Aus dem Institut für Tier-und Umwelthygiene
des Fachbereichs Veterinärmedizin
der Freien Universität Berlin

Transmission processes of ESBL-/AmpC-producing Enterobacteriaceae in the broiler production chain

Inaugural-Dissertation
zur Erlangung des Grades eines
PhD of Biomedical Sciences
an der Freien Universität Berlin

vorgelegt von
Katrin Boll geb. Dähre
Tierärztin
aus Potsdam

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IV List of Abbreviation

bla	beta-lactamase gene
C. freundii	*Citrobacter freundii*
E. coli	*Escherichia coli*
K. pneumoniae	*Klebsiella pneumoniae*
AmpC	AmpC beta-lactamase
APEC	Avian pathogenic *Escherichia coli*
BMBF	Federal Ministry of Education and Research
bp	base pair
BSBL	broad-spectrum beta-lactamases
CA	clavulanic acid
cfu	colony forming units
cgMLST	core genome multi locus sequence type
CS	cloacal swab
CTX-M	cefotaximase
DAEC	Diffusely adherend *Escherichia coli*
DNA	desoxyribonucleic acid
EAEC	Enteroaggregative *Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBL	extended-spectrum beta-lactamase

ExPEC	Extraintestinal pathogenic *Escherichia coli*
FUB	Freie Universität Berlin, Berlin
hrs	hours
Inc	incompatibility
InPEC	Intestinal pathogenic *Escherichia coli*
MALDI-TOF	Matrix Assisted Laser Desorption Time of Flight Mass Spectroscopy
Mbp	mega base pair
MLST	Multi locus sequence type
pAmpCs	plasmid-mediated AmpC beta-lactamases
PCR	polymerase chain reaction
SNPs	single nucleotide polymorphisms
spp	species
TZB	tazobactam
UPEC	Uropathogenic *Escherichia coli*
WGS	whole genome sequencing

1 Introduction

Infectious diseases were the major cause of death at the beginning of the 20th century. But in 1928, an antimicrobial effect of mould derived substances was observed incidentally by Sir Alexander Fleming. Eighteen years later, 1946, penicillin was available in the open market, changing therapeutic options for bacterial infection (Kong et al., 2010). However, resistance against antibiotics were quick to follow. While this problem could be reduced for the fist decades by the constant introduction of new antibiotics these advancements decreased over time and therewith, antibiotic resistance has increased (Martínez and Baquero, 2014). Nowadays, next to others, extended-spectrum beta-lactamase- (ESBL) and AmpC beta-lactamase- (AmpC) producing Enterobacteriaceae represent a problem both in human and veterinary medicine.

The occurrence of ESBL-/AmpC-producing Enterobacteriaceae is widely distributed. This applies to farm and companion animals, wildlife, as well as to retail meat and humans (Carattoli et al., 2005; Costa et al., 2004; Schaufler et al., 2015; Aarestrup et al., 2006; Blanc et al., 2006; Briñas et al., 2005; Meunier et al., 2006; Ghodousi et al., 2015; Leverstein-van Hall et al., 2011; Guenther et al., 2011; Woerther et al., 2013; Livermore, 2012). As an impact of animal-originated resistant bacteria is assumed (Marshall and Levy, 2011; Smet et al., 2010; Dierikx et al., 2013b) and high prevalence of ESBL-/AmpC-producing Enterobacteriaceae were demonstrated especially for broiler chicken (Huijbers et al., 2014; Laube et al., 2013; Smet et al., 2008; Dierikx et al., 2013b) intervention strategies facilitating a reduction of the load of these resistant bacteria in chicken should be considered. Therefore, information on the entry and transmission of ESBL/AmpC-producing Enterobacteriaceae into/within the broiler fattening farms are required, but only rare.

On the one hand, previous studies assumed a vertical transfer from broiler breeding chicken to their offspring (Dierikx et al., 2013b; Nilsson et al., 2014). On the other hand, the transmission on farm level due to contaminated fattening houses was assumed (horizontal transmission) (Laube et al., 2013; Hiroi et al., 2012b; Huijbers et al., 2016).

As much was known about the prevalence at fattening level but little was known about transmission processes, the presented study was aiming at an elucidation of transmission routes for ESBL-/AmpC-producing Enterobacteriaceae along the broiler production chain. Therefore, whole genome sequencing (WGS) as a high-resolution molecular method was used and parent flocks, the corresponding hatching eggs and the hatchlings in the hatchery as well as the respective fattening flocks were investigated for seven fattening chains.

1.1 ESBL-/AmpC-producing Enterobacteriaceae

Beta-lactam antibiotics were the first antibiotics to be described (Queener, 1986) and resistance against them was the first to be understood (Kong et al., 2010). One principle for bacteria to combat these antibiotics is the production of beta-lactamases, enzymes that hydrolyse the beta-lactam ring resulting in a reduced effectiveness (Pfeifer et al., 2010).

1.1.1 Beta-lactam antibiotics

The beta-lactam ring, shown in figure 1, is the characteristic functional core structure in all beta-lactam antibiotics. This beta-lactam ring can bind to the D-alanin-transpeptidase enzymes, which are necessary for the bacterial cell wall synthesis due to cross-linking of polysaccharides to the peptidoglycan murein. The binding of the beta-lactam ring to the transpeptidase inhibits the cell wall synthesis and, therefore, results in the bactericidal effects of beta-lactam antibiotics (Madigan et al., 2013). From a structural point of view, beta-lactam antibiotics can be subdivided into six different groups (Pfeifer et al., 2010), shown in table 1. Clinically and therapeutically a distinction is made between oral and parenteral antibiotics and from a pharmaceutical point of view penicillin, cephalosporins, monobactams and carbapenems are included in the group of ß-lactam antibiotics (Frey and Löscher, 2010). In terms of the antibacterial potency they are classified into narrow, broad, and extended-spectrum antibiotics, and in the case of cephalosporins into the 1st to 5th generation. In addition, there are beta-lactamase inhibitors like clavulanic acid, sulbactam and tazobactam.

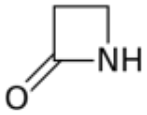

Figure 1: Structure of the beta-lactam ring.

Table 1: Six different structural groups of beta-lactam antibiotics according to Pfeifer et al. (2010).

Structural group	Examples
penams	benzylpenicillin, ampicillin
cephems	1st generation cephalosporins (cefadroxil, cefazolin)
	2nd generation cephalosporins (cefotiam, cefuroxime)
	3rd generation cephalosporins (cefotaxime, ceftazidime)
	4th generation cephalosporins (cefepime)
	5th generation cephalosporins (ceftaroline)
cephamycins	cefoxitin
monobactams	aztreonam
penems	faropenem
carbapenems	imipenem, meropenem

1.1.2 Beta-lactamases

Beta-lactamases are enzymes that cleave the beta-lactam ring of beta-lactam antibiotics and, therefore, reduce their effectiveness (Pfeifer et al., 2010).

In gram-negative bacteria, naturally occurring chromosomally located beta-lactamases are quite common and when produced in small quantities they do not significantly contribute to antibiotic resistance (Liakopoulos et al., 2016). But already in the early 1950s, enteric bacteria with resistance to first penicillins attracted attention (Pfeifer et al., 2010). To combat emerging resistances, new antibiotics were introduced constantly. Within the brief span of eight years (1978-1986) six new classes of beta-lactam antibiotics, natural as well as semisynthetic and synthetic ones, were introduced. During this time, resistance to the newly introduced beta-lactam antibiotics emerged surprisingly rapid (Medeiros, 1997).

1.1.2.1 Extended-spectrum beta-lactamases

The first report of an extended-spectrum beta-lactamase (ESBL) was published in 1983 (Knothe et al., 1983). Since then, ESBL-producing bacteria distributed worldwide and represent a challenging public health problem.

A commonly used definition of ESBLs defines them to be beta-lactamases with resistance against penicillins, 1st, 2nd and 3rd generation cephalosporins and aztreonam (but not against cephamycins or carbapenems) and ESBLs are inhibitor-susceptible (Paterson and Bonomo, 2005; Bauernfeind et al., 1998; Thomson, 2010).

The most frequently occurring ESBL-genes belong to the beta-lactamase families SHV, TEM and CTX-M and are usually multi drug resistant (Bradford, 2001; Paterson and Bonomo, 2005).

One main cause for beta-lactam resistance in enterobacterial species was the expansion of the substrate spectrum of the broad-spectrum beta-lactamases (BSBLs) SHV and TEM (Jarlier et al., 1988; Sirot et al., 1988). The beta-lactamase gene (*bla*) SHV (bla_{SHV}) was first described as a chromosomally encoded beta-lactamase in members of the genus *Klebsiella* (Heritage et al., 1999) and already in the 1980s, $bla_{\text{SHV-1}}$ (BSBL) was found in a variety of plasmid types transferred from several bacterial species collected from a wide geographic range (Matthew et al., 1979). The first ESBL detected in 1983 in Germany was discovered in a *Klebsiella ozeanae* with extended-spectrum properties (Knothe et al., 1983). Sequencing showed that the beta-lactamase differed from $bla_{\text{SHV-1}}$ by replacement of glycin by serin at position 238. This replacement alone accounts for extended-spectrum properties of this beta-lactamase, designated $bla_{\text{SHV-2}}$, an ESBL (Paterson and Bonomo, 2005). Since then, SHV-type ESBLs were detected in human clinical isolates from all over the world (Paterson et al., 2003) as well as in livestock and companion animals. To date, 132 SHV-genes are assigned (http://www.laced.uni-stuttgart.de/).

The BSBL TEM-1 was reported first in 1965 from an *Escherichia coli (E. coli)* isolate from a patient in Athen (Datta and Kontomichalou, 1965). $bla_{\text{TEM-1}}$ only confers resistance to penicillins and early cephalosporins but its descendants expanded the spectrum to 2nd, 3rd

and 4th generation cephalosporins, monobactams and beta-lactamase inhibitors (Salverda et al., 2010) and already in the 1980s, plasmid carrying genes encoding for TEM-types were detected (Sirot et al., 1987; Brun-Buisson et al., 1987). To date, 167 TEM-genes are assigned (http://www.laced.uni-stuttgart.de/).

Later, since the early 1990s, further beta-lactamase-related resistance mechanisms were discovered. The CTX-M family arised in Enterobacteriaceae due to the mobilization of genes coding for enzymes with ESBL-activity from the environmental bacterial genus *Kluyvera* and the name CTX-M originated from the hydrolytic activity against cefotaxime (CTX-M - cefotaximase) (Bonnet, 2004; Pfeifer et al., 2010). CTX-M enzymes can be classified by amino acid sequence similarities into five major groups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. Each subgroup has a natural ancestor represented by the different environmental *Kluyvera* species (Bonnet, 2004). The CTX-M resistance genes are genetically surrounded by specific mobile transposase elements, leading to the worldwide dissemination of $bla_{\text{CTX-M}}$ in various species of the family Enterobacteriaceae (Bonnet, 2004; Cantón et al., 2012).

1.1.2.2 AmpC beta-lactamases

Another important group of beta-lactamases are the AmpC beta-lactamases. They exhibit a hydrolytic profile similar to the ESBLs while having an additional hydrolytic activity towards cephamycins like cefoxitin and cefotelan (Bajaj et al., 2016). AmpC beta-lactamases are not inhibited by the common beta-lactamase inhibitors like clavulanic acid and tazobactam but by cloxacillin and boronic acid (Bradford, 2001; Jacoby, 2009; Thomson, 2010; Helmy and Wasfi, 2014).

The *ampC* gene is included in the chromosome of nearly all enterobacterial species besides *Klebsiella* spp. and *Proteus* spp. and is regulated by complex mechanisms (Pfeifer et al., 2010). In gram-negative organisms, overexpression of AmpC beta-lactamases occurs either by deregulation of the *ampC* chromosomal gene (depressed mutants) or by acquisition of a transferable *ampC* gene on a plasmid derived from several members of the family Enterobacteriaceae or other transferable elements (pAmpCs - plasmid-mediated AmpC beta-lactamases) (Hanson and Sanders, 1999; Bauernfeind et al., 1998; Bush, 2001; Thomson and Smith Moland, 2000; Perez-Perez and Hanson, 2002).

Six families of plasmid-mediated AmpC beta-lactamases were described based on the sequence similarities as CIT, FOX, MOX, DHA, EBC and ACC (Perez-Perez and Hanson, 2002). For *E. coli*, one of the most commonly recognized plasmid-mediated AmpCs is the CMY-2 type belonging to the CIT family and shares homology with chromosomally encoded *ampC* from *Citrobacter freundii* (*C. freundii*) (Sidjabat et al., 2009; Oteo et al., 2010; Helmy and Wasfi, 2014). The majority of plasmid-mediated *ampC* genes are detected in nosocomial isolates of *E. coli* and *Klebsiella pneumoniae (K. pneumoniae)*.

1.1.2.3 Classification of beta-lactamases

Most commonly, beta-lactamases are classified according to two general schemes. One classification is based on the molecular structure and one on the functionality (Paterson and Bonomo, 2005).

The molecular classification scheme according to Ambler (1980) divides beta-lactamases into four major classes (A-D) (Table 2). Classes A, C and D with serine in its active site (serine-beta-lactamases) and class B which needs a bivalent cation (preferentially zinc) to facilitate beta-lactam hydrolysis (metallo-beta-lactamases) (Pfeifer et al., 2010). The most frequently occurring ESBLs belong to Ambler class A.

The functional classification scheme by Bush-Jacoby-Medeiros considers the functional similarities regarding the substrate and inhibitor profiles (Bush and Jacoby, 2010) (Table 3). Therefore, this classification scheme is of particular relevance for clinicians and laboratory microbiologists.

Table 2: Modified classification scheme of beta-lactamases based on the molecular structure according to Ambler (1980) (Pfeifer et al., 2010).

