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

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zur Erlangung des Grades eines
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Michaela Projahn
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ACC	Ambler class C
ACT	AmpC type
AFLP	Amplified fragment length polymorphisms
AIEC	Adherent and Invasive *E. coli*
APEC	Avian pathogenic *E. coli*
BIL-1	Bilal
bla	beta-lactamase gene
BMBF	Bundesministerium für Bildung und Forschung
BSBL	broad-spectrum beta-lactamase
CA	clavulanic acid
cgMLST	core gene MLST
CMY	cephamycin
CRISPRs	Clustered regularly interspaced short palindromic repeats
CTX-M	cefotaximase
DHA	Dhahran hospital in Saudi Arabia
DNA	desoxyribonucleic acid
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EARSS	European Antimicrobial Resistance Surveillance System
EDTA	Ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
ESBL	extended-spectrum beta-lactamase
ETEC	Enterotoxigenic *E. coli*
ExPEC	extraintestinal pathogenic *E. coli*
FOX	cefoxitin
FUB	Freie Universitiät Berlin
GIT	gastrointestinal tract
HUS	hemolytic uremic syndrome
ILH	Institut für Lebensmittelsicherheit und –hygiene
InPEC	intestinal pathogenic *E. coli*
ITU	Institut für Tier- und Umwelthygiene

K. pneumoniae	*Klebsiella pneumoniae*
kDa	kilo Dalton
LAT	latamoxef
Mbp	million base pairs
MIR-1	Miriam Hospital in Providence, R.I.
MLEE	Multi-locus enzyme electrophoresis
MLST	Multi-locus sequence typing
MLVA	Multi-locus variable number tandem repeat analysis
MOX	moxalactam
NGS	Next-generation sequencing
pAmpCs	plasmid-mediated AmpC beta-lactamases
PCR	polymerase chain reaction
PFGE	pulsed-field gel-electrophoresis
RFLP	Restriction length polymorphism
SHV	sulfhydryl reagent variable
SNPs	single-nucleotide polymorphisms
ST	sequence type
TEM	Temoneira
TZA	tazobactam
UPEC	Uropathogenic *E. coli*
UTI	urinary tract infections
wgMLST	Whole genome MLST
WGS	Whole genome sequencing

IV. List of Genes

aatA	outermembrane protein
adk	adenylate kinase
afa/dra	Dr-antigen-binding adhesins
aggR	putative transcriptional activator
arpA	regulator of acetyl-CoA synthetase
astA/East-1	arginine succinyltransferase
bfpM	putative transposase
bmaE	*E. coli* M-agglutinin subunit gene
*cdtB*_III, IV	type III cytolethal distending toxin protein
chuA	outer membrane hemin receptor
cnf1/2/3	cytotoxic necrotizing factor 1/2/3
crl	curli fibre gene
csgA	curli major subunit
cvaC	colicin V synthesis protein
cvi	colicin V immunity protein
daaA-E	fimbrial adhesion operon
eae	*E. coli* attaching and effacing locus
eltB	heat-labile enterotoxin subunit B
escV	type III secretion system
faeC	K88 fimbrial protein
fanA	fimbrial adhesin K ninety-nine
fasA	fimbrial protein
fedA	F18 fimbrial protein
feoA/B	ferrous iron transporter, protein A/B
fim41, 41a	F41 fimbrial protein
fimC	fimbrial chaperone protein
fimH	minor component of type 1 fimbriae
fumC	fumarate hydratase
fyuA	yersiniabactin/pesticin outer membrane receptor
gafD	N-acetyl-D-glucosamine specific fimbrial lectin gene
gimB	dihydrodipicolinate synthase
gyrB	DNA gyrase subunit B
hek/hra	adhesin/virulence factor

*hlyA*_v2	hemolysin transport protein
hlyE/clyA	hemolysin E
ibeA	invasion of brain endothelium
icd	isocitrate dehydrogenase
iha	bifunctional enterobactin receptor/adhesin protein
ipaH	invasion plasmid antigen H
ireA	iron-responsive element
iroN	iron outer membrane receptor
irp2	yersiniabactin biosynthetic protein
iss	inreased serum survival
iucD	L-lysine 6-monooxygenase
iutA	aerobactin receptor
kpsMT_II	group 2 capsular polysaccharide units
malX	maltose and glucose-specific enzyme
matA/ecpR	transcriptional regulator
mdh	malate dehydrogenase
neuC	UDP-N-acetylglucosamine 2-epimerase
nfaE	nonfimbrial adhesin I precursor
ompA	outermembrane protein
papABCDEFGHIJK	fimbrial major pilin protein operon
pks	polyketide synthetase
purA	adenylosuccinate synthetase
recA	recombinase A
sat	serine protease
sfa/foc	S and F1C fimbriae subunits
sitABCD	structural injection transglycosylase
STX1AB, 2AB, 2eAB	Shiga toxin
tia	invasion determinant
traT	conjugal transfer surface exclusion protein
tsh	temperature-sensitive hemagglutinin autotransporter
TSPE4.C2	putative lipase esterase gene
vat	vacuolating autotransporter toxin
yjaA	unknown function
yqi	fimbrial adhesion

1. Introduction

Extended beta-lactamase (ESBL)-/Cephamycinase (AmpC)- producing Enterobacteriaceae evolved as a major problem in human and veterinary health (Ewers et al., 2012; Kaesbohrer et al., 2012). Due to the increased resistance to beta-lactams, one of the most frequently used class of antibiotics, their treatment became challenging. Most prominent are resistant *Escherichia* (*E*.) *coli* and *Klebsiella* (*K*.) *pneumoniae* strains which often occur as multi-drug resistant variants.

ESBL-/AmpC- producing *E. coli* is not only linked to severe infections but are also found as colonizers in the normal gut flora of humans and animals (Smith et al., 2007; Wigley, 2015). Especially the colonization of food-producing animals seemed to be problematic as a possible intake of the resistant bacteria into the food-production chain is frequently discussed (Davis and Price, 2016; Lazarus et al., 2015). Highest numbers of colonization rates with ESBL-/AmpC- producing *E. coli* are reported for broiler chicken (Idelevich et al., 2016; Olsen et al., 2014). Therefore, intensive investigations on the occurrence, epidemiology and spread of these resistant bacteria were conducted during the last years (Brinas et al., 2003; Dierikx et al., 2010; Smet et al., 2008). It was found, that there is a high variability in ESBL-/AmpC-producers (Blaak et al., 2015; Bortolaia et al., 2010; Solà-Ginés et al., 2015) and also samples from (grand-) parent flocks and the processing facilities were positive concerning these resistant bacteria (Agersø et al., 2014; Dierikx et al., 2013b; Mo et al., 2014; Pacholewicz et al., 2015b). Few studies already tested one day old (grand-)parent or broiler chicks positive for ESBL-/pAmpC-producers (Dierikx et al., 2013a; Laube et al., 2013). However, there was a lack of detailed information about the spread and the transmission of ESBL-/AmpC-producing Enterobacteriaceae along the whole broiler production chain and the impact of the hatchery on these processes and the colonization of recently hatched chicken.

Therefore, the aims of the project were as follows:

i. the determination of the molecular epidemiology of ESBL-/AmpC-producing Enterobacteriaceae, especially *E. coli*, in an integrated broiler production in Germany
ii. the investigation of possible vertical top-down transmissions of the resistant bacteria along the single stages of the production chain using highly discriminative molecular typing methods
iii. the identification of virulence-associated factors in high prevalent occurring strains
iv. the determination of further factors that might contribute to higher persistence rates in the broiler production and the environment

The work described here was conducted within the framework of the RESETII- consortium ("ESBL and Fluorchinolon-Resistance in Enterobacteriaceae", BMBF, grant no.: 01KI1313C).

1.1. Resistance to beta-lactam antibiotics

Beta-lactam antibiotics are the most widely used class of antibiotics for the treatment of bacterial infections. However, due to the emergence and worldwide dissemination of resistance mechanisms against these antibiotics and an increasing rate of infections by multidrug resistant (MDR) bacteria, their use in human and veterinary medicine is highly discussed and challenging.

