2 DDD/1000 inhabitants/day [65]. It can, therefore, be speculated that antimicrobial use in humans in the Netherlands is currently prudent. Also the occurrence of cefotaxime-resistance among human clinical isolates, and indicator for ESBL-production, is one of the lowest in Europe [66]. Systematic reporting on antimicrobial consumption and

antimicrobial resistance in companion animals is currently lacking, which is an important data gap. Besides reduction of antibiotic use, control strategies on livestock farms that limit interactions between livestock, companion animals, wildlife, vermin and humans should be considered. Examples include, preventing companion animals from entering animal housing, active control of vermin and strict hygiene protocols for veterinarians or other personnel entering the animal houses. In the case of organic broilers, it was shown that a contaminated environment before the start of the production round and positive day-old chicks both play a role in the introduction and transmission of ESBL/AmpC-producing *E. coli* in the new flock (*Chapter 6*). To prevent introduction through day-old chicks it is necessary to consider all previous steps in the broiler production chain, that is (great)grandparent and parent stock, and hatcheries [6]. Veal calves were also positive upon arrival on the farm [60], which suggests that here previous steps in the production chain also need to be taken into account for control. Future studies should quantify the effectiveness of intervention measures on farms that take into account all different reservoirs of resistance genes, but also at previous points in the animal production chain.

Main conclusions of this thesis

To summarise, all investigated conventional and organic broiler farms were positive for ESBL/AmpC-producing Enterobacteriaceae, and these bacteria were able to persist and transmit in a flock without antibiotic pressure. Prevalence in humans living or working on farms was higher than in the general population. Also, individuals living or working on farms with a high degree of contact with broilers were at an increased risk for carriage compared to those individuals with a low degree of contact. Furthermore, evidence for clonal transmission of ESBL/AmpC-producing *E. coli* between humans and broilers on farms was found. These results suggest that direct contact with broilers contributes to carriage of individuals on farms. As only a very small percentage of the general population is exposed to live broilers, compared to the percentage of individuals owning pets, visiting petting zoos and travelling abroad, direct contact with broilers does not appear to be important for carriage in the general human population. Putative sources and pathways include companion animals, other humans, travel abroad and food products. Their relative contribution to carriage of ESBL-producing Enterobacteriaceae among humans in the general population remains to be elucidated, and more insight in dynamics of transmission in both livestock and other animal populations in close contact with humans is needed.