	ß-lactam class	**ß-lactamase**	**Important examples**	**Preferential occurrence**	**Important phenotypical resistance traits**
Serine-ß-lactamases	A	Broad-spectrum ß-lactamase	TEM-1, TEM-2, SHV-1, SHV-11	Enterobacteriacae and nonfermenters	amipicillin, cephalotin
		ESBL TEM-type	TEM-3, TEM-52		penicillins, 3rd gen. cephalosporins
		ESBL SHV-type	SHV-5, SHV-12		
		ESBL CTX-M-type	CTX-M-1, CTX-M-15		
		Cabapenemases	KPC, GES, SME		all ß-lactams
	C	AmpC cephamycinases (chrmomosomal encoded)	AmpC	*Enterobacter* spp. *Citrobacter* spp.	
	D	AmpC cephamycinases (plasmid encoded)	CMY, DHA MOX FOX, ACC	Enterobacteriaceae	cephamycins (cefoxitin), 3rd gen. cephalosporins
		Broad-spectrum ß-lactamases	OXA-1,-9	Enterobacteriaceae, *A. baumanii*	oxacillin, ampicillin, cephalotin
		ESBL OXA-type	OXA-2,-10		
		Carbapenemases	OXA-48, -23,-24,-58		ampicillin, imipenem, all ß-lactams
Metallo-ß-lactamases	B	Metallo-ß-lactamases (Carbapenemases)	VIM IMP	Enterobacteriaceae and nonfermenters	all ß-lactams

Table 3: Classification scheme of beta-lactamases based on the functionality (Bush, 2013; Bush and Jacoby, 2010; Bush et al., 1995).

Group	Enzyme type	Molecular class	Inhibition by	Substrate	Examples
1	Cephalosporinases	C	No	Cephalosporins	CMY-2, FOX-1
1e	Cephalosporinases	C	No	Cephalosporins	CMY-37
2a	Penicillinases	A	CA/TZE	Penicillins	PC-1
2b	Broad-spectrum	A	CA/TZE		TEM-1, -2, SHV-1
2be	Extended-spectrum	A	CA/TZE	Extended-spectrum Cephalosporins	TEM-3, SHV-2, CTX-M-15
2br	Inhibitor-resistant	A	No	Extended-spectrum Cephalosporins, Monobactams	TEM-50
2ber		A	No	Extended-spectrum Cephalosporins, Monobactams	TEM-50
2c	Carbenicillinase	A	CA/TZE	Carbenecillin	PSE-1, CARB-3
2ce	Carbenicillinase	A	CA/TZE	Carbenecillin, cefepim	RTG-4
2d	Cloxacillinase	D	variable	Cloxacillin	OXA-1, -10
2de	Extended-spectrum	D	variable	Extended-spectrum cephalosporins	OXA-11, -15
2df	Carbapenemases	D	variable	carbapenems	OXA-23, -48
2e	Cephalosporinases	A	CA/TZE	Extended-spectrum cephalosporins	CepA
2f	Carbapenemase	A	variable	Carbapenems	KPC-2 IMI-1
3a	Metalloenzyme	B	EDTA	Carbapenems	IMP-1, VIM-1
3b	Metalloenzyme	B	EDTA	Carbapenems	CphA, Sfh-1

CA, clavulanic acid; TZB, tazobactam, EDTA, ethylenediaminetetraacetic acid

1.1.3 Enterobacteriaceae

The family Enterobacteriaceae of the order Enterobacterales belongs to the gram-negative faculatative anaerobic rod-shaped bacteria. In 2016, the former order "Enterobacteriales" was changed to Enterobacterales. Until 2016, Enterobacteriaceae were the only family in the order "Enterobacteriales" and the systematic changes were connected with a distribution of the so far known taxa to several new families (examples given for new families: Erwiniaceae, Yersiniaceae, Morganellaceae) (Adeolu et al., 2016).

In the presented study, the focus mainly was on resistant isolates of the species *Escherichia (E.) coli*, as they are detected frequently in humans and animals and are often associated with multi-drug resistance. In addition, *Klebsiella (K.) pneumoniae* were of interest in the presented study.

1.1.3.1 *Escherichia coli*

E. coli belongs to the genus *Escherichia* in the family of Enterobacteriaceae (Table 4). The gram-negative, non-sporulating facultative anaerobe bacterium has a size of 1.1-1.5 x 2.0-6.0 µm (Rolle et al., 2007).

Table 4: Taxonomy of *Escherichia coli*.

Systematic	
Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacterales
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*Escherichia coli*

E. coli strains can be classified into three major groups: commensal *E. coli*, intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC) (Russo and Johnson, 2000).

As a widespread commensal of the gastrointestinal tract in all vertebrates, *E. coli* is located in the large intestine, especially in caecum and colon and coexists with its host in good health only causing disease in immunocompromised hosts or when the normal gastrointestinal barriers are damaged (Tenaillon et al., 2010; Russo and Johnson, 2000; Kaper et al., 2004).

The pathogenic strains of *E. coli* represent a common cause of severe infections and in humans they are involved both in community and healthcare settings (European Centre for Disease Prevention and Control, 2019). These strains have acquired specific virulence attributes allowing them to adapt to new niches and cause a broad spectrum of diseases (Kaper et al., 2004).

InPEC strains cause enteric/diarrhoeal diseases and enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherend *E. coli* (DAEC) are known (Nataro and Kaper, 1998).

ExPEC strains mainly cause urinary tract infections (uropathogenic *E. coli* – UPEC), but also diseases of the central nervous system, the circulatory system and the respiratory system (Kaper et al., 2004; Russo and Johnson, 2003).

In poultry, avian pathogenic *E. coli* (APEC) cause extraintestinal infections, primarily respiratory infections, pericarditis and septicaemia (Kaper et al., 2004).

1.1.3.2 *Klebsiella pneumoniae*

K. pneumoniae is a gram-negative, facultative anaerobe, non-motile, non-sporulating, mostly encapsulated, rod-shaped bacterium of the family Enterobacteriaceae (Table 5).

Table 5: Taxonomy of *Klebsiella pneumoniae*.

Systematic	
Domain	Bacteria
Phylum	Proteobacteria
Class	Gammaproteobacteria
Order	Enterobacterales
Family	Enterobacteriaceae
Genus	*Klebsiella*
Species	*Klebsiella pneumoniae*

K. pneumoniae is naturally resistant to penicillins and often carries acquired resistance to multiple antimicrobials (Wyres et al., 2020). The species typically colonizes the gut and respiratory tract of both animals and humans, and frequently causes human nosocomial infections whereby the gastrointestinal tract and the hands of hospital staff act as the main pathogenic reservoirs for transmission. In particular, *K. peneumoniae* accounts for a significant proportion of hospital-acquired urinary tract infections, pneumonia, septicaemia, and wound infections. Particularly at risk are vulnerable patient groups as neonates, the elderly, and immunocompromised patients (Wyres et al., 2020; Podschun and Ullmann, 1998).

In livestock, *K. pneumoniae* are detected in broiler and pig farms (Bródka et al., 2012; Hiroi et al., 2012a) and represent an important cause of mastitis in dairy cows (Timofte et al., 2014; Zadoks et al., 2011). In veterinary clinics, nosocomial events caused by *K. pneumoniae* are indicated and the locations of isolation reflect primarily the situation in human medicine (Ewers et al., 2014).

1.2 ESBL-/AmpC-producing Enterobacteriaceae in public health

Beta-lactamases were first detected in the early 1980s in humans and in that habitat, ESBL-/AmpC-producing bacteria have increasingly been detected since the 1990s. Later, since 2000, these bacteria have also increasingly been detected in animals (Smet et al., 2010). As this also includes food-producing livestock, animals were hypothesized as infection sources for humans (Smet et al., 2010; Carattoli, 2008). Figure 2 illustrates different habitats and possible transmission pathways for antibiotic resistant bacteria between them, demonstrating the relevance for public health.

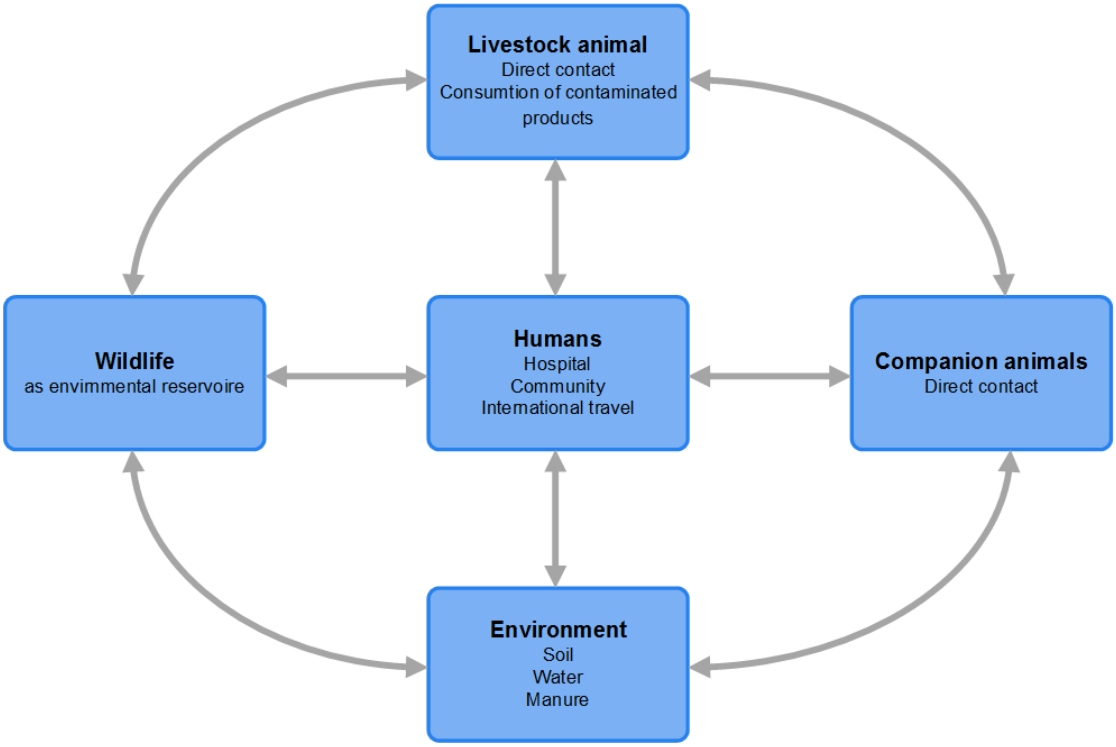

Figure 2: Transmission routes of antibiotic resistant bacteria among different habitats, according to Ewers et al. (2012).

1.2.1 ESBL-/AmpC-producing Enterobacteriaceae in humans

The first ESBL in humans was determined during a *K. pneumoniae* outbreak in a German hospital in 1983 and since the late 1990s, ESBL-/AmpC-producing Enterobacteriaceae have emerged globally and are reported both in clinical settings and in the community (Ewers et al., 2012; Knothe et al., 1983; Pitout et al., 2005). The European Antimicrobial Resistance Surveillance Network reported an increasing quantity of ESBL-/AmpC-producing Enterobacteriaceae, especially in *E. coli* and *K. pneumoniae*, since 2000. These reports also show important geographical differences, ranging from 4.2% to 41.6% for *E. coli* and from 0% to 75.5% for *K. pneumoniae* (Iceland resp. Bulgaria; data from 2016) (Table 6 and Table 7) (European Centre for Disease Prevention and Control, 2017; Coque et al., 2008).

TEM and SHV enzymes were the first variants of ESBLs spreading both in *E. coli* and *K. pneumoniae*. $bla_{\text{SHV-12}}$ and $bla_{\text{TEM-52}}$ represent the most prevalent genes of theses enzyme

families. Since the 1990s, CTX-M-types have become the most common ESBLs, *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-14}}$ being the most prevalent ones in humans, irrespective of the worldwide origin. This is of great interest, as in contrast, the distribution of ESBL-/AmpC-types in animals varies extensively between animal groups and geographical origin (Ewers et al., 2012). In Europe, other CTX-M-types frequently occur locally. For example, CTX-M-9 and CTX-M-10 in Spain, CTX-M-3 in eastern countries and CTX-M-5 in Belarus and Russia (Coque et al., 2008; Hernández et al., 2005; Romero et al., 2005; Empel et al., 2008; Edelstein et al., 2003; European Centre for Disease Prevention and Control, 2017; Ewers et al., 2012).

CMY-2 is the most common enzyme belonging to the AmpC beta-lactamases (European Centre for Disease Prevention and Control, 2017).

An important factor for the global dissemination of ESBLs is the worldwide distribution of the *E. coli* clones of the B2-ST131-O25:H4 group (phylogroup B2, MLST-type 131, O type O25:H4). These *E. coli* are associated with the global occurrence of *bla*$_{\text{CTX-M-15}}$ and are mainly associated with urinary tract infection and bacteraemia (Ewers et al., 2010; European Centre for Disease Prevention and Control, 2017) .

Table 6: Increasing rates in percent (%) of *E. coli* isolates resistant to third-generation cephalosporins in Europe in the years 2001, 2008 and 2016 (https://atlas.ecdc.europa.eu/public/index.aspx).

	E. coli resistant to third-generation cephalosporins, in %		
Country	**2001**	**2008**	**2016**
Austria	0.0	7.4	10.0
Belgium	1.8	4.2	10.5
Bulgaria	6.7	29.3	41.6
Croatia	1.6	3.8	14.7
Cypris	n.d.	19.3	30.2
Czech Republic	2.3	9.9	15.1
Denmark	n.d.	4.1	6.6
Estonia	5.9	4.6	9.0
Finland	0.2	2.1	6.9
France	n.d.	3.8	11.2
Germany	0.7	4.8	11.1
Greece	5.4	9.9	17.6
Hungary	0.4	9.2	16.7
Iceland	0.0	0.8	4.2
Ireland	n.d.	5.9	11.4
Italy	n.d.	15.9	29.8
Latvia	n.d.	11.1	24.1
Lithuania	n.d.	5.6	14.7
Luxembourg	0.5	6.3	13.6
Malta	0.0	21.0	14.6
Netherlands	0.6	4.6	6.4
Norway	0.3	2.6	5.6
Poland	7.1	2.4	13.7
Portugal	2.9	10.1	16.1
Romania	n.d.	23.6	23.4
Slovakia	6.7	n.d.	29.7
Slovenia	0.3	4.2	12.5
Spain	0.6	9.0	15.0
Sweden	0.3	2.3	8.3
United Kingdom	1.2	6.9	9.2

n.d. – not determined

Table 7: Increasing rates in percent (%) of *K. pneumoniae* isolates resistant to third-generation cephalosporins in Europe in the years 2005, 2010 and 2016 (https://atlas.ecdc.europa.eu/public/index.aspx).