1.1.1. Beta-lactam antibiotics

Penicillin, discovered in 1928 by Alexander Flemming, was the first beta-lactam antibiotic which could be clinically used for the treatment of bacterial infections in 1940 (Bush and Macielag, 2010; Ochoa-Aguilar et al., 2016). Since then, further substances were developed as bacteria showing resistance to the respective drugs quickly arose (Medeiros, 1997).

Beta-lactam antibiotics are bactericidal and inhibit the synthesis of the bacterial cell wall (Ochoa-Aguilar et al., 2016). They are on the one hand subdivided based on their chemical structure into penams, penems, carbapenems, cephems, carbacephems and monobactams (Figure 1) (Bush and Macielag, 2010). On the other hand, they are classified according to their antibacterial spectrum into broad spectrum and extended-spectrum beta-lactam antibiotics and in case of cephalosporins into first to fifth generation drugs (Masouda et al., 2014). In addition, there are so-called beta-lactamase inhibitors, which should protect certain beta-lactamase antibiotics from hydrolyses by beta-lactamases (Table 1). Usually, cephalosporins are used as first-line drugs against infections caused by *E. coli*. Due to the increasing resistances, also carbapenems are regarded as "drugs of choice" for the treatment of resistant *E. coli* stains (Pitout, 2012).

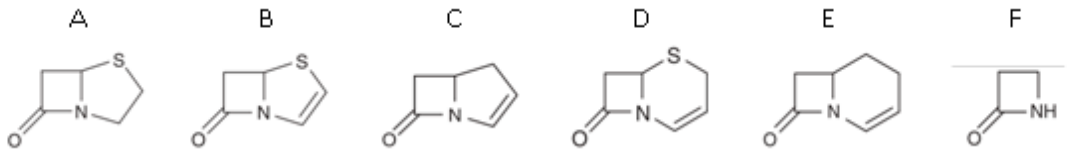

Figure 1: Chemical core structures of beta-lactam antibiotic drugs; A - penams, B - penems, C - carbapenems, D - cephems, E - carbacephems, F - monobactams

Table 1: Examples of beta-lactam antibiotics

Chemical structure	Spectrum	Examples
Penams (Penicillins)	Narrow spectrum (Natural Penicillins)	Penicillin G
	Broad spectrum (Aminopenicillins)	Amoxicillin, Ampicillin
	Extended spectrum	Piperacillin
Carbapenems		Meropenem, Imipenem, Ertapenem
Cephems	First generation	Ceftazolin
(Cephalosporins,	Second generation	Cefoxitin
Caphalomycins)	Third generation	Cefotaxime, Ceftazidime, Ceftiofur*
	Fourth generation	Cefepime
	Fifth generation	Ceftaroline, Ceftolozane
Monobactams		Aztreonam
Inhibitors		Clavulanc acid, Tazobactam

* developed for the use in veterinary medicine

1.1.2. Extended-spectrum beta-lactamases

Almost directly after the introduction of beta-lactam antibiotics for the treatment of bacterial infections, resistant strains were detected that produces enzymes called beta-lactamases. These beta-lactamases hydrolyze beta-lactam molecules and, therefore, inactivate the respective drugs. The increasing numbers of infections caused by beta-lactamase resistant bacteria had become a challenging problem in public health.

Multiple types of the beta-lactamases have been recognized and are, therefore, classified according to different attributes like their hydrolytic activity, susceptibility against inhibition, amino acid sequences, genetic location on plasmids or the chromosome, and their gene ancestry (Ambler et al., 1991; Bush, 2013; Bush et al., 1995; Hall and Barlow, 2005; Livermore, 2008; Philippon et al., 2016; Sykes, 1982).

First description of an extended-spectrum beta-lactamase (ESBL) was done in 1983 (Knothe et al., 1983). The definition of ESBLs varied throughout the years and had to be adjusted due to the detection of further enzyme variants. It is still ambiguous, however, classical ESBLs have the ability to hydrolyze penicillins, cephalosporins and aztreonam, and are usually not resistant to inhibitors like clavulanic acid (CA)(Rubin and Pitout, 2014). They belong to the molecular class A beta-lactamases and are classified as 2be or to a lesser content as 2br or 2ber according to their functional properties (Table2). They are widely detected in Enterobacteriaceae but most prominent in *E. coli* and *K. pneumoniae*. Most ESBLs belong to the gene classes TEM (Temoneira, a patient´s name), SHV

(sulfhydryl reagent variable) and CTX-M (cefotaximase) (Bonnet, 2004; Datta and Kontomichalou, 1965; Heritage et al., 1999; Liakopoulos et al., 2016; Livermore, 2008; Medeiros, 1984; Pitout, 2012; Thomson, 2010).

Table 2: Classification of beta-lactamases according to their functionality (hydrolytic activity and susceptibility against inhibition (Bush, 2013; Bush and Jacoby, 2010; Medeiros, 1997)

Group	Enzyme type	Inhibition by*	Molecular Class	Substrates	Examples
1	Cephalosporinase	No	C	Cephalosporins	CMY-2, FOX-1
1e	Cephalosporinase	No	C	Cephalosporins	CMY-37
2a	Penicillinase	CA or TZB	A	Penicillins	PC1
2b	Broad-spectrum	CA or TZB	A	Penicillins, early cephalosporins	TEM-1, TEM-2, SHV-1
2be	Extended-spectrum	CA or TZB	A	Extended-spectrum cephalosporins, monobactams	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	Inhibitor-resistant	No	A	Penicillins	TEM-30, SHV-10
2ber		No	A	Extended-spectrum cephalosporins, monobactams	TEM-50
2c	Carbacillinase	CA or TZB	A	Carbenicillin	PSE-1, CARB-3
2ce	Carbacillinase	CA or TZB	A	Carbenicillin, Cefepime	RTG-4
2d	Cloaxillinase	Variable	D	Cloxacillin	OXA-1, OXA-10
2de		Variable	D	Extended-spectrum cephalosporins	OXA-11, OXA-15
2df		Variable	D	Carbapenems	OXA-23, OXA-48
2e	Cephalosporinase	CA or TZB	A	Extended-spectrum cephalosporins	CepA
2f	Carbapenemase	Variable	A	Carbapenems	KPC-2, IMI-1
3a	Metalloenzyme	EDTA	B (B1)	Carbapenems	IMP-1, VIM-1
	Metalloenzyme	EDTA	B (B3)	Carbapenems	FEZ-1, CAU-1
3b	Metalloenzyme	EDTA	B (B2)	Carbapenems	Sfh-1

* clavulanic acid (CA), tazobactam (TZA), Ethylenediaminetetraacetic acid (EDTA)

TEM-1 was the TEM beta-lactamase identified in 1963 (Datta and Kontomichalou, 1965). This enzyme confers only resistance to penicillins and early cephalosporins and is, therefore, classified as broad-spectrum beta-lactamase (BSBL) (Salverda et al., 2010). The same applies to TEM-2 and TEM-13, however, there are more than 200 variants detected up to now (Paterson and Bonomo, 2005). These

variants differ in one to five amino acid substitutions compared to the TEM-1 beta-lactamase leading to an extended resistance spectrum in almost all cases. Some variants also gained resistances against inhibitors due to amino acid changes in the respective binding sites (https://www.lahey.org/Studies/; http://www.laced.uni-stuttgart.de). However, most prevalent are gene variants coding for TEM-1, TEM-2, TEM-4, TEM-24 and TEM-52 beta-lactamases in *E. coli* (Cantón et al., 2008; Carattoli et al., 2008; Ewers et al., 2012; Rodriguez et al., 2009).

SHV-1 as progenitor of the SHV-family beta-lactamases also has only broad-spectrum activity. It was first described as chromosomally encoded beta-lactamase gene (*bla*) in *Klebsiella* (Heritage et al., 1999). In 1983, highly similar enzymes were detected in *K. pneumoniae*, *K. ozaenae* and *Serratia* (*S.*) *marcescens*. These enzymes were named SHV-2 but turned out to be encoded on plasmids (Kliebe et al., 1985). Similar to the TEM beta-lactamases multiple variants of bla_{SHV} genes have been detected and enzymes have been evolved that can be sub-categorized as functional groups 2b, 2br and 2be (Liakopoulos et al., 2016). SHV-1, SHV-2, SHV-2a, SHV-5 and SHV-12 beta-lactamases are epidemiologically wide distributed, plasmid-encoded and mostly detected in *Klebsiella* sp. and only rarely found in *E. coli* (Heritage et al., 1999; Liakopoulos et al., 2016).