References

1. Huijbers, P.M.C., Blaak, H., de Jong, M.C.M., *et al*. 2015. Role of the environment in the transmission of antimicrobial resistance to humans: A review. *Environmental Science and Technology*. doi:10.1021/acs.est.5b02566.
2. Laube, H., Friese, A., von Salviati, C., *et al*. 2014. Transmission of ESBL/AmpC-producing *Escherichia coli* from broiler chicken farms to surrounding areas. *Veterinary Microbiology*. 172:519-527.
3. Blaak, H., van Hoek, A.H.A.M., Hamidjaja, R.A., *et al*. 2015. Distribution, numbers, and diversity of ESBL-producing *E. coli* in the poultry farm environment. *PLoS One*. 10:e0135402.
4. Huijbers, P.M.C., de Kraker, M., Graat, E.A.M., *et al*. 2013. Prevalence of extended-spectrum β-lactamase-producing Enterobacteriaceae in humans living in municipalities with high and low broiler density. *Clinical Microbiology and Infection*. 19:E256-259.
5. Dierikx, C.M., van der Goot, J.A., Fabri, T., *et al*. 2013a. Extended-spectrum-β-lactamase-producing *Escherichia coli* in Dutch broilers and broiler farmers. *Journal of Antimicrobial Chemotherapy*. 68:60-67.
6. Dierikx, C.M., van der Goot, J.A., Smith, H.E., *et al*. 2013b. Presence of ESBL/AmpC-producing *Escherichia coli* in the broiler production pyramid: a descriptive study. *PLoS One*. 8:e79005.
7. Huijbers, P.M.C., Graat, E.A.M., Haenen, A.P.J., *et al*. 2014a. Extended-spectrum β-lactamase-producing *Escherichia coli* in broilers and people living and/or working on broiler farms: prevalence, risk factors and molecular characteristics. *Journal of Antimicrobial Chemotherapy*. 69:2669-2675.
8. Huijbers, P.M.C., van Hoek, A.H.A.M., Graat, E.A.M., *et al*. 2014b. Methicillin-resistant *Staphylococcus aureus* and extended-spectrum and AmpC β-lactamase-producing *Escherichia coli* in broilers and in people living and/or working on organic broiler farms. *Veterinary Microbiology*. 176:120-125.
9. Overdevest, ITMA., Willemsen, I., Rijnsburger, M., *et al*. 2011. Extended-spectrum β-lactamase genes of *Escherichia coli* in chicken mean and humans, the Netherlands. *Emerging Infectious Diseases*. 17:1216-1222.
10. CBS (Centraal Bureau voor de Statistiek). 2015. www.cbs.nl (last accessed 29 September 2015).
11. Moodley, A., Guardabassi, L. 2009. Transmission of IncN plasmids carrying $bla_{CTX\text{-}M\text{-}1}$ between commensal *Escherichia coli* in pigs and farm workers. *Antimicrobial Agents and Chemotherapy*. 53:1709-1711.
12. Hammerum, A.M., Larsen, J., Andersen, V.D., *et al*. 2014. Characterization of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* obtained from Danish pigs, pig farmers and their families from farms with high or no consumption of third- or fourth-generation cephalosporins. *Journal of Antimicrobial Chemotherapy*. 69:2650-2657.
13. Dohmen, W., Bonten, M.J.M., Bos, M.E.H., *et al*. 2015. Carriage of extended-spectrum β-lactamases in pig farmers is associated with occurrence in pigs. *Clinical Microbiology and Infection*. doi:10.1016/j.cmi.2015.05.032.
14. Graveland, H., Wagenaar, J.A., Heesterbeek, H., *et al*. 2010. Methicillin-resistant *Staphylococcus aureus* ST398 in veal calf farming: human MRSA carriage related with animal antimicrobial usage and farm hygiene. *PloS One*. 5:e10990.
15. van Cleef, B.A.G.L., Broens, E.M., Voss, A., *et al*. 2010. High prevalence of nasal MRSA carriage in slaughterhouse workers in contact with lives pigs in the Netherlands. *Epidemiology and Infection*. 138:756-763.
16. Mulders, M.N., Haenen, A.P.J., Geenen, P.L., *et al*. 2010. Prevalence of livestock-associated MRSA in broiler flocks and risk factors for slaughterhouse personnel in the Netherlands. *Epidemiology and Infection*. 138:743-755.
17. vSKBN (Vereniging Samenwerkende Kinderboerderijen Nederland). 2014. Branche kinderboerderijen 2014. http://www.kinderboerderijen.nl/kinderboerderijen-in-nederland (last accessed 29 September 2015).
18. Crump, J.A., Sulka, A.C., Langer, A.J., *et al*. 2002. An outbreak of *Escherichia coli* O157:H7 infections among visitors to a dairy farm. *New England Journal of Medicine*. 347:555-560.
19. Goode, B., O'Reilly, C., Dunn, J., *et al*. 2009. Outbreak of *Escherichia coli* O157:H7 infections after petting zoo visits, North Carolina state fair, October-November 2014. *Archives of Pediatrics and Adolescent Medicine*. 163:42-48.
20. FEDIAF (European Pet Food Industry Federation). 2012. The European pet food industry facts and figures. www.fediaf.org/facts-figures (last accessed 29 September 2015).
21. Doljeska, M., Duskova, E., Rybarikova, J., *et al*. 2011. Plasmids carrying $bla_{CTX\text{-}M\text{-}1}$ and *qnr* genes in