	K. pneumoniae, resistant to third-generation cephalosporins, in %		
Country	**2005**	**2010**	**2016**
Austria	5.7	12.6	9.6
Belgium	n.d.	12.6	22.9
Bulgaria	50.0	75.6	72.5
Croatia	45.5.	55.5	48.6
Cypris	n.d.	34.4	30.7
Czech Republic	32.4	48.2	51.8
Denmark	n.d.	10.6	7.5
Estonia	8.1	17.3	32.8
Finland	2.3	4.0	4.1
France	4.1	17.8	28.9
Germany	6.7	12.8	13.6
Greece	60.6	74.6	72.5
Hungary	27.7	45.9	37.5
Iceland	0.0	3.7	0.0
Ireland	7.1	8.5	13.5
Italy	19.5	46.5	55.8
Latvia	n.d.	54.7	47.4
Lithuania	n.d.	50.6	56.7
Luxembourg	n.d.	5.1	35.9
Malta	5.6	12.3	21.6
Netherlands	3.5	7.2	10.3
Norway	2.1	2.1	5.8
Poland	66.0	39.7	64.4
Portugal	n.d.	28.3	46.7
Romania	n.d.	70.6	68.0
Slovakia	n.d.	n.d.	61.3
Slovenia	19.2	22.4	22.8
Spain	7.1	10.2	22.4
Sweden	1.4	2.4	4.9
United Kingdom	12.3	9.7	8.9

n.d. – not determined

1.2.2 ESBL-/AmpC-producing Enterobacteriaceae in companion animals and food producing animals, especially broiler chicken

One of the first detections of a clinical ESBL in animals goes back to 2000. There, a SHV-12 producing *E. coli* was isolated from a dog with urinary tract infection (Teshager et al., 2000). Since then, ESBL-/AmpC-producing Enteroabacteriaceae have been detected in companion animals, in food producing animals as well as in wildlife (Ewers et al., 2011, 2012; Carattoli et al., 2005; Schaufler et al., 2015; Aarestrup et al., 2008; Blanc et al., 2006; Brinas et al., 2003; Briñas et al., 2005; von Salviati et al., 2014; Meunier et al., 2006; Costa et al., 2009; Guenther et al., 2011; Costa et al., 2006; Poeta et al., 2008).

In poultry, ESBL-/AmpC-producing Enterbacteriaceae were first detected in the years 2000 and 2001 in Spain (Brinas et al., 2003). This finding also represents the finding of the beta-lactamase genes *bla*$_{\text{SHV-12}}$, *bla*$_{\text{CMY-2}}$ and *bla*$_{\text{CTX-M-14}}$ for the first time in healthy animals.

bla$_{\text{CTX-M-1}}$ is broadly disseminated among animals in Europe with prevalence of 28% both in companion animals and poultry and 72% in cattle and pigs. *bla*$_{\text{CTX-M-14}}$ and *bla*$_{\text{CTX-M-15}}$, the most common ESBL-genes in humans, are less prevalent in animals in Europe (*bla*$_{\text{CTX-M-14}}$ with prevalence of 4-7% in livestock; *bla*$_{\text{CTX-M-15}}$ only incidentally in poultry, 15% in companion animals and 8% in cattle and pigs). The AmpC beta-lactamase CMY-2 has been described worldwide in companion animals and food producing animals. In poultry, the most common ESBL-/AmpC-resistance genes detected in Europe are *bla*$_{\text{CMY-2}}$ (32%), *bla*$_{\text{CTX-M-1}}$ (28%), *bla*$_{\text{TEM-52}}$ (10%) and *bla*$_{\text{SHV-12}}$ (8%) (Ewers et al., 2012).

High prevalence for ESBL-/AmpC-producing Enterobacteriaceae, up to 100%, was demonstrated especially for broiler fattening farms (Huijbers et al., 2014; Laube et al., 2013; Smet et al., 2008). Concerning broiler, the resistant bacteria were not only detected in broiler farms but also in grandparent and parent breeding chicken as well as in day-old chicken (Nilsson et al., 2014; Laube et al., 2013; Dierikx et al., 2013a,b).

Additionally, ESBL-/AmpC-producing Enterobacteriaceae in fattening chicken also were detected in various European countries on slaughterhouse level and in retail meat (Reich et al., 2013; Börjesson et al., 2016; Kola et al., 2012; Overdevest et al., 2011; Cohen Stuart et al., 2012; Belmar Campos et al., 2014; Zogg et al., 2016). In Germany, for example, ESBL-/AmpC-producers were detected in 89% resp. 53% of carcasses at slaughterhouse (Reich et al., 2013) and in up to 50% in chicken retail meat (Kola et al., 2012).

The occurrence of ESBL-/AmpC-producing Enterobacteriaceae in food producing animals as well as in retail meat assumed an impact of animal originated bacteria on public health. The transmission of resistant bacteria could be plausible via direct contact or due to the consumption of contaminated meat and findings are controversially discussed (Marshall and Levy, 2011; Smet et al., 2010; Ewers et al., 2012; Belmar Campos et al., 2014; Börjesson et al., 2016; Kluytmans et al., 2013; Valentin et al., 2014; Dorado-García et al., 2018).

Considering the facts, that ESBL-/AmpC-producing Enterobacteriaceae frequently occur in broiler chicken as well as in retail meat and that a transmission to human is plausible, intervention strategies enabling a reduction of the load of ESBL-/AmpC-producing Enterobacteriaceae

in chicken should be considered. Therefore, information on potential transmission routes of ESBL-/AmpC-producing Enterobacteriaceae along the broiler production chain are essential. However, those data are only rare. Earlier studies have assumed a vertical transmission of pathogenic *E. coli* from broiler breeding chicken to their offspring (Bortolaia et al., 2010; Petersen et al., 2006; Giovanardi et al., 2005) and first indications for a vertical transfer of ESBL-/AmpC-producing *E. coli* from (grand)parent flocks to the broiler fattening flocks were described (Nilsson et al., 2014; Dierikx et al., 2013b). On the other hand, studies assume that contaminated farm environment could represent a source for ESBL-/AmpC-producing Enterobacteriaceae (horizontal transmission) (Laube et al., 2013; Hiroi et al., 2012b).

To elucidate possible transmission routes of ESBL-/AmpC-producing Enterobacteriaceae we investigated seven broiler fattening flocks along the broiler production chain. Therefore, whole genome sequencing (WGS) as a high-resolution molecular method was used and ESBL-/AmpC-positive parent flocks, the corresponding hatching eggs and the hatchlings in the hatchery as well as the respective fattening flocks were investigated for seven broiler fattening chains.

2 Outline of the study

In the years 2014 to 2017, investigations on ESBL-/AmpC-producing Enterobacteriaceae along the broiler production chain were conducted in the framework of the RESETII consortium "ESBL and Fluorchinolon-Resistance in Enterobacteriaceae" as part of the sub-project "Transmission of ESBL-/AmpC-producing Enterobacteriaceae in the entire production chain of broilers: points of hazard and intervention", funded by the Federal Ministry of Education and Research (BMBF, grand 01KI1313C) (http://reset-verbund.de). The investigations along the production chain included parent flocks, the hatchery and the fattening period and were conducted in collaboration with the doctoral thesis of Michaela Projahn (Institute for Animal Hygiene and Environmental Health, Freie Universität Berlin (FUB)). The thesis of Philine von Tippelskirch (Institute of Food Safety and Food Hygiene, FUB) investigating ESBL-/AmpC-producing Enterobacteriaceae at the slaughterhouse was also part of the RESETII sub-project.

2.1 Samplings

In total, 3137 samples were collected within 53 sampling time points. Seven broiler fattening chains were investigated for ESBL-/AmpC-producing Enterobacteriaceae (Figure 3). Detailed information on the seven investigated fattening flocks are given in table 8 and in Publication I. ESBL-/AmpC-positive parent flocks were selected by an initial screening and their corresponding hatching eggs, the hatchlings as well as the hatchery's environment were investigated. Then, the respective animals were transported to the fattening farm. The transportation vehicles both transporting the hatching eggs from the parent flocks to the hatchery as well as transporting the hatchlings from the hatchery to the fattening farms were sampled as well.

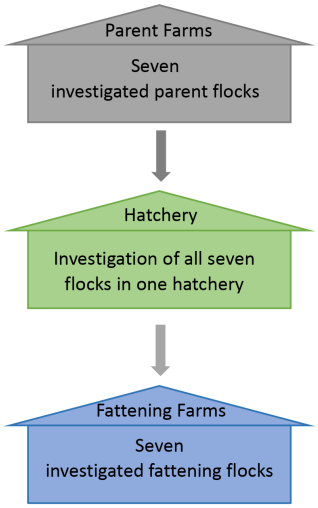

Figure 3: Illustration of the investigated stages of the broiler production chain.

Table 8: Information on the seven investigated broiler fattening flocks (flock A-G) (Daehre et al. 2017).

Chain	No. of farm and barn (farm - barn)	Sampling date	No. of animals	Age of parent flock (weeks)	Age in days, second sampling	Age in days, third sampling	Fattening duration (days)	Antibiotic treatment during fattening
A	1 - 11	11.08.14-05.10.14	22,500	29	19	31	32	Tylosin[c]
B	4 - 41	16.9.14-06.11.14	21,800	57	11	27	32	-
C	4 - 43	15.12.14-09.02.15	21,700	43	17	31	32	-
D	4 - 43	26.01.15-27.04.15	22,000	50	18	30	32	-
E	7 - 74	17.06.15-17.08.15	23,000	51	17	31	38[a]	-
F[b]	7 - 74	29.07.15-28.09.15	22,500	50	19	32	33	-
G	3 - 34	15.01.16-02.03.16	22,000	58	18	34	34	-

a – Preharvesting at day 31
b – Flock F fattened consecutively to flock E in the same barn
c – Tylosin (macrolide antibiotic, treatment on days 26-28, indication: enteritidis caused by clostridia)

On the fattening farms, the investigations were conducted at three different time points during the entire fattening period. The first sampling was carried out at the first day of the fattening period. To get information on the ESBL-/AmpC-status of the barns, the housing environment was investigated just before the arrival of the animals, except for flock C, where the samples were taken in parallel to the chickens' arrival. Directly after the arrival of the day-old chicken on the farm, 40 randomly chosen individuals were sampled by taking cloacal swabs. The second and third sampling on the farm was carried out at the middle and the end of the fattening period whilst both individual animals as well as the housing environment was sampled. For four flocks (D – G), an additional sampling was performed 24 hours after the arrival of the chicken at the farm. At that time, 40 individual animals, pooled faeces and a boot swab was investigated. Two of the investigated flocks (E and F) were fattened consecutively in the same barn. Here, as an additional sample, pasture and soil were investigated at the end of the fattening period from the outside of the barn where the exhaust air was emitted. Detailed information on the investigated samples in the different stages of the broiler production chain are shown in table 9.

Table 9: Investigated samples at the different sampling time points in the different stages of the broiler production chain. Data partially published by Projahn et al. (2017) and Daehre et al. (2017).

Sampling time point	Individual animal samples	Environmental samples	Swab samples
Parent flock		Pooled faeces Boot swab	
Hatchery			
Arrival of eggs	40x outer egg surface 40x egg content 40x inner egg surface	Air samples, feathers, flies	2x truck, wall, ground, drain
After disinfection of eggs	40x outer egg surface	Air samples, feathers	Wall, ground, incubator racks, ventilator
Hatching of chicken	40x cloacal swabs	2x crushed eggshells, chickens' dust	Chicken boxes, discarder of eggshells, 2x band-conveyors during chickens' inspection, chickens after vaccination, transport boxes
Fattening farm			
Arrival on the farm	40x cloacal swabs	Boot swab, feed, litter, drinking water, dust, air sample	2x truck, 2-3x chickens' boxes, feeding trough, water trough, barn wall, hangers of barn equipment, ventilator
24 hours after arrival	40x cloacal swabs	Pooled faeces, boot swabs	

Middle of fattening period	40x cloacal swabs	Pooled faeces, boot swab, feed, litter drinking water, dust, air sample	Feeding trough, water trough, barn wall, hangers of bar equipment, ventilator, chicken
End of fattening period	40x cloacal swabs	Pooled faeces, boot swab, feed, litter, drinking water, dust, air sample	Feeding trough, water trough, barn wall, hangers of barn equipment, ventilator, chicken

2.2 Laboratory methods

All samples were investigated qualitatively and quantitatively for ESBL-/AmpC-producing Enterobacteriaceae (Projahn et al., 2017; Daehre et al., 2017). The isolation of the bacteria was performed on MacConkey agar No. 3 (Oxoid, Wesel, Germany) containing 1 mg/l cefotaxim (AppliChem, Darmstadt, Germany). In addition, for some selected samples, the occurrence of non-resistant Enterobacteriaceae was investigated using the MacConkey agar without the addition of an antibiotic. Colonies with Enterobacteriaceae-like phenotypes were suspected to be ESBL-/AmpC-producers and further investigated. Species were determined using Matrix Assisted Laser Desorption Time of Flight Mass Spectroscopy (MALDI-TOF MS) (MALDI Microflex® LT and Biotyper database®, Bruker Daltonics, Bremen, Germany) and phylogroups of the *E. coli* isolates were defined by performing a multiplex polymerase chain reaction (PCR) (Clermont et al., 2013) with modifications according to Projahn et al. (2017). Phenotypic antimicrobial resistance was tested via agar disk diffusion and, in some cases, via the Vitek®2 system (BioMérieux, Germany). The most common class A beta-lactamase-genes $bla_{\text{CTX-M}}$, bla_{SHV}, bla_{TEM} and CIT-type AmpCs were detected for all ESBL-/AmpC-suspicious enterobacterial isolates and the ESBL-/AmpC-genes were verified by sequencing. As the aim of the study was the elucidation of transmission routes along the production chain, whole genome sequencing (WGS) analysis were performed as well. Therefore, isolates with equal characteristics regarding species, phylogroups and resistance genes from the respective fattening chains were selected. Detailed information on the laboratory methods are displayed in Publication I and Publication II.

2.3 Results

2.3.1 Detection fequencies in ESBL-/AmpC-producing Enterobacteriaceae in parent flocks, in the hatchery, and in fattening farms

In total, 66.7% (24/36) of the samples from the parent flocks, 0.8% (12/1583) of the samples taken in the hatchery and 18.7% (249/1334) of the samples from the fattening period were tested positive (Table 10).