The most prevalent group of ESBLs found in Enterobacteriaceae are CTX-M type beta-lactamases. In the late 1980s non-TEM, non-SHV ESBLs, showing higher levels of resistance to Cefotaxime, were reported from all over the world (Bonnet, 2004; Rossolini et al., 2008). Further descriptions and comparative analyses of CTX-M ESBL variants resulted in the sub-classification into five groups named after the first detected CTX-M beta-lactamase (Figure 2). The three largest groups are the CTX-M-1, CTX-M-2 and the CTX-M-9 cluster (Cantón et al., 2012; Rossolini et al., 2008; Rubin and Pitout, 2014; Walther-Rasmussen and Høiby, 2004). The dissemination of $bla_{CTX\text{-}M}$ genes is based on genetic mobilization events from the chromosomes of *Kluyvera* sp. to plasmids (Paterson and Bonomo, 2005). Especially *bla* genes of the groups CTX-M-1, CTX-M-2, CTX-M-9 and CTX-M-25 are genetically surrounded by certain mobile transposase elements (Cantón et al., 2012). This leads to the worldwide success of CTX-M beta-lactamases which were found not only in *E. coli* and *K. pneumoniae* but also in other Enterobacteriaceae species like *Shigella sonnei*, *Proteus mirabilis*, *Morganella morganii*, *Citrobacter freundii*, *Serratia marcescens*, *Enterobacter aerogenes* (Bonnet, 2004). The predominant detected $bla_{CTX\text{-}M}$ genes encode for CTX-M-1, CTX-M-2, CTX-M-3, CTX-M-9, CTX-M-14 and CTX-M-15 beta-lactamases. CTX-M beta-lactamase-producing strains were isolated from humans as well as animals from every continent of the world and are frequently associated with nosocomial infections (Bonnet, 2004; Cantón et al., 2012; Ewers et al., 2012; Paterson and Bonomo, 2005; Pfeifer et al., 2010)

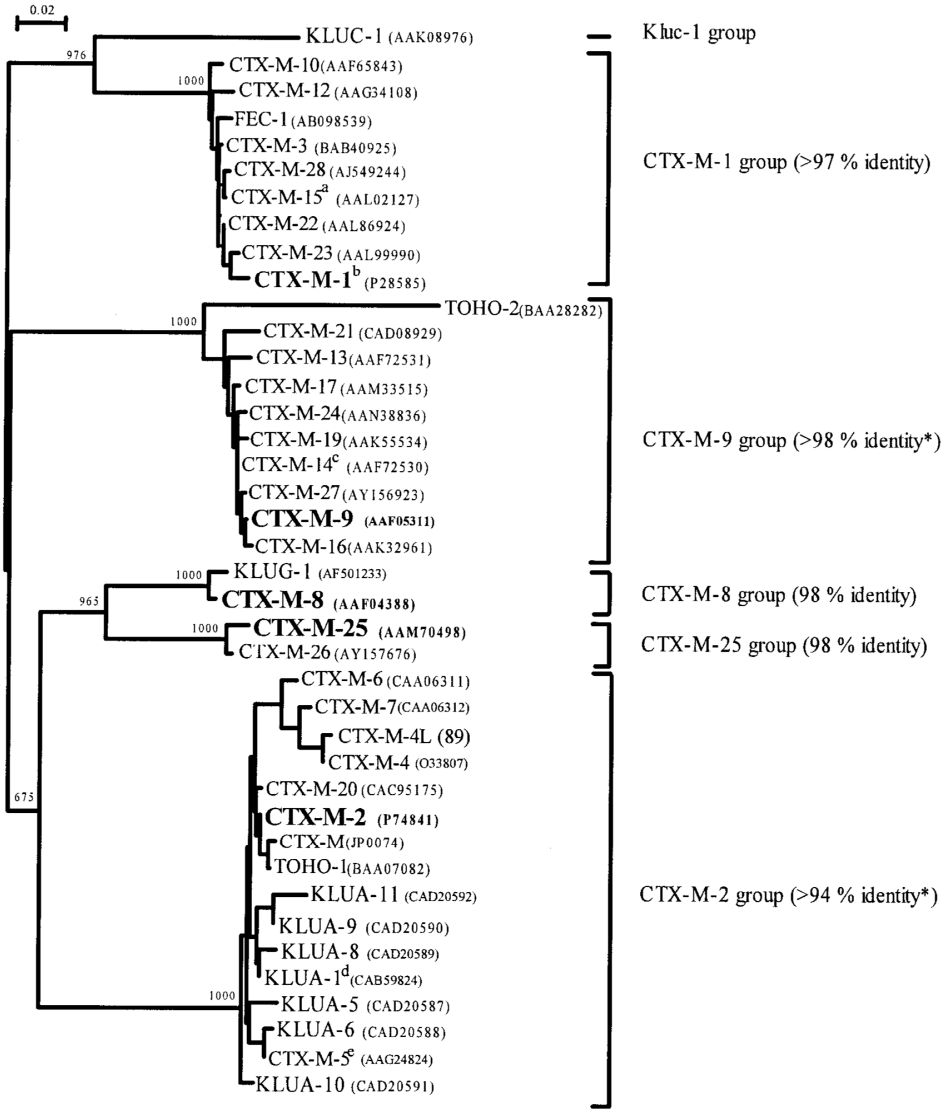

Figure 2: Phylogenetic tree of CTX-M beta-lactamases based on their amino acid sequences (Bonnet 2004)

1.1.3. AmpC beta-lactamases

AmpC beta-lactamases (Cephamycinases) are class C beta-lactamases showing additional hydrolytic activity against Cephamycins like Cefoxitin and are usually not affected by beta-lactam inhibitors (Bajaj et al., 2016; Philippon et al., 2002). AmpC beta-lactamase genes can be located on the chromosome or on plasmids and have been named on the one hand according to the resistance to cephamycins (CMY), cefoxitin (FOX), moxalactam (MOX) or latamoxef (LAT) and on the other hand based on their first discovery (Miriam Hospital in Providence, R.I. (MIR-1); Dhahran hospital in Saudi Arabia (DHA); the patient's name Bilal (BIL-1)) (Figure 3) (Philippon et al., 2002). This nomenclature has inconsistencies especially regarding the gene names AmpC type (ACT) or Ambler class C (ACC) and several enzymes have been renamed. Chromosomal resistance to cephamycins is mostly conferred via an inducible AmpC operon consisting of the genes *ampD, ampG* and *ampR.* This process of substrate induction is a reversible process. In *E. coli ampC* is consecutively expressed at low levels due to the absence of the *ampR* gene, however, resistant *E. coli* can also harbor plasmid-mediated AmpC beta-lactamases (pAmpCs) (Bajaj et al., 2016; Hanson, 2003; Honoré et al., 1986; Philippon et al., 2002; Rubin and Pitout, 2014). Typical pAmpCs belong to the CMY, FOX and DHA families and are derived from chromosomally encoded AmpC genes of *Enterobacter* spp., *Citrobacter freundii, Morganella morganii, Aeromonas* spp. and *Hafnia alvei* (Pitout, 2012; Thomson, 2010). They are not inducible and usually located on large plasmids which contain additional resistances leading to a multi-resistant phenotype (Philippon et al., 2002; Rubin and Pitout, 2014; Thomson, 2001).