Escherichia coli isolates from an equine clinic and horseback riding centre. *Journal of Antimicrobial Chemotherapy*. 66:757-764.
22. Meyer, E., Gastmeier, P., Kola, A., *et al*. 2012. Pet animals and foreign travel are risk factors for colonisation with extended-spectrum β-lactamase-producing *Escherichia coli*. *Infection*. 40:685-687.
23. Johnson, J.R., Owens, K., Gajewski, A., *et al*. 2008. *Escherichia coli* colonization patterns among human household members and pets, with attention to acute urinary tract infection. *Journal of Infectious Diseases*. 197:218-224.
24. Damborg, P., Nielsen, S.S., Guardabassi, L. 2009. *Escherichia coli* shedding patterns in humans and dogs: insights intro within-household transmission of phylotypes associated with urinary tract infections. *Epidemiology and Infection*. 137:1457-1464.
25. Koningstein, M., Leenen, M.A., Mughini-Gras, L., *et al*. 2015. Prevalence and risk factors for colonization with extended-spectrum cephalosporin-resistant *Escherichia coli* in children attending daycare centers: A cohort study in the Netherlands. *Journal of the Pediatric Infectious Diseases Society*. doi: 10.1093/jpids/piv042.
26. Rodríguez-Baño, J., López-Cerero, L., Navarro, M.D., *et al*. 2008. Faecal carriage of extended-spectrum β-lactamase-producing *Escherichia coli*: prevalence, risk factors and molecular epidemiology. *Journal of Antimicrobial Chemotherapy*. 62:1142–1149.
27. Valverde, A., Coque, T.M., Paz Sánchez-Moreno, M., *et al*. 2004. Dramatic increase in prevalence of fecal carriage of extended-spectrum β-lactamase-producing Enterobacteriaceae during nonoutbreak situations in Spain. *Journal of Clinical Microbiology*. 42:4769-4775.
28. Valverde, A., Grill, F., Coque, T.M., *et al*. 2008. High rate of intestinal colonization with extended-spectrum-β-lactamase-producing organisms in household contacts of infected community patients. *Journal of Clinical Microbiology*. 46:2796-2799.
29. Löhr, I.H., Rettedal, S., Natås, O.B., *et al*. 2013. Long-term faecal carriage in infants and intra-household transmission of CTX-M-15-producing *Klebsiella pneumoniae* following a nosocomial outbreak. *Journal of Antimicrobial Chemotherapy*. 68:1043-1048.
30. Hilty, M., Betsch, B.Y., Bögli-Stuber, K., *et al*. 2012. Transmission dynamics of extended-spectrum β-lactamase-producing Enterobacteriaceae in the tertiary care hospital and household setting. *Clinical Infectious Diseases*. 55:967-975.
31. Larsen, J., Petersen, A., Sørum, M., *et al*. 2015. Methicillin-resistant *Staphylococcus aureus* CC398 is an increasing cause of disease in people with no livestock contact in Denmark, 1999 to 2011. *Eurosurveillance*. 20:30021.
32. Titelman, E., Hasan, C.M., Iversen, A., *et al*. 2014. Faecal carriage of extended-spectrum β-lactamase-producing Enterobacteriaceae is common 12 months after infection and is related to strain factors. *Clinical Microbiology and Infection*. 20:O508-O515.
33. Tängdén, T., Cars, O., Melhus, Å., *et al*. 2010. Foreign travel is a major risk factor for colonization with *Escherichia coli* producing CTX-M-type extended-spectrum β-lactamases: a prospective study with Swedish volunteers. *Antimicrobial Agents and Chemotherapy*. 54:3564-3568.
34. Paltansing, S., Vlot, J.A., Kraakman, M.E.M., *et al*. 2013. Extended-spectrum β-lactamase-producing Enterobacteriaceae among travellers from the Netherlands. *Emerging Infectious Diseases*. 19:1206-1213.
35. Woerther, P.L., Burdet, C., Chachaty, E., *et al*. 2013. Trends in human fecal carriage of extended-spectrum beta-lactamases in the community: Toward the globalization of CTX-M. *Clinical Microbiology Reviews*. 26:744-758.
36. Laupland, K.B., Church, D.L., Vidakovich, J., *et al*. 2008. Community-onset extended-spectrum β-lactamase (ESBL) producing *Escherichia coli*: Importance of international travel. *Journal of Infection*. 57:441-448.
37. EFSA/ECDC (European Food Safety Authority / European Centre for Disease Prevention and Control). 2015. EU summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2013. *EFSA Journal*. 13:4036.
38. MARAN. 2015. Monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2014. Central Veterinary Institute of Wageningen UR, Lelystad. www.cvi.wur.nl/ (last accessed 29 September 2015).
39. Cohen Stuart, J,. van den Munckhof, T., Voets, G., *et al*. 2012. Comparison of ESBL contamination in organic and conventional retail chicken meat. *International Journal of Food Microbiology*. 154:212-214.
40. van Hoek, A.H.A.M., Veenman, C., van Overbeek, W.M., *et al*. 2015. Prevalence and characterization of EBSL- and AmpC-producing Enterobacteriaceae on retail vegetables.