Table 10: Detection frequencies of ESBL-/AmpC-producing Enterobacteriaceae in the seven investigated broiler chains. Data partially published by Projahn et al. (2017) and Daehre et al. (2017).

Sampling time point	No. of positive samples/ no. of all samples						
	Chain A	Chain B	Chain C	Chain D	Chain E	Chain F	Chain G
Parent flock							
Boot swab	1/1	1/1	1/1	1/1	3/3	0/3	1/4
Pooled faeces	3/3	3/3	3/3	2/3	3/3	1/3	1/4
Hatchery							
Arrival of eggs							
Outer egg surface	0/40	4/40	0/40	0/40	0/40	1/40	0/40
Egg content	0/40	0/40	0/40	0/40	0/40	0/40	0/40
Inner egg surface	0/40	0/40	0/40	0/40	0/40	0/40	0/40
Env. samples*	0/7	0/9	0/8	0/10	0/11	0/10	0/13
After disinfection of eggs							
Outer egg surface	0/40	1/40	0/40	0/40	0/40	0/40	0/40
Env. samples*	0/8	0/6	0/6	0/6	0/6	0/7	0/10
Hatching of chicken							
Cloacal swabs	0/40	0/40	0/40	0/40	0/40	0/40	0/40
Env. samples*	0/8	3/9	0/8	0/11	0/9	2/14	1/17
Fattening farm							
Arrival on farm							
Cloacal swabs	0/40	2/40	0/40	0/40	0/40	0/40	0/40
Env. samples*	0/14	0/16	3/15	0/15	0/17	2/20	2/28
24 hours after arrival							
Cloacal swabs	n.d.	n.d.	n.d.	0/40	1/40	2/40	2/40
Env. samples*	n.d.	n.d.	n.d.	0/2	0/2	2/2	0/2
Middle of fattening period							
Cloacal swabs	12/40	17/40	40/40	2/40	6/40	2/40	0/40
Env. samples*	4/12	10/12	9/13	3/16	3/16	3/16	2/18
End of fattening period							
Cloacal swabs	35/40	12/40	33/40	1/40	10/40	0/40	0/40
Env. samples*	8/12	6/10	5/13	1/13	4/16	3/16	2/18

n.d. – not determined

* - environmental samples including air and swab samples

2.3.1.1 Hatchery samples

In the hatchery, ESBL-/AmpC-producing Enterobacteriaceae only were detected rarely (Table 10). At the arrival of the eggs, ESBL-/AmpC-producing Enterobacteriaceae only were detected in 1.8% (n=280) on the outer egg surfaces in two flocks (flocks B and F). After the disinfection of the eggs, ESBL-producing Enterobacteriaceae still were detectable on one outer egg surface in flock B (0.4%; n=280). After the hatching of the chicken, samples from the hatchery's environment were tested positive in flock B, F and G. There, ESBL-/AmpC-producing Enterobacteriaceae were detected in the hatchlings dust, in crushed eggshells and in environmental swab samples. The cloacal swabs investigated from the recently hatched chicken in the hatchery all were tested negative for ESBL-/AmpC-producing Enterobacteriaceae and only 1.1% of the recently hatched chicken were tested positive for non-resistant Enterobacteriaceae.

2.3.1.2 Fattening farm

On the fattening farms, ESBL-/AmpC-producing Enterobacteriaceae were detected in the seven investigated flocks at all three sampling time points. At the first sampling, the housing environment was tested positive for ESBL-/AmpC-producing Enterobacteriaceae before the arrival of the chicken in a boot swab and litter sample in one flock (flock F). After the animal's arrival, the antibiotic resistant bacteria were detected in 0.7% and non-resistant Enterobacteriaceae in 25% of the individual animals (cloacal swabs; n=280). At the middle and at the end of the fattening period, ESBL-/AmpC-producing Enterobacteriaceae were detected in all seven investigated flocks, however, the prevalence between the seven flocks showed wide variations from 1.9% to 92.3% regarding all samples and from 0% to 100% regarding the cloacal swabs.

At the additional sampling of the flocks D-G, 24 hours after the chicken's arrival at the farm, 3.1% of the investigated cloacal swabs (n=160) were tested positive for ESBL-/AmpC-producing Enterobacteriaceae and 93.8% were positive for non-resistant Enterobacteriaceae. Both environmental samples (boot swab, litter) were tested positive in one flock at this time point (flock F). The additional sample from the outside environment surrounding the exhaust air ventilators (pasture and soil) of flock F was positive for ESBL-producing Enterobacteriaceae at the end of the fattening period.

Detailed information can be found in Publication I and Publication II.

2.3.2 Isolate characterization

Various strains of ESBL-/AmpC-producing Enterobacteriaceae were detected in the seven investigated broiler chains. Overall, in isolates detected in the parent flocks, the hatchery and the fattening flocks, *E. coli* strains belonging to phylogroups E, E/D, A, F, B1 and B2 were identified and the majority of the ESBL-/AmpC-producing *Escherichia* spp. strains (n=294) harboured the $bla_{\text{CMY-2}}$ gene (76.2%), followed by $bla_{\text{SHV-12}}$ (10.2%), $bla_{\text{CTX-M-1}}$ (7.8%), $bla_{\text{TEM-52}}$ (5.4%) and $bla_{\text{CTX-M-15}}$ (0.3%). Referring only to the fattening farms, the majority

of the ESBL-/AmpC-producing *E. coli* strains (n=251) harboured $bla_{\text{CMY-2}}$ (86.1%) followed by $bla_{\text{SHV-12}}$ (11.6%) and $bla_{\text{CTX-M-1}}$ (1.6%). In addition, in one flock (flock B), SHV-2 producing *K. pneumoniae* were detected in the different stages of the broiler production chain and in another flock, *C. freundii* strains encoding for CIT-type AmpCs were isolated. Details for each flock are shown in table 11.

Table 11: Detected ESBL-/AmpC-producing Enterobacteriaceae in the seven investigated broiler chains with information concerning their species, resistance genes and phylogroups. Data partially published by Projahn et al. (2017) and Daehre et al. (2017).

Broiler Chain	Sampling time point	No. of samples	Species	*bla*-genes	Phylogroup
A	Parent flock	4	*E. coli*	TEM-52	A
	Fattening period	69	*E. coli*	CMY-2	E, E/D, B1, F, A
B	Parent flock	8	*E. coli*	CTX-M-1	A, A/C, B1
		4	*E. fergusonii*	TEM-52	neg.
	Hatchery I	4	*E. coli*	CTX-M-1	B1
	Hatchery II	1	*E. fergusonii*	TEM-52	neg.
	Hatchery III	1	*E. coli*	CMY-2	E/D
		2	*K. pneumoniae*	SHV-2	neg.
	Fattening farm	12	*E. coli*	CMY-2	E/D, A/C
		41	*K. pneumoniae*	SHV-2	neg.
C	Parent flock	2	*E. spp*	CMY-2	neg./B1
		1	*E. fergusonii*	TEM-52	neg.
	Fattening farm	117	*E. coli*	CMY-2	F, A, B2
D	Parent flock	4	*E. coli*	CMY-2	A, F
		1	*E. coli*	CTX-M-1	F
	Fattening farm	8	*E. coli*	CMY-2	B1, B2
E	Parent flock	6	*E. coli*	CTX-M-1	F
	Fattening farm	10	*E. coli*	CMY-2	F, E/D
		17	*E. coli*	SHV-12	B1
F	Parent flock	1	*E. coli*	CTX-M-15	F
	Hatchery I	1	*C. freundii*	CMY	neg.
	Hatchery III	1	*E. coli*	SHV-12	F
	Fattening farm	12	*E. coli*	SHV-12	B1, F
		4	*E. coli*	CTX-M-1	a
G	Parent flock	4	*E. coli*	TEM-52	A/C, B1
		1	*E. coli*	CMY-2	B1
	Hatchery III	1	*E. coli*	TEM-52	A
	Fattening farm	7	*C. freundii*	CMY	neg.
		1	*E. fergusonii*	TEM-52	neg.

Hatchery I – arrival of eggs; Hatchery II – after disinfection of eggs; Hatchery III – hatching of chicken

In **flock E** and **flock F**, the two flocks fattened consecutively in the same barn, isolates with equal characteristics concerning their species, phylogroups and resistance genes were detected. *E. coli* isolates harbouring *bla*$_{\text{SHV-12}}$ (phylogroup B1) were detected during the fattening period of flock E. Isolates with those characteristics were also found in samples from the housing environment (boot swab and litter) before the animals of the consecutively fattened flock F arrived at the farm (first sampling). Following, the *E. coli* isolates harbouring *bla*$_{\text{SHV-12}}$ (phylogroup B1) were detected throughout the entire fattening period of flock F, both in individual animals and in samples from the housing environment. Furthermore, these isolates also were detected in the sample from the outside environment surrounding the exhaust air ventilator (soil and pasture). To verify the assumed phylogenetic relationship of the isolates of the two consecutively fattened flocks, 13 *E. coli* isolates (phylogroup B1, resistance gene *bla*$_{\text{SHV-12}}$) of both flocks were used for WGS. Those isolates were selected both from individual animals as well as from environmental samples/swabs from different sampling time points. Based on the WGS-data, all 13 isolates were assigned to the multi locus sequence type (MLST) ST2307. The analyses of the single nucleotide polymorphisms (SNPs) in the core genome showed a close relationship between isolates of these two consecutively fattened flocks with maximum differences of 43 SNPs. Detailed information on the SHV-12 producing *E. coli* from the two flocks are shown in table 12 and in Publication I.

Table 12: Thirteen *E. coli* isolates from the consecutively fattened flocks E and F investigated by WGS. All isolates harbour bla$_{\text{SHV-12}}$, belong to the phylogroup B1 and to MLST type ST2307.

Flock	Sampling time point	Samples
E	End of fattening period	Boot swab, pooled faces, litter, 3x CS[a], env. swab[b]
F	Before arrival	Litter, boot swab
	24 h after arrival	1x CS[a]
	Middle of fattening period	Boot swab, 1x CS[a]
	End of fattening period	Outside sample[c]

[a] - cloacal swab
[b] - environmental swab
[c] - pasture and soil from the outside of the barn where the exhaust air was emitted

In **flock B**, *K. pneumoniae* isolates harbouring *bla*$_{\text{SHV-2}}$ were detected in the hatchery, in the transportation truck and during the fattening period. Interestingly, isolates with these characteristics also were detected in another parent flock (parent flock Z) during the initial screening. An essential information is the fact, that the eggs of both parent flocks (B and Z) were bred at the same time in the hatchery and, therefore, the chicken also hatched at the same time. To get more information on these strains and on possible transmission events, 41 isolates detected in samples from the parent flock (flock Z), the hatchery (flock Z and B), the transportation vehicle (flock B) and the fattening flock (flock B) were further characterized by WGS. All these *K. pneumoniae* isolates harbour *bla*$_{\text{SHV-2}}$, belong to the previously unknown *K. pneumoniae* MLST type ST3128, show phenotypical resistance against various antimicrobials

including third and fourth generation cephalosporins and core genome MLST (cgMLST) analyses revealed 100% similarity. Plasmid types IncR, IncFIB, IncFII and IncHI1B, known for the presence of ESBL-encoding genes were detected in all strains. Additionally, the DNA sequences of the *bla*$_{\text{SHV-2}}$ carrying contigs of approximately 7400 base pair (bp) length were homologous to published plasmids (AF550679, AJ245670). Detailed information is given in Publication II.

3 Publications

3.1 Publication I

Daehre, Katrin; Projahn, Michaela; Semmler, Torsten; Roesler, Uwe; Friese, Anika (2017): **Extended-Sprectrum Beta-Lactamase-/AmpC Beta-Lactamase-Producing Enterobacteriaceae in Broiler Farms: Transmission Dynamics at Farm Level.** In: *Microbial Drug Resistance* 24(4):511-518. DOI: 10.1089/mdr.2017.0150.

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http://doi.org/10.1089/mdr.2017.0150

3.2 Publication II

Daehre Katrin; Projahn, Michaela; Friese, Anika; Semmler, Torsten; Guenther, Sebastian; Roesler, Uwe (2018): **ESBL-Producing *Klebsiella pneumoniae* in the Broiler Production Chain and the First Description of ST3128.** In: Frontiers in Microbiology 9:2302. DOI:10.3389/fmicb.2018.02302.

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ESBL-Producing *Klebsiella pneumoniae* in the Broiler Production Chain and the First Description of ST3128

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ESBL-producing *Klebsiella pneumoniae (K. pneumoniae)* represent an increasing problem both in human and veterinary medicine. As SHV-2 - encoding *K. pneumoniae* were recently detected in the broiler production we were interested in investigating a possible transmission along the broiler production chain and furthermore, in evaluating their possible impact on human health. Therefore, 41 ESBL-producing *K. pneumoniae* originating from a parent flock, from the hatcherys' environment during the hatching of that parent flocks' chickens, and from an associated fattening flock were investigated on an Illumina Miseq. Whole genome sequences were analyzed concerning their MLST-type, cgMLST-type, genotypic and phenotypic resistance, plasmid profiles and virulence genes. Irrespective of the origin of isolation all investigated isolates were multi-drug resistant, harbored the same ESBL-gene bla_{SHV-2}, shared the same sequence type (ST3128) and displayed 100% similarity in core genome multilocus sequence typing (cgMLST). In addition, *in silico* plasmid typing found several Inc/Rep types associated with ESBL-plasmids. Summarizing, identical clones of SHV-2—producing *K. pneumoniae* were detected in different stages of the industrial broiler production in one out of seven investigated broiler chains. This proves the possibility of pseudo-vertical transmission of multi-resistant human pathogens from parent flocks to hatcheries and fattening flocks. Furthermore, the importance of cross-contamination along the production chain was shown. Although the ESBL-producing *K. pneumoniae* clone detected here in the broiler production has not been associated with clinical settings so far, our findings present a potential public health threat.