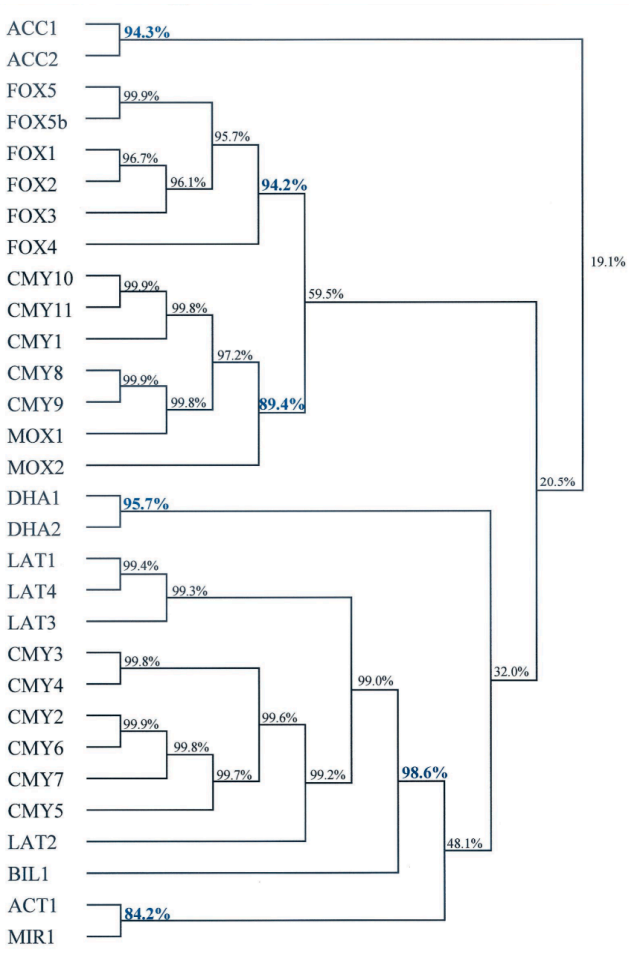

Figure 3: Phylogenetic tree based on the sequence homology of AmpC beta-lactamase genes (Pérez-Pérez and Hanson 2002)

1.2. ESBL-/AmpC-producers in Public Health

Bacteria, resistant to beta-lactam antibiotics, were immediately detected after the introduction of these antibiotics into the clinics for the treatment of bacterial infections. Since then, further beta-lactam substances were developed and beta-lactam antibiotics are now one of the most widely used antibacterial classes in human and veterinary medicine. However, at the same time, more and more bacteria were isolated which express different variant of beta-lactamases and are, therefore, not susceptible to the beta-lactam antibiotics anymore. Especially, ESBL and pAmpC beta-lactamases occurred as a major problem in public health, as these enzymes confer also resistance to third and fourth generation beta-lactams. ESBL-/AmpC-producers occur in human, animals and the environment, however, with mostly different characteristics and clinical impact.

1.2.1. ESBL-/AmpC-producers in humans

Since 2000, increasing numbers of ESBL-/AmpC-producing Enterobacteriaceae in Europe, particularly *E. coli* and *K. pneumoniae*, have been reported by the European Antimicrobial Resistance Surveillance System (EARSS) (Table 3) (Coque et al., 2008; Pitout and Laupland, 2008). Certain types of *bla* genes are widespread and associated with specific endemic clones of *E. coli* and *K. pneumoniae*, respectively. The beta-lactamases TEM-3 and TEM-4 often occur in *K. pneumoniae*, whereas TEM-52 is more frequently found in *E. coli* isolated from UTI patients. The most prevalent bla_{SHV} genes encodes for SHV-12 beta-lactamases, which are detected in both *E. coli* and *K. pneumoniae* and are often associated with additional fluoroquinolone resistance genes. Most alarming are increasing numbers of CTX-M beta-lactamase variants and their spread. CTX-M-1 and CTX-M-15 are found to be prevalent in nearly all European countries. CTX-M-14 and the CTX-M-9 cluster beta-lactamases were predominant in Spain, CTX-M-3 mainly detected in isolates from Eastern Europe countries, whereas the small clusters comprising CTX-M-8 and CTX-M25 were only found in the United Kingdom (UK) (Coque et al., 2008; Ewers et al., 2012; Pitout and Laupland, 2008). The most prominent example of globally distributed ESBL-producing *E. coli* strains are members of the ST131-C2/H30-Rx group. They are classified as phylogroup B2, MLST type ST131 and produces a CTX-M-15 beta-lactamase. Additionally, these strains show high numbers of VAGs and belong to the ExPEC pathotype. ST131 strains are frequently isolated from human infections and rarely found in animal and environmental samples. However, isolates were also detected in wild birds and dogs (Dautzenberg et al., 2016; Ewers et al., 2010; Nicolas-Chanoine et al., 2014; Peirano et al., 2013; Pitout and DeVinney, 2017; Pomba et al., 2009; Pomba et al., 2014; Totsika et al., 2011).

Prevalence data concerning AmpC beta-lactamases in humans are limited as there are no standard investigation procedures established in the clinics. However, strains expressing pAmpCs are often co-

resistant to other antibiotic classes like fluoroquinolones and aminoglycosides (Alonso et al., 2016; Izdebski et al., 2013; Rodriguez-Bano et al., 2012).

Table 3: Increasing proportion of *E. coli* and *K. pneumoniae* isolates resistant to Third-generation cephalosporins in Europe in 2001 and 2015 and 2005 and 2015, respectively (http://atlas.ecdc.europa.eu/public/index.aspx)

Country	Resistant (R) isolates proportion (%)			
	E. coli		*K. pneumoniae*	
	2001	2015	2005	2015
Austria	0,0	9,7	5,7	8,4
Belgium	1,8	9,7	-	19,7
Bulgaria	6,7	38,5	50,0	75,0
Cyprus	nd	28,5	-	43,5
Czech Republic	2,3	14,5	32,4	54,1
Germany	0,7	10,4	6,7	10,1
Denmark	-	7,5	-	7,8
Estonia	5,9	11,4	8,1	23,7
Greece	5,4	19,8	60,6	69,5
Spain	0,6	11,6	7,1	20,3
Finland	0,2	6,1	2,3	3,0
France	-	11,0	4,1	30,5
Croatia	1,6	12,5	45,5	46,8
Hungary	0,4	16,7	27,7	37,2
Ireland	-	11,4	7,1	14,7
Iceland	0,0	1,7	0,0	0,0
Italy	-	30,1	19,5	55,9
Lithuania	nd	16,0	nd	51,7
Luxembourg	0,5	12,7	-	28,3
Latvia	nd	17,9	-	47,0
Malta	0,0	11,8	5,6	15,9
Netherlands	0,6	5,7	3,5	8,6
Norway	0,3	6,0	2,1	5,0
Poland	7,1	11,9	66,0	64,2
Portugal	2,9	16,1	-	40,4
Romania	nd	26,8	-	70,7
Sweden	0,3	6,2	1,4	3,3
Slovenia	0,3	13,7	19,2	22,8
Slovakia	6,7	30,0	-	67,2
United Kingdom	1,2	11,3	12,3	10,5

nd – no data provided

1.2.2. ESBL-/AmpC-producers in companion animals

There are only few studies on companion animals like cats, dogs and horses that investigate the occurrence of ESBL-/pAmpC-producing Enterobacteriaceae including the respective molecular typing of putative resistant strains. However, numbers of studies are increasing as, especially ESBL-/pAmpC-producing *E. coli*, were also found in healthy animals (Albrechtova et al., 2012; Ewers et al., 2012; Frommel et al., 2013; Rubin and Pitout, 2014). In Europe, most commonly found ESBL strains from clinical specimen as well as healthy animals harbor CTX-M genes like $bla_{\text{CTX-M-1}}$ and $bla_{\text{CTX-M-15,}}$ but also TEM-52, SHV-12 and CMY-2 beta-lactamases were identified. Zoonotic transmissions of ESBL-/pAmpC-producers are discusses as some genetically related strains were found in both, humans and animals, however, to a rare amount (Been et al., 2014; Bélanger et al., 2011; Ewers et al., 2012; Ewers et al., 2010).

1.2.3. ESBL-/AmpC-producers in farm animals especially broiler chicken

Despite, companion animals, ESBL-/pAmpC-producing Enterobacteriaceae were detected also in farm animals. Few studies reported mainly CTX-M-1 and CTX-M-9 to be found in cattle and pigs (Geser et al., 2011; Hansen et al., 2013; Paivarinta et al., 2016). In contrast to that, numerous investigations were carried out in broiler chickens due to high colonization rates and broiler retail meat because of assumptions of a possible impact on food production and human health (Costa et al., 2009; Egea et al., 2012; Egervarn et al., 2014; Ghodousi et al., 2015; Kojima et al., 2005; Kola et al., 2012; Randall et al., 2011; Smet et al., 2008).