International Journal of Food Microbiology. 204:1-8.
41. Reuland, E.A., al Naiemi, N., Raadsen, S.A., *et al*. 2014. Prevalence of ESBL-producing Enterobacteriaceae in raw vegetables. *European Journal of Clinical Microbiology and Infectious Diseases*. 33:1843-1846.
42. Depoorter, P., Persoons, D., Uyttendaele, M., *et al*. 2012. Assessment of human exposure to 3rd generation cephalosporin resistant *E. coli* (CREC) through consumption of broiler meat in Belgium. *International Journal of Food Microbiology*. 159:30-38.
43. Tschudin-Sutter, S., Frei, R., Stephan, R., *et al*. 2014. Extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae: A threat from the kitchen. *Infection Control and Hospital Epidemiology*. 35:581-584.
44. Franz, E., van Bruggen, A.H.C. 2008. Ecology of *E. coli* O157:H7 and *Salmonella enterica* in the primary vegetable production chain. *Critical Reviews in Microbiology*. 34:143-161.
45. Søraas, A., Sundsfjord, A., Sandven, I., *et al*. 2013. Risk factors for community-acquired urinary tract infections caused by ESBL-producing Enterobacteriaceae - A case-control study in a low prevalence country. *PloS One*. 8:e69581.
46. Frank, C., Werber, D., Cramer, J.P., *et al*. 2011. Epidemic profile of Shiga-toxin-producing *Escherichia coli* O104:H4 outbreak in Germany. *New England Journal of Medicine*. 365:1771-1780.
47. Buchholz, U., Bernard, H., Werber, D., *et al*. 2011. German outbreak of *Escherichia coli* O104:H4 associated with sprouts. *New England Journal of Medicine*. 365:1763-1770.
48. Leverstein-van Hall, M.A., Dierikx, C.M., Cohen Stuart, J., *et al*. 2011. Dutch patients, retail chicken meat and poultry share the same ESBL genes, plasmids and strains. *Clinical Microbiology and Infection*. 17:873-880.
49. de Been, M., Lanza, V.F., de Toro, M., *et al*. 2014. Dissemination of cephalosporin resistance genes between *Escherichia coli* strains from farm animals and humans by specific plasmid lineages. *PLoS Genetics*. 10:e1004776.
50. van Duijkeren, E., van Overbeek, W.M., Huijbers, P.M.C., *et al*. 2015. Inoculation of mice with avian $bla_{CTX\text{-}M\text{-}1}$- or $bla_{CMY\text{-}2}$- carrying *Escherichia coli* strains does not lead to long-term colonization. *Journal of Antimicrobial Chemotherapy*. doi: 10.1093/jac/dkv216.
51. Smet, A., Rasschaert, G., Martel, A., *et al*. 2011. *In situ* ESBL conjugation from avian to human *Escherichia coli* during cefotaxime administration. *Journal of Applied Microbiology*. 110:541-549.
52. Pires, S.M., Evers, E.G., van Pelt, W., *et al*. 2009. Attributing the human disease burden of foodborne infections to specific sources. *Foodborne Pathogens and Disease*. 6:417-424.
53. Barco, L., Barrucci, F., Olsen, J.E., *et al*. 2013. Salmonella source attribution based on microbial subtyping. *International Journal of Food Microbiology*. 163:193-203.
54. van Pelt, W., van de Giessen, A.W., van Leeuwen, W.J., *et al*. 1999. Oorsprong, omvang en kosten van humane salmonellose. Deel 1. *Infectieziekten Bulletin*. 10:240-243.
55. Hald, T., Vose, D., Wegener, H.C., *et al*. 2004. A Bayesian approach to quantify the contribution of animal-food sources to human salmonellosis. *Risk Analysis*. 24:251-265.
56. Evers, E.G., van der Fels-Klerx, H.J., Nauta, M.J., *et al*. 2008. Campylobacter source attribution by exposure assessment. *International Journal of Risk Assessment and Management*. 8:174-190.
57. Horton, R.A., Randall, L.P., Snary, E.L., *et al*. 2011. Fecal carriage and shedding density of CTX-M extended-spectrum β-lactamase-producing *Escherichia coli* in cattle, chickens and pigs: implications for environmental contamination and food production. *Applied and Environmental Microbiology*. 77:3715-3719.
58. Pacholewicz, E., Liakopoulos, A., Swart, A., *et al*. 2015. Reduction of extended-spectrum-β-lactamase- and AmpC-β-lactamase-producing *Escherichia coli* through processing in two broiler chicken slaughterhouses. *International Journal of Food Microbiology*. 215:57-63.
59. Hansen, K.H., Damborg, P., Andreasen, M., Nielsen, S.S., Guardabassi, L. 2013. Carriage and fecal counts of cefotaxime M-producing *Escherichia coli* in pigs: a longitudinal study. *Applied and Environmental Microbiology*. 79:794-798.
60. Hordijk, J., Mevius, D.J., Kant, A., *et al*. 2013. Within-farm dynamics of EBSL/AmpC-producing *Escherichia coli* in veal calves: a longitudinal approach. *Journal of Antimicrobial Chemotherapy*. 68:2468-2476.
61. Baede, V.O., Wagenaar, J.A., Broens, E.M., *et al*. 2015. Extended-spectrum beta-lactamase and AmpC-producing Enterobacteriacae in household dogs: a longitudinal study. *Antimicrobial Agents and Chemotherapy*. doi:10.1128/AAC.04576-14.
62. Speksnijder, D.C., Mevius, D.J., Bruschke, C.J., *et al*. 2015. Reduction of veterinary antimicrobial

use in the Netherlands. *The Dutch success model. Zoonoses and Public Health*. 62:79-87.

63. Andersson, D.I., Hughes, D. 2010. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology*. 8:260-271.
64. Fischer, E.A., Dierikx, C.M., van Essen-Zandbergen, A., *et al*. 2014. The IncI1 plasmid carrying the $bla_{CTX-M-1}$ gene persists in *in vitro* culture of a *Escherichia coli* strain from broilers. *BMC Microbiology*. 14:77.
65. ECDC (European Centre for Disease Prevention and Control). 2013. Consumption of antimicrobials of antibacterials for systemic use (ATC group J01) in the community (primary care sector) in Europe, reporting year 2013. Available at: http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/esac-net-database/Pages/Antimicrobial-consumption-rates-by-country.aspx (last accessed 29 September 2015).
66. ECDC (European Centre for Disease Prevention and Control). 2014. Antimicrobial resistance surveillance in Europe 2013. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). Stockholm, Sweden.