Keywords: extended-spectrum-beta-lactamases, ESBLs, *Klebsiella pneumoniae*, broiler production, broiler chicken

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INTRODUCTION

The emergence of extended-spectrum beta-lactamase-producing Enterobacteriaceae has been of particular interest for years, in both human and veterinary medicine. Especially *Klebsiella pneumoniae* (*K. pneumoniae)*, causing community and nosocomial infections of the respiratory and urinary tract as well as bloodstream infections are of critical concern. Resistance against

antimicrobials leads to limited therapeutic options, resulting in increasing difficulties of treatments. In contrast to ESBL-producing *E. coli* which have become very common in veterinary medicine, especially in livestock, companion animals and wildlife, ESBL-producing *K. pneumoniae* were detected rarely in healthy broiler (Hiroi et al., 2012; Yossapol et al., 2017; Mahanti et al., 2018), diseased horses (Vo et al., 2007) dairy cows (Locatelli et al., 2010) and in companion animals (Ewers et al., 2014).

In general, the zoonotic impact of animal-originated pathogens on public health via direct contact or due to the consumption of contaminated meat is assumed (Smet et al., 2010; Marshall and Levy, 2011). This warrants the importance of investigations concerning multi-drug resistant bacteria in food-producing animals at different levels of production, to characterize the impact on humans. In previous studies, we detected ESBL-producing *K. pneumoniae* in a German hatchery (Projahn et al., 2017) as well as in a connected broiler farm (Daehre et al., 2017) in one out of seven investigated broiler chains. To characterize and compare those ESBL-producing *K. pneumoniae* strains detected at different levels of the broiler production we used whole genome sequencing assuring a high resolution of the clonal relationship. We are aiming at revealing possible transmission routes of ESBL-producing *K. pneumoniae* along the production pyramid as well as at assessing a possible impact on human health.

MATERIALS AND METHODS

Samples/Flock

In the years 2014–2016, seven German parent flocks, their corresponding hatchlings in the hatchery and later on at the fattening farms as well as the environment of the hatchery and the respective farm were investigated for the occurrence of ESBL-/AmpC-producing Enterobacteriaceae as published by Projahn et al. (2017) and Daehre et al. (2017). There, in one fattening chain (chain B), SHV-2 -producing *K. pneumoniae* strains were detected and those stored bacterial isolates were retrospectively characterized in the present study. In detail, various samples were taken after the hatching of the chicken in the hatchery such as dust, air and swabs from the hatchery's environment. On the fattening farm individual animal samples as well as samples from the housing environment were collected at three different samplings (first day, middle and end of fattening period). In detail, 40 individual animals (cloacal swabs), pooled feces, boot swabs, litter, dust, and air were collected and several surfaces in the barn were swabbed (environmental swabs).

Within this study, additional isolates of a boot swab and a pooled feces sample from another parent flock (flock Z) were investigated as well. This was done due to the fact that the eggs of both parent flocks (B and Z) were bred in the hatchery at the same time. Furthermore, we analyzed isolates originating from environmental swabs from the truck that transported the chicken of parent flock B from the hatchery to the farm. **Figure 1** schematically illustrates the origin of the bla_{SHV-2} - positive isolates. Detailed information on the investigated isolates are shown in **Table 1** and in **Table S1**.

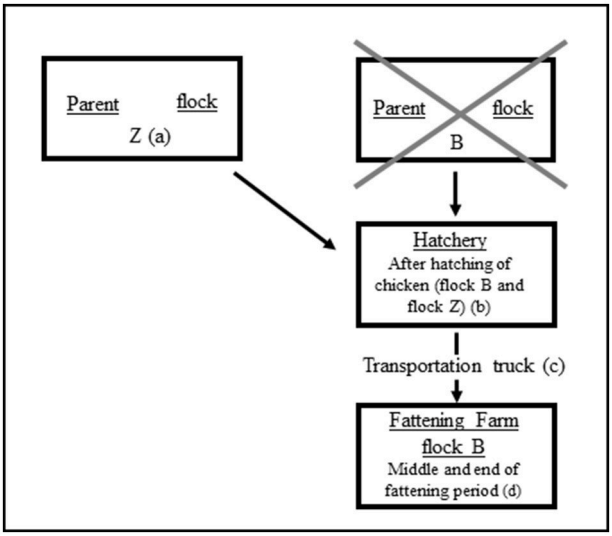

FIGURE 1 | Schematic diagram of stages of the broiler production chain tested positive for SHV-2-producing *Klebsiella pneumoniae.* Parent flock B *was* negative. Positive samples: (a)-pooled feces; (b)-egg shells, environmental swab; (c)-swabs; (d)-cloacal swabs, pooled feces, boot swab, litter, dust, air, environmental swabs.

Laboratory Methods

All samples were processed as described by Projahn et al. (2017) and Daehre et al. (2017). Finally, the samples were streaked out on MacConkey No. 3 (Oxoid, Wesel, Germany) agar plates with the addition of 1 mg/l cefotaxime (AppliChem, Darmstadt, Germany). The species of all isolates with Enterobacteriaceae-like phenotypes were determined by MALDI-TOF analyses. The detection of the most common class A beta-lactamase-genes including bla_{SHV} was performed as described by Roschanski et al. (2014) and verified by sequencing using the same primer set as published by Projahn et al. (2017).

Forty-one *K. pneumoniae* isolates with bla_{SHV-2} - genes from 26 different samples (up to three isolates per sample) were chosen for further characterization. These isolates originated from the other parent flock Z, the hatchery, the truck (transport of chicken from hatchery to farm) as well as from samples from the middle and the end of the fattening period on the farm (cloacal swabs and samples from the housing environment; **Figure 1**, **Table 1**, and **Table S1**).

The Vitek®2 system (BioMérieux, Germany; card GN38) was used to determine phenotypic antimicrobial resistance to various ß-lactam-antibiotics and other classes of antimicrobials.

To get more information concerning the phylogenetic relationship of the different samples, whole genome sequencing (WGS) was performed. Therefore, DNA was extracted with the MasterPure™ DNA purification kit (Epicenter, Illumina) and Illumina MiSeq 300-bp paired-end with a coverage between 50x and 120x was used. Following a quality control performed with the NGS tool kit (Patel and Jain, 2012) high quality reads were defined (minimum of 70% of bases having a phred score higher than 20) and *de novo* assembled into contiguous sequences

TABLE 1 | Information on seven *K. pneumoniae* isolates (ST3128) including sampling timepoint, sample type, isolate ID, plasmid type, genotypic, and phenotypic resistance.

Sampling timepoint	Sample type	Isolate ID	Plasmid type	Genotypic resistance	Phenotypic resistance
I	PF	ITU10028	IncR, IncFIB, IncFII, IncHI1B, Col	bla_{SHV-2}, bla_{SHV-1}, *fos*A-like, *sul*1, *dfr*A12, *tet*(D), *aad*A1, *aad*A2, *aac*(3)-like, oqxA-like, *oqx*B-like, *parC* S80I mutation	AMX, AMC, AMP, SAM, CFR, CFL, CLT, CFZ, CFM, CPZ, CTX, CFV, CFP, CPD, CAZ, CEX, CFX, DOX, ENR, GEN, MAR, PIP, PUFX, TET, TOB, SXT
II	EggS	ITU10022	IncR, IncFIB, IncFII, IncHI1B, IncX1, IncX3, Col	bla_{SHV-2}, bla_{SHV-1}, *fos*A-like, *sul*1, *dfr*A12, *tet*(D), *aad*A1, *aad*A2, *aac(3)-like*, oqxA-like, *oqx*B-like, *parC* S80I mutation	
III	EnvS	ITU10024,	IncR, IncFIB, IncFII, IncHI1B, Col	bla_{SHV-2}, bla_{SHV-1}, *fos*A-like, *sul*1, *dfr*A12, *tet*(D), *aad*A1, *aad*A2, *aac*(3)-like, *oqxA-like, oqxB-like, parC* S80I mutation	
IV	CS	ITU3949	IncR, IncFIB, IncFII, Col	bla_{SHV-2}, bla_{SHV-1}, *fos*A-like, *sul*1, *dfr*A12, *tet*(D), *aad*A1, *aad*A2, *aac*(3)-like, *oqx*A-like, *oqx*B-like, *parC* S80I mutation	
	Litter	ITU3854	IncR, IncFIB, IncFII, IncHI1B, Col	bla_{SHV-2}, bla_{SHV-1}, *fos*A-like, *sul*1, *dfr*A12, *tet*(D), *aad*A1, *aad*A2, *aac*(3)-like, *oqxA*-like, *oqxB*-like, *parC* S80I mutation	
V	CS 18	ITU4179	IncR, IncFIB, IncFII, IncHI1B, Col	bla_{SHV-2}, bla_{SHV-1}, *fos*A-like, *sul*1, *dfr*A12, *tet*(D), *aad*A1, *aad*A2, *aac*(3)-like, *oqx*A-like, *oqx*B-like, *Qnr*S1-like, *parC* S80I mutation	
	BS	ITU4097	IncR, IncFIB, IncFII, IncHI1B, Col	bla_{SHV-2}, bla_{SHV-1}, *fos*A-like, *sul*1, *dfr*A12, *tet*(D), *aad*A1, *aad*A2, *aac*(3)-like, *oqx*A-like, *oqx*B-like, *parC* S80I mutation	

I, parent flock Z; II, hatchery, after hatching; III, truck (from hatchery to farm); IV, fattening farm, middle of fattening period; V, fattening farm, end of fattening period; PF, pooled feces; EggS, pooled eggshells; EnvS, environmental swab; CS, cloacal swab, BS, boot swab; ITU, Institut für Tier- und Umwelthygiene; AMX, amoxicillin; AMC, Amoxicillin/clavulanic acid; AMP, ampicillin; SAM, ampicillin/sulbactam; CFR, cefadroxil; CFL, cefalexin; CLT, cefalotin; CFZ, cefazolin; CFM, cefixime; CPZ, cefoperazone; CTX, cefotaxime; CFV, cefovecin; CFP, cefpirome; CPD, cefpodoxime; CAZ, ceftazidime; CEX, ceftiofur; CFX, cefuroxime; DOX, doxycyclin; ENR, enrofloxacin; GEN, gentamicin; MAR, marbofloxacin; PIP, piperacillin; PUFX, prulifloxacin; TET, tetracyclin; TOB, tobramycin; SXT, trimethoprim/sulfamehoxazol.

(contigs) using CLC Genomics workbench 9.0 (Qiagen, Venlo, Netherlands). These sequence data have been deposited at DDBJ/ENA/GenBank and the accession numbers can be found in the **Table S2**.

The bla_{SHV} genes were sequenced using the same primer set as published by Projahn et al. (2017) and evaluated with BioNumerics 6.6.

WGS data were used for genotypic characterization utilizing the Center for Genomic Epidemiology (Center for Genomic Epidemiology, 2018): multi-locus sequence types [MLST; MLSTFinder 1.8 (Larsen et al., 2012)], plasmids [PlasmidFinder 1.3 (Carattoli et al., 2014)] and resistance genes [ResFinder3.0 (Zankari et al., 2012)] were determined. Additionally, core genome MLST (cgMLST) typing was performed using the cgMLST.org Nomenclature Server[1] and Ridom Seqsphere 4.1 (Ridom GmbH, Muenster, Germany). Within cgMLST, for *K. pneumoniae,* 2358 conserved genome-wide genes are compared, resulting in a very high discriminatory power.

BLAST analyses of the assembled contigs were done and the accordance of bla_{SHV}-carrying contigs with SHV-encoding plasmids described for *K. pneumoniae* (JX461340.1, CP025463, CP025458, DQ449578, JN247852, and others) were checked using the European nucleotide archive[2], the European Center for Biotechnology Information[3] and features of Geneious v. 7.1.2 (Kearse et al., 2012) and DNASTAR® Lasergene 11 SeqMan Pro™ (version 11.2.1). The genetic vicinity of the bla*SHV* region as well as the occurrence of genes known for the association with virulent *K. pneumoniae* (*magA*, *rmpA*, *entB*, *iutA*, *YbtS*, *Kfu*, *allS*, *mrkD*, *wzi*; Compain et al., 2014) were investigated using the same tools.

RESULTS AND DISCUSSION

SHV-2 -encoding *K. pneumoniae* strains were detected in all investigated stages of the broiler production chain: 25% of the

[1]"cgMLST.org Nomenclature Server,". Available online at: http://www.cgmlst.org/ncs (Accessed April 05, 2018).

[2]European Nucleotide Archive. Available online at: https://www.ebi.ac.uk/ena (Accessed April 05, 2018).

[3]"National Center for Biotechnology Information,". Available online at: https://www.ncbi.nlm.nih.gov/ (Accessed April 05, 2018).

samples from parent flock Z (1/4) (but not in samples from parent flock B), 22.2% from the hatchery's environment (2/9) and 28.8% of individual animals (23/80) resp. 68.2% of samples from the housing environment (15/22) of flock B (middle and end of fattening period) were tested positive. All 41 *K. pneumoniae* isolates were multi-drug resistant, harbored the genes for the ESBL beta-lactamase SHV-2 and were assigned to the newly described MLST type ST3128. cgMLST typing revealed 100% similarity. Therefore, a clonal relationship between the isolates detected at the different stages of the broiler production chain can be stated.

Antibiotic resistant *K. pneumoniae* isolates, especially ESBL–and/or carbapenemase–producers with resistance toward third/fourth generation cephalosporins and carbapenems are of great concern in both human and veterinary medicine. To our knowledge, this is the first finding of ESBL-producing *K. pneumoniae* detected in various stages within the same broiler production chain. To get more information on these strains, 41 isolates detected in samples from a parent flock, the hatchery, the transportation vehicle and a fattening flock of one out of seven investigated broiler chains were further characterized.