ESBL-/pAmpC-producing Enterobacteriaceae, mostly *E. coli*, were frequently investigated in broiler fattening farms and found to be high prevalent. Up to 100% of the investigated farms in Germany were positive concerning the resistant bacteria in Germany (Friese et al., 2013; Laube et al., 2013). First detections of *E. coli* resistant to beta-lactam antibiotics were carried out from investigation of broiler flocks affected by colibacillosis (Ginns et al., 1996). However, lots of studies detected ESBL-/pAmpC resistant *E. coli* isolates in fecal samples from healthy broiler chickens (Ben Sallem et al., 2012; Bortolaia et al., 2010; Brinas et al., 2003; Dahms et al., 2015; Dierikx et al., 2010; Jafari et al., 2016; Kameyama et al., 2013; Randall et al., 2011; Reich et al., 2013; Smet et al., 2008). It was found, that most of the ESBL-producers were capable to express TEM-52, SHV-2, SHV-12, CTX-M-1 or CTX-M-15 beta-lactamases, respectively. However, in contrast to humans and other companion and farm animals, CMY-2 positive strains were highly prevalent in broiler chickens (Ewers et al., 2012). Further investigations showed that not only broiler fattening farms but grandparent and parent breeder flocks are also affected (Dierikx et al., 2013b; Ginns et al., 1996; Giovanardi et al., 2005) and even one day old chicks were tested positive for the resistant bacteria (Dierikx et al., 2013a; Laube et al.,

2013). As it was shown that APEC strains from parent broiler chicken were transferred to their offspring (Giovanardi et al., 2005), it was hypothesized that the same might be true for commensal *E. coli* strains. Therefore, further studies were performed during the last years including that one, described here, to determine a potential transmission of ESBL-/pAmpC-producing Enterobacteriaceae along the broiler production chain (Huijbers et al., 2016a; Mo et al., 2014; Mo et al., 2016; Poulsen et al., 2017). As these resistant bacteria were also found in retail chicken meat, studies were conducted at the beginning of the last decade with the aim to investigate the origin and genetic background of these resistant bacteria and their impact on human health. Therefore, isolates from clinical and environmental samples from human and broiler chickens as well as chicken retail meat were investigated (Dierikx et al., 2013a; Dolejska et al., 2011; Egea et al., 2012; Evers et al., 2017; van Hoek et al., 2015; van Hoek et al., 2016; Kluytmans et al., 2013; Leverstein-van Hall et al., 2011; Overdevest et al., 2011; Platteel et al., 2013; Roderova et al., 2016; Thorsteinsdottir et al., 2010; Valentin et al., 2014; Wu et al., 2013). Findings are controversially discussed as some of the studies found different types of *E. coli* between humans and broiler chickens/broiler retail meat as well as different distributions of the *bla* resistance genes (de Been et al., 2014; Belmar Campos et al., 2014; Carmo et al., 2014; Evers et al., 2017; Ewers et al., 2012; Valentin et al., 2014). In addition, up to now there is only one study which investigated the dissemination of ESBL-/AmpC-producing *E. coli* along the processing line of the slaughtering of broiler chicken (Pacholewicz et al., 2015b) which is of great interest in estimating the risk of human exposure to resistant *E. coli* strains. *E. coli* is one of the most important bacterial species with regard to ESBL-/pAmpC-resistance (Liakopoulos et al., 2016; Pitout and Laupland, 2008). However due to the high genetic variability of this bacterial species it is a challenging problem to determine possible transmission routes of a certain strain or clonal complex. Therefore, we used in this study a variety of molecular typing methods in a hierarchical approach that are described in detail in chapter 1.4. Furthermore, it seems that certain bacterial virulence factors are associated with distinct *E. coli* lineages (Bélanger et al., 2011). In poultry, avian pathogenic *E. coli* (APEC) strains cause colibacillosis because of distinct virulence factors and host predisposing factors (Dho-Moulin and Fairbrother, 1999; Ewers et al., 2003). It is obvious, that some of these factors might also contribute to an enhanced survival of commensal strains in the environment of the broiler production chain and a preferential colonization of broiler chicken. Therefore, in the study also virulence factors as well as the biofilm formation were investigated in selected resistant *E. coli* isolates.

1.2.4. ESBL-/AmpC-producers in the environment

ESBL-/pAmpC-producing Enterobacteriaceae were not only found in samples from humans or animals but were also detected in various environmental settings. Clinical studies also investigated the environment of certain hospitals to identify certain contamination sources for the patients (Hu et al., 2013; Jorgensen et al., 2017). On the one hand, certain hospital-associated materials or certain virulence-associated properties might contribute to a survival of ESBL-/pAmpC-producing Enterobacteriaceae in clinics (Boll et al., 2016; Starlander et al., 2014). On the other hand, wastewater from hospitals seemed to have a great impact on the spread of the resistant bacteria but also waste water from the general population are found to be contaminated (Drieux et al., 2016; Ojer-Usoz et al., 2017; Roderova et al., 2016). Furthermore, ESBL-/pAmpC-producing Enterobacteriaceae, mainly *E. coli,* were increasingly detected also in other sources like surface water and rivers (Franz et al., 2015; Guyomard-Rabenirina et al., 2017; Jang et al., 2013; Kittinger et al., 2016). These data assume that there is an ongoing introduction of the resistant bacteria from the hosts into the environment (Gomi et al., 2017). Other studies found that also various wild animals were colonized with ESBL-/pAmpC-producing Enterobacteriaceae (Cristovao et al., 2017; Radhouani et al., 2013) indicating that these animals were either affected by the spread of the resistant bacteria from humans or farm animals into the environment, or that they can act as contamination source for the community (Guenther et al., 2011).

Comparative analyses of ESBL-/pAmpC-producing Enterobacteriaceae from livestock/farm animals and their respective farmers revealed certain similarities between the investigated strains (Dierikx et al., 2013a; Dorado-Garcia et al., 2017). ESBL-/pAmpC-producing Enterobacteriaceae from different food-producing animals were also found to be spread into the farm environment (Blaak et al., 2015; Gao et al., 2015; Hartmann et al., 2012; Laube et al., 2014; Ma et al., 2012). ESBL-/pAmpC-producing *E. coli* was found in air samples from the inside of a chicken barn as well as in boot swabs from the surrounding area of a fattening farm (Laube et al., 2014). These data lead to the assumption of an airborne transmission of the resistant *E. coli* from the farms into the environment. Therefore, in this study also various environmental samples and environmental swabs from different areas in the broiler production chain were investigated concerning ESBL-/pAmpC-producing *E. coli* to identify possible contamination sources and the respective transmission routes along the broiler production chain.

1.3. *Escherichia coli*

Bacterial strains of the species *E. coli* are of major interest in ESBL-/AmpC-research as they occur in high frequencies in humans and animals and are often associated with multidrug resistance. Therefore, also the work in the RESETII-project mainly focuses on resistant isolates of this species.

1.3.1. Taxonomy

E. coli belongs to the family of Enterobacteriaceae (Table 4) and is a common resident of the gastrointestinal tract (GIT) of humans and animals (Tenaillon et al., 2010). The genus *Escherichia* is subdivided into five different species: *E. albertii, E. coli, E. fergusonii, E. hermanii* and *E. vulneris* (Brenner et al., 1982; Gaastra et al., 2014; Huys et al., 2003; Kiernicka et al., 1999; Walk et al., 2009). *E. coli* is a gram-negative, facultative anaerobic and nonspore forming bacterium. It is rod-shaped and has a size of up to 2 µm in length.