Summary

The emergence of antibiotic resistance both in humans and in animals requires urgent attention. Antibiotic resistance in animals becomes a public health issue when there is transmission of antibiotic resistant bacteria, or their resistance genes, from animals to humans. β-lactam antibiotics are critically important for the treatment of human bacterial infections, and resistance to this class of antibiotics mediated by extended-spectrum β-lactamases (ESBL) and AmpC β-lactamases has emerged in Gram-negative bacteria. Poultry, in particular broilers, might play a role in the transmission of resistance genes to humans due to the high prevalence of ESBL/AmpC-producing Enterobacteriaceae among their intestinal biome, compared to other livestock species, companion animals, and wildlife. Poultry meat has been identified as a possible source for human infections, and transmission to humans via the food chain was hypothesised. This is only one possible route, however, by which ESBL-producing Enterobacteriaceae might be exchanged between broilers and humans. Transmission by close physical contact and via the environment should also be considered as broiler farms, their immediate surroundings, but also the wider natural environment could be contaminated with ESBL-producing Enterobacteriaceae, potentially leading to transmission at sites relevant for human exposure.

The aim of this thesis was to investigate transmission of antibiotic resistant bacteria between animals and humans, and more specifically the transmission of ESBL-producing *E. coli* between broilers, and between broilers and humans in varying degrees of contact with these animals. In order to gain insight into transmission routes, studies were conducted answering key questions on (1) the role of the natural environment in the transmission of clinically relevant antimicrobial resistant (AMR) bacteria to humans; (2) prevalence of, and risk factors for, carriage of ESBL-producing Enterobacteriaceae in the general human population living in areas with high and low broiler densities, and humans on conventional and organic broiler farms; and (3) prevalence of carriage, and transmission dynamics among broilers on a broiler farm under field conditions.

Systematically collected and categorised evidence from literature showed that clinically relevant AMR bacteria are present in the natural environment, more specifically in soil, water, air, and wildlife. No direct evidence was found showing transmission of AMR bacteria to humans, although one study did find genetically related isolates from humans, animals and the surrounding environment suggesting transmission is plausible. Quantitative data and epidemiological studies further suggest this (*Chapter 2*). The occurrence of clinically relevant AMR bacteria in the environment leads to the hypothesis that individuals in areas with high broiler densities might have an increased risk for carriage of ESBL-producing Enterobacteriaceae. This hypothesis was rejected, however, as the risk was lower (3.6% vs. 6.7%; OR=0.45; P=0.009) for these individuals (*Chapter 3*). Mean distance to the nearest broiler farm was smaller for individuals in municipalities with high broiler densities (2.2 km), compared with low broiler densities (6.2 km; P≤0.0001). The overall high broiler density in

the Netherlands (1301 broilers/km^2) and mobility of individuals between municipalities and provinces, may have disturbed a possible relationship between broiler density and ESBL-carriage, however. Owning a horse increased the risk for ESBL-carriage (15.6% vs. 4.4%; OR=4.69; P≤0.0001). This might not be solely attributable to owning or having contact with a horse, as prevalence increased from 4% in individuals without companion animals to almost 12% in individuals who owned more than four different species of companion animals (*Chapter 3*). Contact with multiple species of companion animals might play a role in transmission, but information on the mutual exchange of ESBL-producing Enterobacteriaceae between companion animals and their owners is needed.

Not only transmission via the environment, but close physical contact with broilers might lead to increased risk for carriage of ESBL/AmpC-producing *E. coli* among humans. Compared to humans in the general population (5.1%; *Chapter 3*) and Dutch patients (4.9%), prevalence of carriage among farmers, their family members and employees on both conventional (19.1%; *Chapter 4*) and organic (18.5%; *Chapter 5*) broiler farms was higher. Evidence for clonal transmission of ESBL/AmpC-producing *E. coli* between humans and broilers was found on conventional farms (*Chapter 4*), and horizontal gene transfer was suspected on both conventional and organic farms (*Chapters 4 and 5*). An increased risk of carriage was shown among farmers and employees compared to partners and family members (27.1% vs. 14.3%; OR=2.5; P=0.08), suggesting an increased risk of exposure for humans on broiler farms and that this risk is larger for individuals in close contact with broilers. Transmission through direct contact with broilers and via the farm environment therefore results in human carriage.

Due to differences in management practices between conventional and organic farms, especially antibiotic use, the prevalence of ESBL/AmpC-producing *E. coli* carriage among broilers, and humans living and/or working on organic broiler farms were compared to results from conventional farms. All investigated broiler farms were positive for ESBL/AmpC-producing *E. coli*, and there appeared to be no difference in sample-level prevalence between conventional (96.4%; *Chapter 4*) and organic (94.3%; *Chapter 5*) farms at five weeks of age, which was just before slaughter on conventional farms. On organic farms, the sample level prevalence decreased to 80.0% at slaughter age, which is approximately 70 days (*Chapter 5*). The decrease might also be the case for broilers on conventional farms if the production round would be longer.