All *K. pneumoniae* isolates harbor the $bla_{\mathrm{SHV-2}}$ - gene, belong to the previously unknown *K. pneumoniae* MLST type ST3128 and show phenotypical resistance against various antimicrobials including third and fourth generation cephalosporins, but not against carbapenems (**Table 1** and **Table S1**). With regard to the fluoroquinolone resistance, all 41 isolates have a mutation (S80I) in the *parC* gene known for fluoroquinolone-resistant *K. pneumoniae* (Correia et al., 2017) but mutations in the QRDR-region of the *gyrA* gene were not detected. All investigated *K. pneumoniae* isolates possess *entB* and *mrkD*, but none of the other genes, associated with virulence of *K. pneumoniae* (*magA*, *rmpA*, *iutA*, *YbtS*, *Kfu*, *allS*, *wzi).* Enterobactin *(entB)* is a prototypical catecholate siderophore as part of iron acquisition systems. But *entB* only is known for virulence when occurring in a combination with iron acquisition systems (*iutA, YbtS, Kfu*). The same applies to *mrkD*. *mrkD* is believed to function as the type 3 fimbrial adhesion and mediates binding to extracellular matrix (Jagnow and Clegg, 2003) but in virulent *K. pneumoniae* strains only occurs in combination with other virulence factors, which were tested negative in our isolates. Therefore, the 41 ESBL-producing *K. pneumoniae* strains, detected in healthy broiler chicken, do not harbor virulence genes that were described in any clinical association. Inc typing using plasmidFinder found the plasmid types IncR, IncFIB, IncFII, and IncHI1B known for the presence of ESBL-encoding genes in all strains (**Table 1** and **Table S1**). The DNA sequences of the $bla_{\mathrm{SHV-2}}$ carrying contigs of ∼7,400 bp length were homologous to plasmid p1658/97 from *Eschericia coli* (accession number: AF550679) and plasmid pSEM from *Salmonella enterica* (AJ245670), containing a *recF* gene upstream and a *deoR* gene downstream from the $bla_{\mathrm{SHV-2}}$. These genes are also present in a bla_{SHV}-carrying plasmid of *K. pneumoniae* published by Yu et al. (2006). The *recF* gene may contribute to the mobilization of the *bla* gene to a plasmid via the *recF* recombination pathway (Kolodner et al., 1985).

Additionally, a bla-SHV-2 carrying fragment (∼3,500 bp) of the contigs was detected in plasmids of *K. pneumoniae* (JX461340.1, CP025463, CP025458, DQ449578, JN247852) (more than 99% identity). The adjacent DNA sequence was identical to other plasmids (CP027613, LT985275, and others). These findings make the location of the $bla_{\mathrm{SHV-2}}$ on a plasmid very likely.

To elucidate the epidemiological relationship and, therefore, the transmission dynamics along the production chain, cgMLST was performed. cgMLST, comparing 2358 genes for *K. pneumoniae*, revealed 100% similarity. This demonstrates that identical clones of *K. pneumoniae* (ST3128), encoding for SHV-2 were detected in the different stages of the broiler production chain pointing toward ongoing transmission processes.

The circulation of ESBL-/AmpC-producing Enterobacteriaceae along the broiler production process was described in previous studies (Dierikx et al., 2013; Nilsson et al., 2014). Projahn et al. (2017) conducted transmission investigations with a special focus on the hatchery and *Escherichia coli (E. coli)*. There, the introduction of ESBL-producing *E. coli* strains directly from the parent flock into the hatchery, despite the eggs' disinfection, was shown. Additionally, a pseudo-vertical transmission, in detail, the introduction of ESBL-producing Enterobacteriaceae into the hatchery and the chickens' colonization by the uptake of resistant bacteria from the environment of the hatchery was discussed.

Our results reinforce these hypotheses. Parent flock Z (the other parent flock) was tested positive for SHV-2–producing *K. pneumoniae*. In the hatchery, clones of this strain were detected after the chickens hatching in the hatchery's environment (environmental swab and eggshells). We showed that the chicken of flock B, that hatched at the same time in the hatchery were colonized with those clones. Furthermore, the SHV-2-producing *K. pneumoniae* were detected during the whole fattening period of flock B both, in individual animals as well as in samples from the environment. This clearly demonstrates a transmission from parent flocks, via the hatchery, into the fattening flocks. This is in accordance to Projahn et al. (2018). They showed that ESBL-producing *E. coli* were already present in the hatchery and colonized the recently hatched chickens. Therefore, they also confirmed the hatchery as a contamination source for the fattening period.

In our study, clones of the SHV-2-producing *K. pneumoniae* were detected in parent flock Z, in the hatchery after the chickens hatching and during the fattening period of flock B. As the eggs of parent flock Z were bred at the same time as the eggs of flock B, the chicken of flock Z and B hatched at the same time in the same surrounding. This means the original source of the ESBL-producing *K. pneumoniae* strains was not the respective parent flock B but an unrelated parent flock. This clearly emphasizes the importance of cross-contamination via the environment, especially at hatchery-level.

As described by Dierikx et al. (2013) the broiler production system seems rather simple: only a few breeding companies produce broilers for many farms. The breeding eggs of several parent flocks are processed in a few hatcheries and the hatched broiler chicken are delivered to various fattening farms. Thus, the introduction of resistant bacteria from one parent flock into the hatchery can cause the spread of these strains in several

fattening farms. Consequently, the absence of antibiotic resistant bacteria in parent flocks is essential to minimize the occurrence of these bacteria in the production pyramid and one high prevalent parent flock has the potential to contaminate all hatchlings of one course of hatching.

Besides, *K. pneumoniae* clones were also detected in the truck, transporting the chicken from the hatchery to the farms (environmental swabs from the truck's ground and walls). Projahn et al. (2018) also detected ESBL- producing Enterobacteriaceae in a transportation truck and indicated a transmission of resistant bacteria via the transportation process. As the trucks transport animals for several farms, cross-contamination even during the transport could be of importance as well.

As described above, the *K. pneumoniae* isolates were detected during the investigation on ESBL-/AmpC-producing Enterobacteriaceae in parent flocks, the hatchery and at fattening farms (Daehre et al., 2017; Projahn et al., 2017). Thus, next to the *K. pneumoniae*, several ESBL-/AmpC- producing *E. coli* were detected. These *E. coli* isolates encoded for SHV-12, CMY-2 or CTX-M-1, but not for SHV-2, in contrast to the *K. pneumoniae* isolates. Therefore, with our investigated isolates we cannot show any association between ESBL- producing *E. coli* and ESBL-producing *K. pneumoniae* in the broiler production chain.

The detected antibiotic resistant *K. pneumoniae* strains of ST3128 have not been reported in clinical settings, yet, and, therefore, did not have an impact on human health so far. However, plasmids with resistance genes are transferable between strains and species. Therefore, resistance-carrying plasmids detected in food-producing animals always pose a possible risk for human health.

In summary, our results demonstrate the presence of SHV-2–producing *K. pneumoniae* clones in several stages of the broiler production pyramid. A pseudo-vertical transmission of ESBL-producing *K. pneumoniae*, resulting in a positive fattening flock caused by the uptake of bacteria that were introduced into the hatchery by another parent flock was shown for the first time. This also indicates the importance of cross-contamination. As *K. pneumoniae* of ST3128 have not been known previously, clones of our strains have never been reported in clinical associations. Therefore, to date, these strains did not have an impact on human health. Nevertheless, a reduction of antibiotic-resistant bacteria in food-producing animals should be achieved, in order to not worsen the situation in human and veterinary medicine.

AUTHOR CONTRIBUTIONS

UR and AF designed the study. KD and MP performed the samplings and the laboratory work. TS and SG performed bioinformatic analysis. KD analyzed the data and wrote the manuscript. All authors have read and approved the final draft of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02302/full#supplementary-material

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4 Unpublished data

4.1 Colonisation of broiler chicken with Enterobacteriaceae in first three days of life

4.1.1 Introduction

Previous studies reported high prevalence for ESBL-/AmpC-producing Enterobacteriaceae especially in broiler fattening farms (Huijbers et al., 2014; Laube et al., 2013; Smet et al., 2008). Additionally, the occurrence of the antibiotic resistant bacteria could even be shown in day-old chicken, partially with high prevalence (Laube et al., 2013; Dierikx et al., 2013b). Laube et al. (2013) demonstrated a prevalence of 51% for ESBL-/AmpC-producing Enterobacteriaceae in day-old chicken. They defined the sampling time point for the day-old chicken with a timeframe of three to 36 hours after the animal's arrival on the farm but no information on the period of time between their hatching and the arrival on the farm are given. In the study presented above, the chicken from seven broiler fattening flocks were first sampled by taking cloacal swabs directly after their hatching in the hatchery (n=280). Secondly, cloacal swabs were taken directly after the chicken's arrival on the farm (n=280) between their third and 12^{th} hour of life and for four flocks (flocks D-G), the broiler chicken also were investigated 24 hours after their arrival on the farm (n=160).

In that study, in the hatchery, ESBL-/AmpC-producing Enterobacteriaceae could not be detected in any cloacal swab (0%). At the first sampling on the fattening farm, 0.7% and 24 hours later 3.3% of the investigated day-old chicken were tested positive for ESBL-/AmpC-producing Enterobacteriaceae. Interestingly, even the detection rate of non-resistant Enterobacteriaceae was rare during the sampling in the hatchery and the first sampling at the farm (1.1% respectively 25%). Twenty-four hours after the arrival on the farm, non-resistant Enterobacteriaceae were detected in 93.8% of the animals (Table 13).

Table 13: Colonization of day-old broiler chicken with ESBL-/AmpC-producing Enterobacteriaceae and non-resistant Enterobacteriaceae at three different time points: directly after the hatching in the hatchery ([a]), directly after the arrival on the farm ([b]) and 24 hours after the arrival on the farm ([c]). Data partially published by Projahn et al. (2017)and Daehre et al. (2017).

	Prevalence of (ESBL-/AmpC-producing) Enteorbacteriaceae in cloacal swabs	
Sampling time point	**ESBL-/AmpC-producing Enterobacteriaceae**	**Non-resistant Enterobacteriaceae**
Hatchery[a]	0%	1.1%
Arrival on farm[b]	0.7%	25%
24 hours[c]	3.3%	93.8%

This information has raised the question from which point in time the ESBL-/AmpC-producing Enterobacteriaceae can be detected by using cloacal swabs. Additionally, we also wanted to determine from which point in time non-resistant Enterobacteriaceae can be detected in recently hatched chicken by using cloacal swabs. Therefore, in April and May 2017, we investigated three broiler flocks intensively in the first three days of life for the occurrence of non-resistant Enterobacteriaceae and for the occurrence of ESBL-/AmpC-producing Enterobacteriaceae. Each 120 animals per sampling time point (40 animals per flock, each) were investigated directly at their arrival on the farm (0 hrs) and 8, 24, 32, 48, 56 and 72 hours (8, 24, 48, 56, 72 hrs) after their arrival. In addition, at the end of the fattening period the barns were investigated for the ESBL-/AmpC-status by taking boot swabs.

Laboratory methods were used as described above.

4.1.2 Results

4.1.2.1 ESBL-/AmpC-producing Enterobacteriaceae

ESBL-/AmpC-producing Enterobacteriaceae could not be detected in any of the 840 investigated cloacal swabs in the first three days of life. The boot swabs, investigated at the end of the fatting periods, were tested negative as well.

4.1.2.2 Non-resistant Enterobacteriaceae

At the arrival on the farm (0 hrs), non-resistant Enterobacteriaceae were detected in 5.8% of the investigated cloacal swabs (7/120). Eight hours after the arrival on the farm (8 hrs) 23.3% (28/120), 24 hours after the arrival (24 hrs) 95.8 % (115/120) and 32 hours after the arrival (32 hrs) 98.3% (118/120) of the investigated animals were tested positive. From 48 hours after the arrival on the farm (48 hrs) onwards, non-resistant Enterobacteriaceae were detected in all investigated cloacal swabs (100%). Figure 4 illustrates the prevalence and the bacterial count of the Enterobacteriaceae.

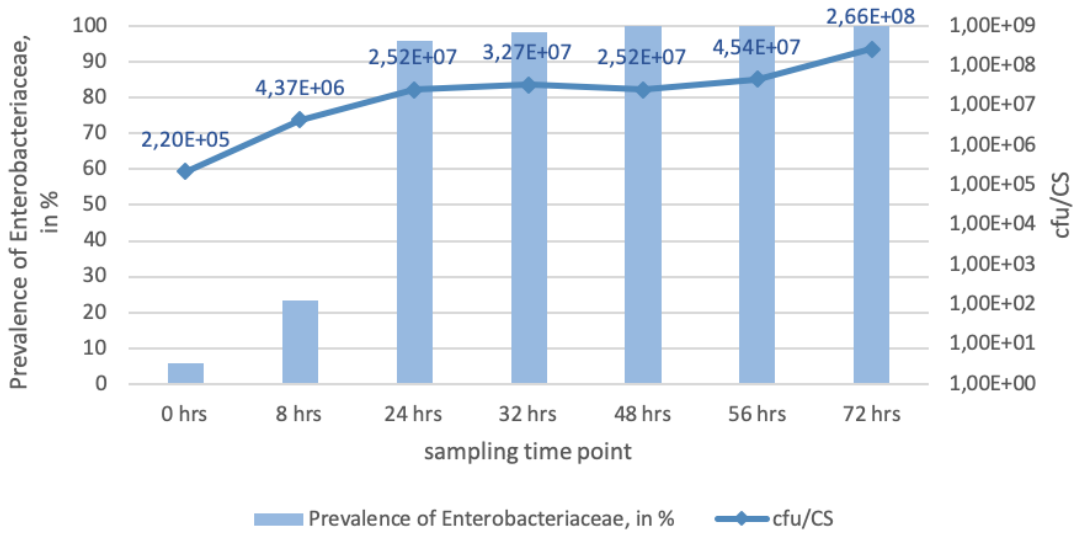

Figure 4: Detection of non-resistant Enterobacteriaceae in broiler chicken detected by cloacal swabs (CS) in the first three days of life. Prevalence of Enterobacteriaceae in percent (%) and colony forming units per clocal swab (cfu/CS). Each 120 animals were sampled per time point (n=120 per time point; n=840 in total).

Taken together, ESBL-/AmpC-producing Enterobacteriaceae were not detected in the three investigated flocks. Nevertheless, we could show an increase of the detection of Enterobacteriaceae in the first hours of live in broiler chicken using cloacal swabs. Whilst the detection rate for the time points 0 hrs and 8 hrs was low with 5.8% and 23.3%, on the second day of life (24 hrs, 32 hrs) 95.8% resp. 98.3% were tested positive. From 48 hrs after arrival on the farm onwards, Enterobacteriaceae were detected in 100 % of the cloacal swabs from the chicken. An increase of the bacterial count was shown as well.