Table 4: Taxonomy levels of *E. coli*

Level	
Domain	Bacteria
Phylum	Proteobacteria
Class	γ-Proteobacteria
Order	Enterobacteriales
Family	Enterobacteriaceae
Genus	*Escherichia*
Species	*Escherichia coli*

1.3.2. Pathotypes

E. coli is one of the predominant bacteria that colonizes the gut of healthy vertebrates usually living in a symbiotic relationship with their hosts. However, *E. coli* strains are highly diverse and complex and can be classified according to their clinical characteristics into commensals, intestinal pathogenic *E. coli* (InPEC) and extraintestinal pathogenic *E. coli* (ExPEC) strains (Smith et al., 2007).

Commensal strains are part of the GIT microbiome of humans, animals and birds, only causing disease in immunocompromised hosts (Russo and Johnson, 2003). InPEC strains lead to enteric or diarrheagenic diseases like colitis or gastroenteritis. Well-known representatives are Enterohemorrhagic *E. coli* (EHEC), which cause bloody diarrhea or the hemolytic uremic syndrome (HUS) and are able to produce Shiga toxins (Stx) (Nguyen and Sperandio, 2012). Further subgroups of InPEC, depending on their specialized virulence traits, are Enteropathogenic *E. coli* (EPEC),

Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC) and Adherent and Invasive *E. coli* (AIEC) (Kalita et al., 2014).

ExPEC, on the one hand, can colonize the gut of healthy hosts without causing disease. On the other hand, these strains can cause meningitis, skin structure infections or sepsis through entry into extraintestinal sites (Smith et al., 2007). Most prevalent are *E. coli* which lead to urinary tract infections (UTI) in humans and, therefore, are called Uropathogenic *E. coli* (UPEC) (Marrs et al., 2005). ExPEC strains that cause systemic infections or colibacillosis in birds are classified as Avian pathogenic *E. coli* (APEC) (Ewers et al., 2003). ExPEC differ genetically and epidemiologically from commensals and InPEC. However, the distinct classification of isolates as ExPEC is only done by molecular methods. Currently, ExPEC are defined based on the presence of two or more of the following virulence markers: *papA* (P fimbriae structural subunit) and/or *papC* (P fimbriae assembly), *sfa/foc* (S and F1C fimbriae subunits), *afa/dra* (Dr-antigen-binding adhesins), *kpsMT* II (group 2 capsular polysaccharide units), and *iutA* (aerobactin receptor) (Johnson et al., 2003).

1.3.3. Genotypes

The mean size of the *E. coli* genome is about 5 million base pairs (Mbp) comprising the chromosomal DNA as well as further structures like plasmids and prophages. Since the publication of the first complete genome of the *E. coli* strain K12 in 1997 (Blattner et al., 1997), a multitude of strains were sequenced leading to the finding that the actual genome size of an *E. coli* can range between 4.6 Mbp and 5.9 Mbp (Robins-Browne et al., 2016).

Only approx. 20% of the genome belongs to the so-called core genome which comprises genes that are found in all *E. coli* strains. The remaining 80% of the DNA contents belong to genes and accessory elements that are highly flexible and can be acquired or exchanged via horizontal gene transfer by conjugation or transduction. These accessory elements usually are associated with pathogenicity islands, transposons, bacteriophages, virulence traits and resistances to antimicrobials (Robins-Browne et al., 2016). Due to this high diversity phylogenetic analyses and strain comparisons for pathogen tracing and outbreak investigations are much more complicated. Despite phenotypic characterizations like serotyping (variations in the surface O- polysaccharide and H- flagella antigens) (Orskov et al., 1977) a variety of genetic classification methods have been developed which provide different discriminatory power and are used for different aspects of strain investigations (Table 5) (Chaudhuri and Henderson, 2012; Clermont et al., 2015; Fratamico et al., 2016; Karama and Gyles, 2010; Metzgar et al., 2001). Methods used for the investigation of *E. coli* isolated derived during this study are mentioned in the following sections.

Table 5: Typing methods for the determination of certain pheno- or genotypes of *E. coli*

Method	Phenotype	Genotype
Amplified fragment length polymorphisms (AFLP)		X
Bacteriophage-typing	X	
Clustered regularly interspaced short palindromic repeats (CRISPRs)		X
Luminex-based suspension assay	X	X
Microarrays		X
Multi-locus enzyme electrophoresis (MLEE)	X	
Multi-locus sequence typing (MLST)		X
Multi-locus variable number tandem repeat analysis (MLVA)		X
Phylogrouping using multiplex PCR		X
Pulsed-field gel electrophoresis (PFGE)		X
rep-PCR		X
Restriction length polymorphism (RFLP)		X
Ribotyping		X
Serotyping of O-/H-antigen	X	
Single Nucleotide polymorphisms (SNPs)		X

1.4. Molecular typing methods

In the RESETII-project a hierarchical approach was applied to the detected *E. coli* isolates. In a first screening step, suspected resistant isolates were investigated concerning their phylogenetic group. For further detailed molecular investigations high resolution methods were applied to selected isolates. The methods used for the investigation of the "Molecular Epidemiology and Vertical Transmission of ESBL-/AmpC-producing Enterobacteriaceae along the Broiler Production Chain" are described in more detail in the next chapters.

1.4.1. Phylogroups

The classification into phylogroups originate from the multi-locus enzyme electrophoresis (MLEE) method (Milkman, 1973) which differentiates *E. coli* strains into four groups: A, B1, B2 and D. Using a multiplex PCR to detect fragments of the genes *arpA* (regulator of acetyl-CoA synthetase), *chuA* (outer membrane hemin receptor), *yjaA* (unknown function) and TSPE4.C2 (putative lipase esterase gene) (Clermont et al., 2013) revised and expanded the scheme into the phylogroups A, B1, B2, C, D, E and F.

Despite the genetic classification, many studies reported not only correlations with other typing methods but also with certain pathotypes. More precisely, it was found out that commensal *E. coli* strains usually belong to the phylogroups A and B1 whereas most of the human pathogenic ExPEC are classified as B2 strains (Smith et al., 2007). In contrast, APEC strains were found to be of phylogroups C, E or F and only to a less extent to phylogroup B2 (Logue et al., 2017). However, due to the high variation in the genomes of *E. coli* this classification is more or less a rough estimation of the evolutionary relationship between different *E. coli* strains.

1.4.2. Multi-locus sequence typing

Multi-locus sequence typing (MLST) uses the occurrence of single-nucleotide polymorphisms (SNPs) in certain house-keeping genes for the determination of allelic variations of these genes. Each gene allele is assigned by a number in order to their first discovery. The unique combination of the gene allele numbers leads to the definition of a respective sequence type (ST). Three different MLST schemes have been developed for *E. coli* using seven to 11 different house-keeping genes which are common in all *E. coli* strains (Jaureguy et al., 2008; Reid et al., 2000; Wirth et al., 2006). The most widely used scheme for *E. coli* was published in 2006 by Wirth et al. based on the seven genes *adk* (adenylate kinase), *fumC* (fumarate

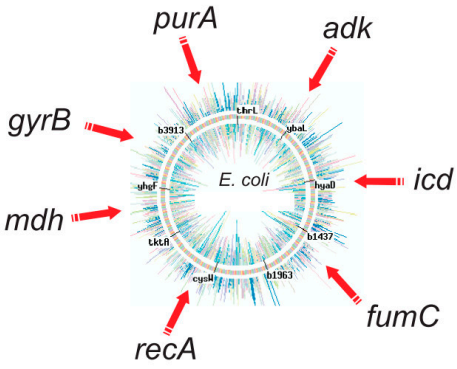

Figure 4: Genomic locations of the seven housekeeping genes used for MLST classification by Wirth et al. 2006

hydratase), *gyrB* (DNA gyrase subunit B), *icd* (isocitrate dehydrogenase), *mdh* (malate dehydrogenase), *recA* (recombinase A), and *purA* (adenylosuccinate synthetase) (Figure 4). Up to now, 7537 different STs were identified in *E. coli* strains by this MLST method. There are certain STs that got famous due to their connection to important *E. coli* outbreaks. Strains of the ST131 are globally spread and associated with a variety of human infections mostly in combination with multidrug resistances (Pitout and DeVinney, 2017). Other STs like ST393, ST69, ST95 and ST73 also occur in higher content in healthcare-associated infections (Riley, 2014). Previous studies found out that *E. coli* strains of several STs can be isolated from humans, animals and environmental samples. Especially the ST410 strains seemed to be successful in interspecies transmissions and usually harbor certain antimicrobial resistance genes (Schaufler et al., 2016b; Timofte et al., 2016). However, the determination of the ST number does not provide a direct phylogenetic relationship between strains of different STs nor gives detailed information about strains of the same ST. Therefore, further typing methods were developed and are commonly used especially in outbreak investigations. The availability of fast whole genome sequencing (WGS) lead to the development of the core gene MLST (cgMLST) scheme (see 4.3.5. Whole genome sequencing) (Maiden et al., 2013).