ESBL/AmpC-producing *E. coli* are able to transmit and persist on organic farms, even without selective pressure from antibiotics, as indicated by a reproduction ratio of 1.70 for total ESBL/AmpC-producing *E. coli* (*Chapter 6*). This does not hold true for individual phylogenetic groups as indicated by reproduction ratios less than one for groups separately, however. This might mean that persistence of total ESBL/AmpC-producing *E. coli* is determined by repeated shifts from one phylogenetic group to another during the period between arrival at the farm and slaughter age. Furthermore, the environment was relatively more important in the

transmission of the A1 phylogenetic group and direct contact between broilers was relatively more important for the transmission of the B1 phylogenetic group, for those broilers that were not previously infected. This suggests that the contaminated environment before the start of the production round and positive day-old chicks both play a role in the introduction and transmission of ESBL/AmpC-producing *E. coli* in the new flock.

The results of this thesis show that direct contact between humans, broilers and the broiler farm environment, as experienced by people living or working on these farms, contributes to human carriage. In the general population, not living on broiler farms, different sources and pathways might contribute to carriage among humans such as occupational or domestic exposure to animals, human-human transmission, travel abroad and consumption of food products, although the contribution of each remains to be elucidated.

About the author

Curriculum Vitae

Patricia Maria Catharina Huijbers was born on January 21st, 1985 in Abu Dhabi, United Arab Emirates. She attended international schools in Luxembourg and France, and received her bilingual International Baccalaureate Diploma from the American School of The Hague. Between 2003 and 2009 she obtained her Bachelor and Master degrees in 'Animal Sciences' at Wageningen University. She specialised in animal health, and wrote two theses: one on the effect of oral vaccination against White Spot Syndrome Virus on virus transmission in shrimp and another on the effect of early nutrition on development of the immune system in broilers. She also spent 5 months in Canada with the Bovine Genomics Group at the University of Alberta. After obtaining her Master degree in 2009, Patricia began her professional career at Wageningen University. First she was coordinator of the European Master in Animal Breeding in Genetics and then executive secretary of the Wageningen Institute of Animal Sciences (WIAS). In November 2011, Patricia was appointed as a PhD candidate at Wageningen University and the National Institute for Public Health and the Environment (RIVM) to investigate transmission of antibiotic resistant bacteria between animals and humans, and more specifically the transmission of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* between broilers, and between broilers and humans in varying degrees of contact with broilers. Within this project she worked with different groups at Wageningen University and the RIVM but also with members of the poultry industry, and policy makers. Her work has been presented at international conferences and published in peer-reviewed journals. During her PhD, Patricia wrote her own research proposal, took courses to develop both professional and in-depth competences, was a member of the PhD council and was involved in the supervision of MSc and BSc students. Furthermore, she was awarded a WIAS PhD Fellowship so that she might pursue a three-month project at the 'Epidemiology and Zoonoses' unit of the Federal Institute for Risk Assessment in Berlin, Germany.

Publications

Refereed scientific journals

Huijbers, P.M.C., Graat, E.A.M., van Hoek, A.H.A.M., Veenman, C., de Jong, M.C.M., van Duijkeren, E. Transmission dynamics of extended-spectrum β-lactamase and AmpC β-lactamase-producing *Escherichia coli* in a broiler flock without antibiotic use. *Submitted.*

Huijbers, P.M.C., Blaak, H., de Jong, M.C.M., Graat, E.A.M., Vandenbroucke-Grauls, C.M.J.E., de Roda Husman, A.M. 2015. Role of the environment in the transmission of antimicrobial resistance in humans: a review. *Environmental Science and Technology*. 49:11993-12004.

van Duijkeren, E., van Overbeek, W.M., **Huijbers, P.M.C.**, van de Giessen, A.W., van Hoek, A.H.A.M. 2015. Inoculation of mice with avian $bla_{CTX\text{-}M\text{-}1}$- or $bla_{CMY\text{-}2}$- carrying *Escherichia coli* strains does not lead to long-term colonization. *Journal of Antimicrobial Chemotherapy*. 70:3164-3165.

Huijbers, P.M.C., van Hoek, A.H.A.M., Graat, E.A.M., Haenen, A.P.J., Florijn, A., Hengeveld, P.D., van Duijkeren, E. 2014. Methicillin-resistant *Staphylococcus aureus* and extended-spectrum and AmpC beta-lactamase-producing *Escherichia coli* in broilers and people living and/or working on organic broiler farms. *Veterinary Microbiology*. 176:120-125.

Huijbers, P.M.C., Graat, E.A.M., Haenen, A.P.J., van Santen, M.G., van Essen-Zandbergen, A., Mevius, D.J., van Duijkeren, E., van Hoek, A.H.A.M. 2014. Extended-spectrum beta-lactamase-producing *Escherichia coli* in broilers and people living and/or working on broiler farms: prevalence, risk factors, and molecular characteristics. *Journal of Antimicrobial Chemotherapy*. 69:2669-2679.

Huijbers, P.M.C., de Kraker, M., Graat, E.A.M., van Hoek, A.H.A.M., van Santen, M.G., de Jong, M.C.M., van Duijkeren, E., de Greeff, S.C. 2013. Prevalence of extended-spectrum beta-lactamase-producing Enterobacteriaceae in human living in municipalities with high and low broiler density. *Clinical Microbiology and Infection*. 19:E256-E259.