5 Discussion

Many studies showed that ESBL-/AmpC-producing Enterobacteriaceae occur in broiler fattening farms with high prevalence (Huijbers et al., 2014; Laube et al., 2013; Dierikx et al., 2013a) and both vertical and horizontal transmissions were assumed (Laube et al., 2013; Huijbers et al., 2016; Nilsson et al., 2014; Dierikx et al., 2013b). As detailed investigations on transmission processes of ESBL-/AmpC-producing Enterobacteriaceae along the production pyramid were only rare, the presented study was conducted to elucidate possible transmission routes. Therefore, seven broiler fattening chains were investigated for ESBL-/AmpC-producing Enterobacteriaceae. ESBL-/AmpC-positive parent flocks were determined, the corresponding breeding eggs and hatchlings were investigated in the hatchery and the fattening flocks were traced in a long-term investigation. Additionally, within the RESETII-consortium (subproject at FUB) the same flocks were investigated in the slaughterhouse, thus, the introduction of ESBL-/AmpC-producing Enterobacteriaceae from the fattening farms into the slaughterhouse could be assessed as well (von Tippelskirch et al., 2018).

A detailed description on the investigations on ESBL-/AmpC-producing *E. coli* in parent flocks and the hatchery within the RESETII-project was published by Projahn et al. (2018). Here, the focus is on the processes occurring on the level of the fattening farms and, additionally, on the pseudo-vertical transmission of resistant *K. pneumoniae* from a parent flock into the fattening farm.

Within the presented study, various strains of ESBL-/AmpC-producing Enterobacteriaceae were detected in all investigated stages of the broiler production chain (parent flocks, hatchery, fattening period) with wide varying prevalence. Using WGS-data, both the horizontal and the pseudo-vertical transmission route could be identified.

5.1 Prevalence of ESBL-/AmpC-producing Enterobacteriaceae and detected resistance genes in the fattening farms

5.1.1 Prevalence of ESBL-/AmpC-producing Enterobacteriaceae

The prevalence of ESBL-/AmpC-producing Enterobacteriaceae during the fattening period showed wide variations between the seven flocks and even a flock without any positive cloacal swab was detected. However, in this flock the environment was contaminated, indicating that ESBL-/AmpC-positive housing environment does not necessarily result in high-prevalent flocks.

On the fattening farms, the first cloacal swabs were taken directly after the chickens' arrival, between two and six hours after their hatching. At that time, 0.7% of the day-old-chicken were

tested positive for ESBL-/AmpC-producing Enterobacteriaceae and non-resistant Enterobacteriaceae were detected in 25.7%. This is in contrast to Laube et al. (2013). They tested 51% of the investigated day-old chicken positive for ESBL-/AmpC-producing Enterobacteriaceae by cloacal swabs three to 36 hours after arrival on the farm. However, the time frame from the oral uptake to the colonization of the chickens' intestine and to the faecal shedding of commensal ESBL-/AmpC-producing *E. coli* is not known yet. Therefore, the sampling time point in the first hours/days of life influences the detection of ESBL-/AmpC-producing Enterobacteriaceae. To assess the detectability for Enterobacteriaceae, both resistant and non-resistant, in day-old chicken using cloacal swabs, we investigated three broiler flocks intensively in the first three days of life. Therefore, the chicken were sampled directly at their arrival on the farm (0 hrs) and 8, 24, 32, 48, 56 and 72 hours (8, 24, 32, 48, 56, 72 hrs) after their arrival on the farm. Unfortunately for that study, ESBL-/AmpC-producing Enterobacteriaceae were not detected in the investigated animals and the negative boot swabs taken at the end of the fattening periods proved this negative ESBL-/AmpC-status of the three investigated flocks. Non-resistant Enterobacteriaceae in day-old chicken were detected in only 5.8% at 0 hrs and 23.3% at 8 hrs. At 24 hrs and 32 hrs after the arrival on the farm, Enterobacteriaceae were detected with prevalence of 95.8% and 98.3% and from 48 hrs onwards, all investigated animals were tested positive for Enterobacteriaceae using cloacal swabs. This is in accordance with Coloe et al. (1984). They could not detect any bacteria in the chickens' gut after hatching even though the animals were dissected but at day three bacteria were isolated. Considering that ESBL-/AmpC-producing Enterobacteriaceae normally account for about 1% of the total Enterobacteriaceae, the possibility of detecting resistant Enterobacteriaceae at time points when even the non-resistant Enterobacteriaceae cannot be reliably detected is probably rather low. Therefore, samplings in recently restocked chicken for the detection of ESBL-/AmpC-producing Enterobacteriaceae using cloacal swabs should not be performed within the first 24 hours of life.

5.1.2 The use of antibiotics as cause of the occurrence of ESBL-/AmpC-producing Enterobacteriaceae

Several studies assume the use of antibiotics to be responsible for the occurrence of antibiotic resistant bacteria both in veterinary medicine (Dierikx et al., 2013b; Cavaco et al., 2008) as well as in human medicine (Asensio et al., 2000; Lautenbach E. et al., 2001). For example, Dierikx et al. (2013b) assume that the use of antibiotics can select and maintain resistant isolates in the broiler production. In our study, only one of the seven investigated flocks (flock A) was treated with a macrolide antibiotic at the end of the fattening period (day 26-28). But even this flock was tested positive for ESBL-/AmpC-producing Enterobacteriaceae before the antibiotic treatment (day 19). This is in accordance to Huijbers et al. (2016), as they detected ESBL-/AmpC-producing Enterobacteriaceae even in organic farms without any antibiotic treatment. Furthermore, Guenther et al. (2017) detected ESBL-producing *E. coli* also in wild birds that are normally not exposed to antimicrobial agents. On the other hand, the uptake of antibiotics even without an antibiotic treatment could be causative for the occurrence of antibiotic resistant bacteria as well. Hamscher et al. (2003) detected antibiotics in dust

samples originating from pig fattening farms and assumed that the permanent exposure to subtherapeutic concentrations of antibiotics represent ideal conditions for the development or the selection of antibiotic resistance. Additionally, Schulz et al. (2016) demonstrated the occurrence of cultivatable antimicrobial-resistant *E. coli* in sedimentation dust samples for more than 20 years and indicates that dust from/in livestock farms may lead to transmission to other flocks. Therefore, both the presence of antibiotic residues in environmental samples and antibiotic-resistant bacteria in dust samples as risk factors for the occurrence of antibiotic resistant bacteria in livestock farming would be interesting for future studies.

5.1.3 Detected resistance genes in fattening flocks

Overall, *E. coli* strains belonging to the phylogroups E, E/D, A, F, B1 and B2 were detected. The majority of the ESBL-/AmpC-producing strains detected in the fattening flocks (86.1%) harboured $bla_{\text{CMY-2}}$, followed by $bla_{\text{SHV-12}}$ (11.6%) and only 1.6% harboured the $bla_{\text{CTX-M-1}}$ gene. The dominating prevalence of $bla_{\text{CMY-2}}$ is in accordance to Ewers et al. (2012). In their review they summarized published data on ESBL-/AmpC-producing Enterobacteriaceae within the habitats "animal" and "human" and showed that the most common ESBL-/AmpC-genes in poultry in Europe are $bla_{\text{CMY-2}}$ and $bla_{\text{CTX-M-1}}$ (32% resp. 28%). In contrast, in human, $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-15}}$ are the most common beta lactamase resistance genes (Ewers et al., 2012). These genes are also found in chicken, but with low prevalence. Nevertheless, an impact of animal-originated resistant bacteria on public health is assumed by several authors. They mention a possible transmission of resistant bacteria from animals to humans via direct contact or due to the consumption of contaminated meat (Marshall and Levy, 2011; Smet et al., 2010; Leverstein-van Hall et al., 2011; Kluytmans et al., 2013; de Been et al., 2014). To get more information on possible transmission routes from farm animals to human via meat, the research within the RESETII-consortium also included the investigation of the seven flocks at slaughterhouse level (von Tippelskirch et al., 2018). In the slaughterhouse, several environmental samples were also investigated before the arrival of the respective flocks. On the one hand, the results of this study show indications for the introduction of ESBL-/AmpC-producing Enterobacteriaceae from the fattening farms into the slaughterhouse (for flock F, isolates with identical characteristics concerning species, phylogroup and resistance genes were detected during the fattening period as well as in samples taken in the slaughterhouse after the slaughter of the corresponding herd). On the other hand, the results indicate cross-contamination of ESBL-/AmpC-producing Enterobacteriaceae on slaughterhouse level (for flock G, resistant isolates with equal characteristics as detected in the slaughterhouses' environment before the arrival of the animals also were detected in skin and fillet samples).

Dorado-García et al. (2018) analysed and compared data on ESBL-/AmpC-producing *E. coli* obtained from 35 studies in the Netherlands. They showed that most livestock or food associated reservoirs did not show a high level of similarity in their gene profiles in comparison with humans both from the general and the clinical populations. Therefore, they suggest that poultry and poultry meat are not major contributors to ESBL-/AmpC-occurrence in humans. Additionally, they found a high similarity in gene distributions between farmers and their animals as a result

of intensive and direct contact; supporting the hypothesis that direct contact is the most important transmission route.

5.2 Transmission routes

In our study, for ESBL-/AmpC-producing Enterobacteriaceae both the horizontal transmission on farm level as well as the pseudo-vertical transmission from prior stages of the production chain into the fattening farms was proven.

5.2.1 Horizontal transmission

Previous studies assumed a horizontal transmission of ESBL-/AmpC-producing Enterobacteriaceae in broiler fattening farms (Huijbers et al., 2016; Laube et al., 2013). Within our study, we reinforced this hypothesis as we demonstrated a direct influence of a previous fattened flock on the ESBL-/AmpC-status on a following broiler flock due to contaminated housing environment, despite the cleaning and disinfection procedure.

The housing environment itself seems to play an important role in the occurrence of resistant bacteria in broiler flocks. Already 1986, Maris (1986) proposed that the all-in all-out method is one of the important requirements for hygiene in broiler barns. And twenty years ago, it was recommended to avoid the contact of new birds and day-old chicken with droppings, feathers, dust and debris left over from the previous flocks as some disease-causing microorganisms may survive for a long period (Jeffrey, 1997). Meroz and Samberg (1995) recommended that dry cleaning, wet cleaning, disinfection and the monitoring of the cleaning and disinfection procedures should be performed between the production cycles, each. Nowadays, these hygiene measurements including the all-in all-out management and cleaning and disinfection between the production cycles are performed as standard. Nevertheless, the occurrence of ESBL-/AmpC-producing Enterobacteriaceae in broiler flocks due to contaminated housing environment was assumed in different studies (Huijbers et al., 2014; Laube et al., 2013; Hiroi et al., 2012a). If resistant bacteria occur in a broiler flock and cannot be eliminated during the cleaning and disinfection procedure between two flocks in one barn, remaining bacteria could be causative for the colonization of the chicken of consecutively fattened flocks. Robé et al. (2019) demonstrated that even a very low colonization dosage of 10^1 colony forming units (cfu) ESBL-/AmpC-producing *E. coli* lead to the colonization of broiler chicken. Therefore, even a very low load of remaining bacteria after cleaning and disinfection could result in positive flocks fattened consecutively in the same barn. In the presented study, we confirmed the hypothesis of a circulation of ESBL-/AmpC-producing Enterobacteriaceae between following fattening flocks by determining close epidemiologic relationship between isolates from two consecutively fattened flocks (flocks E and F). The flocks E and F derived from different parent flocks and were fattened consecutively in the same barn. In both flocks, *E. coli* isolates of phylogroup B harbouring the $bla_{\text{SHV-12}}$ gene were detected. In the previous fattened flock E, these isolates were detected during the fattening period and in the barn's environment the resistant bacteria also were detected after cleaning and disinfection and preparation for the following flock. In

the consecutively fattened flock F, isolates with these characteristics also were found in animal and environmental samples. WGS has shown that all these isolates (*E. coli*, phylogroup B, $bla_{\text{SHV-12}}$) belong to MLST 2307 and differ in at most 43 SNPs in total. On basis of the *E. coli* genome of approximately 5 mega base pair (Mbp), the 43 SNPs (8.6 SNPs per Mbp) represent 0.0012% divergence only. Some isolates from flock E and flock F differed only 1.7 or 2.0 SNPs per Mbp. Compared to a described German outbreak of EHEC strains with 1.8 SNPs per Mbp (de Been et al., 2014; Grad et al., 2013), a direct clonal relationship of isolates both from flock E and flock F was proven.

Thus, we demonstrated a direct influence of a previous fattened flock on the ESBL-/AmpC-status on a following broiler flock due to contaminated housing environment, despite the cleaning and disinfection procedure. As this horizontal transmission was proved, our working group conducted investigations on management measures for cleaning and disinfection (data not published yet). In general, cleaning and disinfection should reduce and minimize the load of bacteria in the barns and, therefore, should minimize the load of ESBL-/AmpC-producing Enterobacteriaceae. As a colonization dose of 10^2 cfu ESBL-/AmpC-producing *E. coli* (inoculating one out of five animals) was shown to be sufficient to colonize all broiler chicken of the group (Robé et al., 2019), it is a major challenge to achieve such a low load of ESBL-/AmpC-producers to eliminate or to significantly reduce the colonization of the chicken and, therefore, the spread of these bacteria in broiler flocks.

Interestingly, in flock F, closely related *E. coli* strains (phylogroup B1, $bla_{\text{SHV-12}}$, MLST type 2307) as detected during the fattening period were also detected in a sample from the outside environment (pasture and soil) surrounding the ventilators exhausting air. This indicates a possible airborne contamination of surrounding areas with ESBL-/AmpC-producing Enterobacteriaceae, as also shown by Laube et al. (2014). They also detected similar ESBL-producing *E. coli* isolates both inside and outside of a broiler barn. Others showed that *E. coli* is significantly reduced outdoors, especially when exposed to direct daylight and increasing temperatures (Handley and Webster, 1995). This minimizes the public-health risk of ESBL-/AmpC-producing Enterobacteriacae from an airborne contamination of surrounding areas of barns. Nevertheless, a recurring entry of resistant bacteria from the barns immediate surrounding is possible due to contaminated shoes or equipment, and therefore, may support a horizontal circulation of ESBL-/AmpC-producing Enterobacteriaceae in livestock farms.

5.2.2 Pseudo-vertical transmission

Indications for the vertical transmission of pathogenic *E. coli* from broiler breeding chicken to their offspring was described (Giovanardi et al., 2005; Petersen et al., 2006) and concerning ESBL-/AmpC-producing Enterobacteriaceae, already day-old chicken were tested positive and vertical transmission was assumed (Laube et al., 2013; Nilsson et al., 2014; Dierikx et al., 2013b). Within the presented study, the pseudo-vertical transmission was proven, as ESBL-producing *K. pneumoniae* from a parent flock resulted in a positive fattening flock caused by the uptake of these bacteria inside the hatchery (flock B). This was the first time that the pseudo-vertical transmission was shown for *K. pneumoniae*.