1.4.3. Pulsed-field gel electrophoresis

Pulsed-field gel-electrophoresis (PFGE) is based on the enzymatic cutting (XbaI macrorestriction) of whole genomic DNA into large fragments (Arbeit et al., 1990; Flanagan et al., 1989; Hunter et al., 2005). Between ten to 25 fragments of different sizes are produced by this method, depending on the investigated *E. coli* strain. These fragments are then electrophoretically separated from each other leading to a specific band pattern. PFGE is considered to be a "gold standard" for the subtyping of *E. coli* strains due to a high discriminatory power. A Dice coefficient of at minimum 80% of the band profiles or a difference of at maximum three bands is usually supposed as close relationship between two strains (Karama and Gyles, 2010). However, in contrast to the PCR methods phylogrouping and MLST, the results of the PFGE are hard to compare between different labs. There are no distinct PFGE-types or standardized guidelines defined for the comparison of PFGE profiles especially when dealing with minor differences in the fragment patterns (Karama and Gyles, 2010; Lukinmaa et al., 2004). Nevertheless, PFGE is widely used in outbreak investigations and gives a good indication for the determination of a close or possible phylogenetic relationship between strains (Gerner-Smidt et al., 2006; Karama and Gyles, 2010).

1.4.4. Single nucleotide polymorphism

The occurrence of specific single nucleotide polymorphisms (SNPs) can be used to develop a phylogenetic classification method for bacteria. SNPs appear as synonymous (silent) mutations which

have no effect on the translated protein, or as non-synonymous mutation leading to a change in the respective protein sequence. Synonymous SNPs are powerful identifiers for evolutionary processes as they are usually not driven by any selection pressure but occur spontaneously (Kimura, 1979). There are well established SNP-typing methods for bacteria with low genetic variations like *Mycobacterium tuberculosis*, *Yersinia pestis* or *Francisella tularensis* (Filliol et al., 2006; Gagneux and Small, 2007; Gutacker et al., 2006; Morelli et al., 2010; Vogler et al., 2009). As *E. coli* strains are highly variable (mutation rate of approx. 1-2x10^{-3} mutations per generation per genome) and horizontal gene transfer is frequently detected in these strains, a sound SNP-classification based on few SNPs is not very likely (Barrick et al., 2009; Foster et al., 2015; Whittam et al., 1998). Several studies investigated the usage of SNPs for the subclassification of certain *E. coli* lineages using lineage specific genes or large numbers of SNP mutations detected from whole genomic sequences (see 4.3.5. Whole genome sequencing) indicating that SNPs can be used for further detailed comparisons of strains of the same PFGE pattern or isolates derived from outbreak investigations (Haugum et al., 2011; Jung et al., 2013; Lager et al., 2016; Lee et al., 2017; Norman et al., 2012; Tartof et al., 2007; Turabelidze et al., 2013).

1.4.5. Whole genome sequencing

During the last years, whole genome sequencing (WGS), also called next-generation sequencing (NGS), has become a popular tool for analyzing bacterial species. Since 2004, the introductions of new sequencing technologies lead to new insights not only in bacterial genome research (Diaz-Sanchez et al., 2013; Mardis, 2017; Snyder et al., 2009) (Table 6).

Ongoing advancements in these technologies provide now fast and high-throughput sequence data of whole bacterial genomes. However, these sequence data need to be further processed as most platforms only produce short sequence reads. Different algorithms and computational tools were developed for the alignment of short reads into large consensus sequences using either a reference genome or the *de novo* assembling approach (Bao et al., 2011). Usually, NGS does not provide a complete circular bacterial genome as certain DNA structures like repetitive elements ore gene isoforms are a challenging problem for the sequencing methods as well as bioinformatic programs (Liang et al., 2016; Treangen and Salzberg, 2011). Furthermore, the discrimination of consensus sequences originating from the bacterial chromosome and plasmidic structures without a suitable reference genome bears some problems (Brolund et al., 2013; Rozov et al., 2017). Therefore, most of the published bacterial genomes are so-called draft genomes providing nearly all essential genetic information but lacking some information about the exact arrangement of the consensus sequences in the genome (Bao et al., 2011; Forde and O'Toole, 2013; Hu et al., 2011; Mardis, 2017).

Table 6: Overview of available WGS technologies from 2006-2016 (Mardis 2017)

Company	Read length	Applications
454/Roche	400 bp (single end)	Bacterial and viral genomes, multiplex-PCR products, validation of point mutations, targeted somatic-mutation detection
Illumina	150–300 bp (paired end)	Complex genomes (human, mouse and plants) and genome-wide NGS applications, RNA-seq, hybrid capture or multiplex-PCR products, somatic-mutation detection, forensics, noninvasive prenatal testing
BI SOLiD	75 bp (single end) or 50 bp (paired end)	Complex genomes (human, mouse, plants) and genome-wide NGS applications, RNA-seq, hybrid capture or multiplex-PCR products, somatic-mutation detection
Pacific Biosciences	Up to 40 kb (single end or circular consensus)	Complex genomes (human, mouse and plants), microbiology and infectious-disease genomes, transcript-fusion detection, methylation detection
Ion Torrent	200–400 bp (single end)	Multiplex-PCR products, microbiology and infectious diseases, somatic-mutation detection, validation of point mutations
Oxford Nanopore	Depends on library preparation (1D or 2D reads)	Pathogen surveillance, targeted mutation detection, metagenomics, bacterial and viral genomes
Qiagen GeneReader	107 bp (single end)	Targeted mutation detection, liquid biopsy in cancer

With the increasing amount of sequence data also more and more bioinformatic tools and platforms were developed to analyze these sequence data and extract as much information as possible. Using WGS data from *E. coli* it is possible to *in silico* determine for example the respective MLST type (Larsen et al., 2012) and the serotype (Joensen et al., 2015) instead of performing the conventional laboratory methods. Further platforms like EnteroBase hosted from the Warwick Medical School in the UK (https://enterobase.warwick.ac.uk) or the Center for Genomic Epidemiology (CGE) hosted in Denmark (http://www.genomicepidemiology.org) provide servers for the analyses of WGS data. EnteroBase can be used to determine the cgMLST or whole genome MLST (wgMLST) type whereas the CGE offer batch analyses of multiple strains to simultaneously analyze the MLST type, the serotype, the *fimH* (minor component of type 1 fimbriae) type as well as the occurence of certain virulence and antibiotic resistance genes (Thomsen et al., 2016). In addition, there exist various tools for the multiple alignment, SNP discovery and phylogenetic analyses of bacterial strains (Altmann et al., 2012; Olson et al., 2015). These tools are also increasingly used for the investigation and analyses of *E. coli* outbreaks (Figure 5), surveillance studies and the evolution on certain *E. coli* lineages (Barrick et al., 2009; Bletz et al., 2013b; Eppinger et al., 2011; Holmes et al., 2015; Lindsey et al.,

2016; Nadon et al., 2017; Roer et al., 2017; Rusconi et al., 2016; Schürch and van Schaik, 2017; Sherry et al., 2013; Taboada et al., 2017; Zankari et al., 2013; Zhang et al., 2006). However, due to the big amount of different algorithms for the assembly, the SNP calling and the prediction of the phylogeny, the outcome of certain analyses can vary between the tools. Usually, SNP discovery in *E. coli* is based on genes of the core genome. This defined core genome can vary between different analyses tools and different sets of *E. coli* strains as these might share a differing number of common genes. This has to be kept in mind when comparing WGS data of *E. coli* strain collections from diverse studies.