Contributions to conferences and seminars

Huijbers, P.M.C., Graat, E.A.M., van Hoek, A.H.A.M., Veenman, C., de Jong, M.C.M., van Duijkeren, E. Transmission dynamics of ESBL-*E. coli* in a broiler flock without antibiotic pressure. 14th International Symposium on Veterinary Epidemiology and Economics, Merida, Mexico, 3-7 November 2015. *Oral presentation.*

Huijbers, P.M.C., Graat, E.A.M., van Hoek, A.H.A.M., de Jong, M.C.M., van Duijkeren, E. Transmission dynamics of ESBL/AmpC-producing *E. coli* on a broiler farm. 3rd International One Health Congress, Amsterdam, The Netherlands, 15-18 March 2015. *Poster presentation.*

Huijbers, P.M.C., Graat, E.A.M., van Hoek, A.H.A.M., van Santen, M.G., Hengeveld, P.D., Haenen, A.P.J., de Greeff, S.C., Schouls, L.M., van de Giessen, A.W., van Duijkeren, E. High prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* in people living and/or working on Dutch broiler farms. Annual Conference of the Netherlands Epidemiology Society (WEON) on Epidemiology in Global Health: Challenges and Methods, Utrecht, The Netherlands, 6-7 June 2013. *Oral presentation.*

Huijbers, P.M.C., Graat, E.A.M., van Hoek, A.H.A.M., van Santen, M.G., Hengeveld, P.D., Haenen, A.P.J., Schouls, L.M., van de Giessen, A.W., van Duijkeren, E. Prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* in people living and/or working on Dutch broiler farms. Voorjaarsvergadering van de Nederlandse Vereniging voor Medische Microbiologie (NVMM) en de Koninklijke Nederlandse Vereniging voor Microbiologie (KVvM), Papendal, The Netherlands, 16-17 April 2013. *Oral presentation.*

Huijbers, P.M.C., Graat, E.A.M., van Duijkeren, E., de Greeff, S.C., de Jong, M.C.M. Transmission of ESBL-producing bacteria between poultry and humans – A human and veterinary perspective. WIAS Science Day, Wageningen, The Netherlands, 28 February 2013. *Oral presentation.*

Huijbers, P.M.C., de Kraker, M., Graat, E.A.M., van Hoek, A.H.A.M., van Santen, M.G., Huijsdens, X.W., van Duijkeren, E., de Jong, M.C.M., de Greeff, S.C. Prevalence of extended-spectrum β-lactamases in humans living in municipalities with high or low broiler density. 3rd American Society for Microbiology Conference on Antimicrobial Resistance in Zoonotic Bacteria and Foodborne Pathogens in Animals, Humans, and the Environment, Aix-en-Provence, France, 26-29 June 2012. *Oral presentation.*

WIAS Training and Supervision Plan

The Basic Package (3.0 ECTS)	**Year**
WIAS Introduction Course	2012
WIAS Ethics and Philosophy in Life Sciences	2012

Scientific Exposure (11.4 ECTS)

International Conferences	
3rd ASM Conference on Antimicrobial Resistance in Zoonotic Bacteria and Foodborne Pathogens in Animals, Aix-en-Provence, France	2012
Voorjaarsvergadering van de NVMM en KVvM, Papendal, the Netherlands	2013
WEON Epidemiology in Global Health, Utrecht, the Netherlands	2013
International One Health Congress, Amsterdam, the Netherlands	2015
Conference of the International Society for Veterinary Epidemiology and Economics, Yucatan, Mexico	2015

Seminars and Workshops	
SWAB Symposium European Antibiotics Day, Bilthoven, the Netherlands	2011
Study Day Dutch Society for Veterinary Epidemiology and Economics, Utrecht, the Netherlands	2011
ESBL Symposium: Voorkomen van ESBLs in de Voedselketen en bij de Mens, Utrecht, the Netherlands	2012
Antibiotic Resistance: An Ecological Perspective, Amsterdam, the Netherlands	2013
Epidemiology of ESBLs in Animals, Humans and Healthcare, Utrecht The Netherlands	2013
8e Nationale Zoonosen Symposium, Bilthoven, the Netherlands	2014
WIAS Science Day, Wageningen	2012, 2013, 2015

Presentations	
3rd ASM Conference on Antimicrobial Resistance in Zoonotic Bacteria and Foodborne Pathogens in Animals, Aix-en-Provence, France (oral)	2012
WIAS Science Day, Wageningen, the Netherlands (oral)	2013
Voorjaarsvergadering van de NVMM en KVvM, Papendal, the Netherlands (oral)	2013
International One Health Congress, Amsterdam, the Netherlands (poster)	2015
Conference of the International Society for Veterinary Epidemiology and Economics, Yucatan, Mexico (oral)	2015