In general, the emergence of ESBL-/AmpC-producing Enterobacteriaceae has been of particular interest for years, as described above. Especially, *K. pneumoniae*, causing community and nosocomial infections are of critical concern. In veterinary medicine, up to date, ESBL-producing *K. pneumoniae* have rarely been detected in animals (Hiroi et al., 2012a; Yossapol et al., 2017; Mahanti et al., 2018; Locatelli et al., 2010; Vo et al., 2007; Ewers et al., 2014). Therefore, the finding of SHV-12 producing *K. pneumoniae* strains in various stages of the broiler production chain within our study is of importance and we were aiming at revealing possible transmission routes of ESBL-producing *K. pneumoniae* along the production chain as well as at assessing a possible impact on human health.

The circulation of ESBL-/AmpC-producing Enterobacteriaceae along the broiler production process was described in previous studies (Nilsson et al., 2014; Dierikx et al., 2013b). And Projahn et al. (2017) showed the introduction of ESBL-producing *E. coli* strains directly from the parent flock into the hatchery, despite the eggs' disinfection and a pseudo-vertical transmission. In detail, the introduction of ESBL-producing Enterobacteriaceae into the hatchery and the chickens' colonization by the uptake of resistant bacteria from the environment of the hatchery was discussed. The presented study reinforces these hypotheses. We demonstrated the occurrence of SHV-2 producing *K. pneumoniae* strains in different stages of the broiler production chain in a timely relation to the production cycle of one herd (detected in parent flock Z, in hatchery during hatch of flock B and Z (both bred in the hatchery at the same time), in transportation vehicle of flock B, during fattening period of flock B). WGS revealed that these isolates harbour the $bla_{\text{SHV-2}}$ gene, belong to the previously unknown *K. pneumoniae* MLST type ST3128 and show identical antimicrobial resistance pattern. Due to analyses of the SHV-carrying contigs concerning their genetic vicinity and their comparability with other known and published plasmids, the location of the $bla_{\text{SHV-2}}$ in our isolates on a plasmid is very likely (detailed information in Publication II). To elucidate the epidemiological relationship and, therefore, the transmission dynamics along the production chain, cgMLST was performed. As cgMLST analyses, comparing 2358 genes for *K. pneumoniae*, revealed 100% similarity, all investigated isolates are identical clones of the ESBL-producing *K. pneumoniae*. This points towards ongoing transmission processes along the broiler production pyramid from the parent flocks via the hatchery to fattening flocks and, therefore, supports the hypothesis of a pseudo-vertical transmission.

As the eggs of parent flock Z were bred at the same time as the eggs of flock B, the chicken of

flock Z and B hatched at the same time in the same surrounding. This means, the original source of the ESBL-producing *K. pneumoniae* strains was not the respective parent flock B but an unrelated parent flock. This clearly emphasizes the importance of cross-contamination, especially at hatchery level.

Dierikx et al. (2013b) described the broiler production system to be rather simple: only a few breeding companies produce the animals for many broiler farms. In a few hatcheries, the breeding eggs of several parent flocks are processed, and the broiler chicken are delivered to various fattening farms. Therefore, the introduction of resistant bacteria from one parent flock into the hatchery can cause the spread of these strains in several fattening farms. Consequently, the absence of antibiotic resistant bacteria in grandparent and parent flocks is essential to minimize the occurrence of these bacteria in the production pyramid and one high prevalent (grand)parent flock has the potential to contaminate all hatchlings of one course of hatching. In addition, we also showed that cross-contamination even during the transport could be of importance as well, as *K. pneumoniae* clones also were detected in the truck, transporting the chicken from the hatchery to the farms.

6 Conclusion

In our study, seven broiler fattening chains were investigated for ESBL-/AmpC-producing Enterobacteriaceae. ESBL-/AmpC-positive parent flocks were selected, the corresponding hatchings eggs as well as the hatchlings were examined in the hatchery and the respective broiler chicken were traced during the entire fattening period at the farm. In addition, the subproject of the RESETII consortium also included investigations of these seven broiler flocks at the slaughterhouse, conducted by the Institute of Food Safety and Food Hygiene, FUB.

This set-up of the investigation including the use of whole genome sequencing as a high-resolution molecular method was intended to elucidate transmission routes of ESBL-/AmpC-producing Enterobacteriaceae along the entire broiler production chain.

We were able to show that the transmission and entry of ESBL-/AmpC-producing Enterobacteriaceae occurs at different stages of the broiler production chain.

On the one hand, we demonstrated the pseudo-vertical transmission of ESBL-producing Enterobacteriaceae. This means the introduction of the bacteria from parent flocks into the hatchery und the subsequent colonization of fattening flocks due to the uptake of the resistant bacteria in the hatchery (Publication II). As only a few breeding companies produce broilers for many farms, hatcheries represent a bottleneck and can result in the spread of a single strain from a parent flock into several fattening farms.

On the other hand, we demonstrated the horizontal transmission. This means that a contaminated housing environment can result in the subsequent flock being positive for the resistant Enterobacteriaceae (Publication I). Therefore, the optimization for cleaning and disinfection procedures on farms is essential.

Additionally, due to the investigations of the seven flocks at slaughterhouse level within the framework of the RESETII-consortium, indications for transmission processes introducing ESBL-/AmpC-producing Enterobacteriaceae from the fattening farms into the slaughterhouse as well as cross-contamination at slaughterhouse level were shown.

Taken together, the occurrence of these transmission processes of ESBL-/AmpC-producing Enterobacteriaceae revealed in the presented PhD-thesis and the subproject of the RESETII-consortium implies the need for intervention strategies at all stages of the broiler production chain.

7 Summary

ESBL-/AmpC-producing Enterobacteriaceae represent a problem both in human and veterinary medicine. High prevalence for these resistant bacteria especially were demonstrated for broiler fattening farms and also on slaughterhouse level as well as in chicken retail meat. In chicken, $bla_{\text{CTX-M1}}$ and $bla_{\text{CMY-2}}$ are the most frequent detected ESBL-/AmpC-resistance genes. In contrast, the most determined beta-lactamase genes in humans are $bla_{\text{CTX-M14}}$ and $bla_{\text{CTX-M15}}$. These genes are also found in chicken, but with low prevalence. Nevertheless, an impact of animal-originated bacteria on public health is assumed and might occur due to direct contact or the consumption of contaminated meat. Regarding these facts, intervention strategies facilitating a reduction of the load of ESBL-/AmpC-producing Enterobacteriaceae in chicken should be considered. Therefore, information on transmission pathways of these bacteria into the broiler farms are needed, however, little was known about it. Earlier studies have assumed the vertical transmission of pathogenic *E. coli* and as ESBL-/AmpC-producing Enterobacteriaceae even were detected in day-old chicken and (grand)parent flocks, first indications for the vertical transfer of these resistant bacteria were given. On the other hand, other studies assume that contaminated farm environment could represent a source for ESBL-/AmpC-producing Enterobacteriaceae. This transmission is defined as horizontal transmission.

The presented study within in RESETII-consortium intended to elucidate possible transmission routes of ESBL-/AmpC-producing Enterobacteriaceae along the entire broiler production chain. Therefore, seven ESBL-/AmpC-positive parent flocks, their corresponding hatching eggs and hatchlings in the hatchery as well as the respective fattening chicken were investigated. For detailed information on the horizontal transmission, two flocks fattened consecutively in the same barn were included in this study. Additionally, within the RESETII-consortium, the same flocks were also investigated at the slaughterhouse, enabling an assessment of transmission routes including that stage of the broiler production chain (Institute of Food Safety and Food Hygiene, FUB, thesis of Philine von Tippelskirch).

By using whole genome sequencing to determine phylogenetic relationships we showed that the transmission and entry of ESBL-/AmpC-producing Enterbacteriaceae occurs at different stages of the broiler production chain. On the one hand, the pseudo-vertical transmission route was demonstrated. Thereby the introduction of resistant strains from a parent flock into the hatchery occurs which can result in a positive fattening flock due to the uptake of the bacteria in the hatchery (Publication II). On the other hand, we demonstrated the horizontal transmission route. This transmission pathway occurs due to contaminated housing environment resulting in the subsequent flock being positive for the resistant Enterobacteriaceae (Publication I). Due to the intensive investigation of broiler chicken in their first three days of life concerning the detectability of Enterobacteriaceae we showed that samplings in recently restocked chicken for

the detection of ESBL-/AmpC-producing Enterobacteriaceae using cloacal swabs should not be performed within the first 24 hours of life (Unpublished data).

Indications for the subsequent transmission of resistant bacteria from fattening farms into the slaughterhouse as well as cross-contamination at slaughterhouse level were shown as well within the RESETII-consortium at the Insitute of Food Safety and Food Hygiene, FUB.

In summary, transmission pathways of ESBL-/AmpC-producing Enterobacteriaceae in the broiler production chain are immensely complex and occur in all stages of the production chain. Therefore, intervention strategies to reduce the load of ESBL-/AmpC-producing Enterobacteriaceae in chicken should be considered at all levels.

8 Zusammenfassung

Übertragungswege ESBL-/AmpC-produzierender Enterobacteriaceae in der Masthähnchen-Produktionskette

ESBL-/AmpC-produzierende Enterobacteriaceae stellen sowohl in der Human- als auch in der Tiermedizin eine Herausforderung dar. Für diese resistenten Bakterien wurden hohe Prävalenzen vor allem in Masthähnchenfarmen nachgewiesen, aber ebenso auf Schlachthofebene sowie auf Hähnchenfleisch aus dem Einzelhandel. Bei Hühnern sind $bla_{\text{CTX-M1}}$ und $bla_{\text{CMY-2}}$ die am häufigsten nachgewiesenen ESBL-/AmpC-Resistenzgene. Im Gegensatz dazu sind die am häufigsten nachgewiesenen Beta-Laktamase-Gene beim Menschen $bla_{\text{CTX-M14}}$ und $bla_{\text{CTX-M15}}$. Diese Gene werden auch bei Hühnern detektiert, allerdings mit geringen Prävalenzen. Es wird dennoch ein Einfluss von Bakterien tierischen Ursprungs auf die öffentliche Gesundheit vermutet, der durch direkten Kontakt oder den Verzehr von kontaminiertem Fleisch auftreten könnte. Für Interventionsstrategien in der Geflügelhaltung werden Informationen zu den Übertragungswegen antibiotikaresistenter Bakterien in der Masthähnchenhaltung benötigt. Frühere Studien ließen die vertikale Übertragung pathogener *E. coli* vermuten. Andere Studien nahmen eine kontaminierte Stallumgebung als mögliches Reservoir für ESBL-/AmpC-produzierende Enterobacteriaceae und entsprechend eine horizontale Übertragung an.

Die hier vorgestellte Studie im Rahmen des BMBF-geförderten Konsortiums RESET II hatte die Aufklärung möglicher Übertragungswege von ESBL-/AmpC-produzierenden Enterobacteriaceae entlang der Masthähnchenproduktionskette zum Ziel. Dazu wurden sieben ESBL-/AmpC-positive Elternherden, die entsprechenden Bruteier und Schlupfküken in der Brüterei sowie die daraus aufgezogenen Masttiere untersucht. Für detaillierte Informationen zur horizontalen Übertragung wurden zwei der untersuchten Herden direkt nacheinander im gleichen Stall gemästet wurden. Zusätzlich wurden im Rahmen des RESETII-Konsortiums durch das Institut für Lebensmittelqualität und -sicherheit der Freien Universität Berlin (Dissertation von Philine von Tippelskirch, 2018) dieselben Herden auch im Schlachthof untersucht, um eine Bewertung der Übertragungswege auch auf dieser Stufe der Masthähnchenproduktion zu ermöglichen.

Mittels Ganzgenomsequenzierung als hochauflösende molekulare Methode zur Bestimmung der phylogenetischen Verwandtschaftsbeziehungen konnten wir zeigen, dass die Übertragung und der Eintritt von ESBL-/AmpC-produzierenden Enterobacteriaceae auf verschiedenen Stufen der Masthähnchenproduktionskette erfolgt.

Zum einen wurde eine pseudo-vertikale Übertragung aufgezeigt. Dabei kommt es zum Eintrag resistenter Stämme aus einem Elterntierbestand in die Brüterei. Durch Aufnahme der Bakterien durch die geschlüpften Küken in der Brüterei und anschließende Kolonisierung der Tiere kann dies zu einem hochprävalent positiven Masthähchenbestand führen. Andererseits konnten wir

auch die horizontale Übertragung eindeutig aufzeigen. Bei diesem Übertragungsweg führt eine aus dem vorherigen Mastdurchgang mit ESBL-/AmpC-produzierenden Enterobacteriaceae kontaminierte Stallumgebung zu einer mit diesen resistenten Bakterien kolonisierten nachfolgenen Masthähnchenherde. Aufgrund der intensiven Untersuchungen von Masthähnchen in den ersten drei Lebenstagen hinsichtlich der Nachweisbarkeit von Enterobacteriaceae konnten wir zudem zeigen, dass Probenahmen zum Nachweis von ESBL-/AmpC-produzierenden Enterobacteriaceae mittels Kloakentupfern nicht innerhalb der ersten 24 Lebensstunden durchgeführt werden sollten.

Hinweise auf die anschließende Übertragung resistenter Bakterien aus Mastbetrieben in den Schlachthof sowie auf eine Kreuzkontamination auf Schlachthofebene wurden zusätzlich im Rahmen der Untersuchungen des Instituts für Lebenmittelqualität und -sicherheit der Freien Universität Berlin (Dissertation von Philine von Tippelskirch, 2018) gezeigt.

Zusammenfassend lässt sich sagen, dass die Übertragung der ESBL-/AmpC-produzierenden Enterobacteriaceae in der Masthähnchenproduktionskette sehr komplex ist, sowohl horizontale als auch pseudo-vertikale Transmissionsrouten enthält und auf allen Stufen der Masthähnchenproduktion auftritt. Daher sollten Interventionsmaßnahmen zur Reduzierung von ESBL-/AmpC-produzierenden Enterobacteriaceae beim Masthähnchen auf allen Stufen der Produktion entwickelt und implementiert werden.

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X Selbstständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbstständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfsmittel verwendet habe.

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Katrin Boll