In-Depth Studies (6.5 ECTS)	**Year**
Disciplinary and Interdisciplinary Courses	
Risk Assessment of Infectious Agents, Utrecht, the Netherlands	2015
Advanced Statistics Courses	
Statistics for the Life Sciences, Wageningen	2015
MSc Level Courses	
Capita Selecta Laboratory of Microbiology, MIB-50403	2015
Professional Skills Support Courses (3.3 ECTS)	
Project and Time Management, Wageningen	2015
Career Orientation, Wageningen	2015
WGS PhD Workshop Carousel, Wageningen	2015
Research Skills Training (8.0 ECTS)	
Preparing own PhD Research Proposal	2011-2012
External Training Period Federal Institute for Risk Assessment, Berlin, Germany (3.5 months)	2015
Didactic Skills Training (4.4 ECTS)	
Supervising Theses	
MSc Thesis, Major	2012
BSc Thesis	2015
Tutorship	
Praktijkproject Inleiding Dierwetenschappen	2012, 2014
Management Skills Training (10.0 ECTS)	
Membership of Boards and Committees	
Member of WAPS Council	2012, 2013
Education and Training Total (46.6 ECTS)	

Acknowledgements

As I near the milestone of defending the thesis that lies before you, it is time to thank the people who had an important impact on me and the project these past four years. As they say, there is no “I” in team, and that is certainly true when writing a PhD thesis.

First I would like to thank my supervisory committee. Mart, thank you for giving me the opportunity to embark on this PhD and for contributing your vast knowledge and critical eye to the project. I learned a lot from our collaboration which will serve me well in my future career. Lisette, thank you for being there every step of the way from the critical review of manuscripts to early mornings collecting samples in the broiler house to providing guidance along the PhD path. You also taught me some interesting expressions which I am sure will stay with me in times to come. Engeline, when I started this project I was a layperson regarding veterinary microbiology but our discussions on this topic and your helpful comments on manuscripts allowed me to learn in leaps and bounds. You were never too busy to sit down with me and talk about the project and for that I am grateful.

A big thank you also goes to all the people I had the good fortune to collaborate with during my PhD. Sabine, I want to thank you for your sincere interest in my progress throughout the PhD and your willingness to give advice and guidance. Marlieke, because of your excellent preparation of the first experiment I was able to hit the ground running and submit my first article within a year after starting my PhD. Thank you. Anja, thank you for helping me with all my questions about the farm studies. Hetty, Ana Maria, Christina, it was a pleasure to collaborate with you on our review article. At times I honestly did not know if we would get it done but we managed to deliver a great result. Arjen, although our contact moments were few I always felt that I could come to you for questions and advice. Thank you. Fachgruppe 43, thank you for a wonderful three months at the Federal Institute for Risk Assessment in Berlin and for sharing all your interesting projects with me. Laura, Sander, thank you both for your contributions to this thesis through your Master and Bachelor thesis work. Thijs, Rosan, the best broiler catchers I know, thank you for all your help taking samples!

When I started my PhD I was all thumbs in the lab and I want to thank a number of people for helping me to develop my skills in this area. Angela, your clear and precise explanations about the different steps needed for isolating, identifying and characterising ESBL-producing bacteria were invaluable to the project and to me. Wendy, Christiaan and Paul, I am grateful for all the hours you spent helping me and explaining how things work in a microbiological lab. Many thanks to all of you!

A nice working environment starts with nice colleagues and I have been fortunate to get to know many during my PhD, both at Wageningen University and the National Institute for Public Health and the Environment. Dividing my time between two institutes and five different groups was not easy but each group made me feel welcome. A big thank you to everybody at QVE, ADP, ABG, EPI and Z&O for all the nice conversations during coffee breaks, lunches, dinners, drinks and walks! I especially want to thank my fellow PhD students at both institutes for our discussions on the process of thesis writing, shared frustrations, and above all, laughs You made my PhD a less solitary experience. Ladies at the WIAS office, thank you for your support and giving me the opportunity to go for a PhD fellowship abroad.

Dear family and friends, there are countless ways in which you have helped, supported and encouraged me over the years. I know I can count on you now and in the future and for that I am extremely grateful. Brenda and Maaike, thank you for agreeing to stand by me in the Aula and the pep talks that I am quite sure will be needed before my defence. Miranda, thank you for designing an awesome cover. And finally, Michael, thank you for always being there for me and listening to the endless (in my experience) PhD tales. I know we are headed towards a bright future and I cannot wait to get started!

In my dogged attempt to be complete in these acknowledgements I might have inadvertently forgotten somebody. You know me though and that is something I would never do intentionally. So before I leave you with the rest of this thesis, one more BIG thank you to everybody!

Colophon

Financial support from the Quantitative Veterinary Epidemiology Group of Wageningen University and the National Institute for Public Health and the Environment for printing this thesis is gratefully acknowledged.

Printed by: Gildeprint, Enschede, The Netherlands
Thesis layout: N. Nijhuis, Gildeprint
Cover design: M. Schut