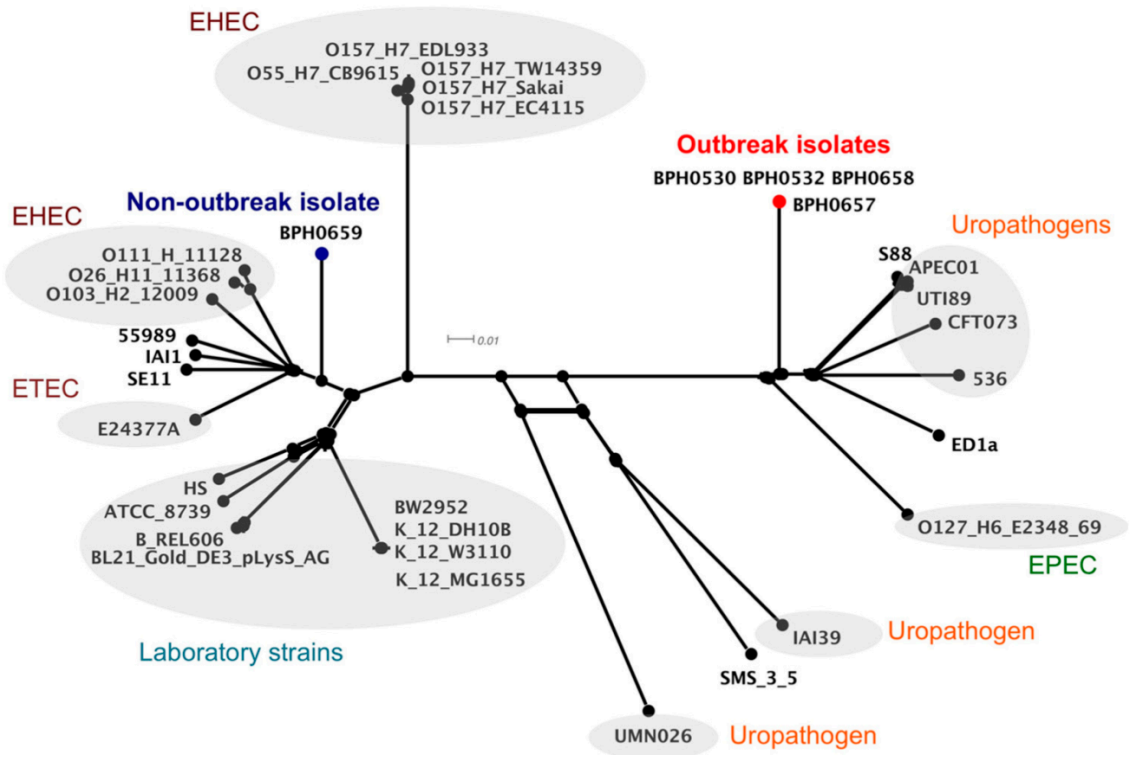

Figure 5: Phylogenetic tree based on discovered SNPs during *E. coli* outbreak investigations by Sherry et al. 2013

1.5. Virulence and environmental adaptation

E. coli usually colonizes the GIT of human and animals as part of the normal commensal bacterial flora. However, there are several facultative and obligate pathogenic lineages, which lead to intestinal or extraintestinal infections (see 1.3.2. Pathotypes). Especially virulence factors are increasingly investigated in isolates from humans and broiler chicken as some of these factors might contribute to a zoonotic potential (Ewers et al., 2003; Rodriguez-Siek et al., 2005). Another important factor in infectious diseases is the ability of *E. coli* strains to form biofilms (Hall-Stoodley et al., 2004). It seemed to be very likely that biofilm formation also plays an important role in the environment of the broiler production and the slaughterhouse due to the protective function against environmental stress (Flemming and Wingender, 2010).

1.5.1. Virulence-associated genes

Especially ExPEC strains, as facultative pathogenic *E. coli* causing UTI, newborn meningitis or sepsis, harbor a variety of virulence-associated genes (VAGs) (Clermont et al., 2017; Dobrindt and Hacker, 2008; Ewers et al., 2014). However, due to the increasing investigations of VAGs in pathogenic and commensal strains isolated from healthy as well as infected humans and animals, certain virulence traits are increasingly considered as fitness factors. Several studies found out that genes like *astA* (heat stable cytotoxin associated with enteroaggregative *E. coli*), *chuA* (heme receptor gene), *fimC* (Type I fimbriae), *iha* (iron regulated adhesin), *ireA* (iron-responsive element), *irp2* (iron repressible protein), *iss* (increased serum survival), *iucD* (aerobactin synthesis), *neuC* (K1 capsular polysaccharide), and *tsh* (temperature-sensitive hemagglutinin) or *vat* (vacuolating autotransporter toxin) are not exclusively detected in pathogenic but also in commensal strains (Carli et al., 2015; van Hoek et al., 2016; Kemmett et al., 2013; Paixao et al., 2016; Silveira et al., 2016). Fitness factors are usually associated with adherence and nutrient acquisition, and support the survival in the host without directly leading to disease and under unfavorable conditions in the environment (Smith et al., 2007; Vila et al., 2016). Nevertheless, ExPEC strains show higher levels of VAGs than commensal strains (Clermont et al., 2017; Fu et al., 2017; Silveira et al., 2016; Skjot-Rasmussen et al., 2012). Genes, that are usually investigated and reported as important virulence factors are listed in table 7 and were also investigated in this study (Bélanger et al., 2011; Ewers et al., 2007; Frommel et al., 2013; Guenther et al., 2017; Maciel et al., 2017; Masters et al., 2011; Rendón et al., 2007; Schierack et al., 2013; Silveira et al., 2016).

In poultry, APEC can cause avian colibacillosis leading to high mortality and morbidity as well as economic losses in the industry. The VAGs *iroN* (Salmochelin siderophore receptor gene), *ompT* (episomal outer membrane protease gene), *hlyF* (putative avian hemolysin) and *sitAP* (plasmid-encoded iron transport protein) or *iss* and *iutA* are used to predict and verify APEC strains as these

strains have also the potential to be transmitted zoonotically (Dissanayake et al., 2014; Guabiraba and Schouler, 2015; Johnson et al., 2008; Schouler et al., 2012; Solà-Ginés et al., 2015; Wigley, 2015).

Table 7: Overview of investigated ExPEC (n=70) and InPEC (n=19) virulence factors

ExPEC genes						InPEC genes
Adhesion	**Iron uptake**	**Invasion**	**Protection**	**Toxin**	**Miscellaneous**	
aatA	*chuA*	*pks*	*cvi*	*astA (East-1)*	*vat*	*daaA-E*
afaA/ draB	*fyuA*	*tia*	*kpsMT_II*	*cnf1-3*	*malX*	*eae*
bfpM	*feoA/B*	*gimB*	*neuC*	*cdtB_III*		*faeC*
bmaE	*ireA*	*ibeA*	*ompA*	*cdtB_IV*		*fanA*
csgA	*iroN*		*traT*	*cdtB*		*fasA*
crl	*irp2*		*iss*	*cvaC*		*fedA*
fimC, fimH	*iucD*			*hlyA_v2*		*fim41, 41a*
gafD	*iutA*			*hlyE (clyA)*		*escV*
hek/hra	*sitABCD*			*sat*		*est1a*
iha						*STX2eAB*
matA (ecpR)						*STX1AB*
nfaE						*STX2AB*
papABCDEFGHIJK						*aggR*
sfaSX						*ipaH*
focCDG						*eltB*
tsh						
yqi						

In this study, virulence factors were determined based on whole genome sequences of selected resistant isolates. Therefore, an in-house script from the Robert Koch-Institute was applied to the WGS data to identify the genetic sequences of the virulence genes by the Basic Local Alignment Search Tool (BLAST) method (Publication II).

1.5.2. Biofilm production

E. coli cells appear not only as single "planktonic" cells but have the ability to form complex biofilm structures. These biofilms have the advantages to protect bacteria against the host immune system, against antibiotics as well as environmental stress and, therefore, can contribute to severe infections (Flemming and Wingender, 2010; Hall-Stoodley et al., 2004; McCrate et al., 2013). Biofilms are build up on extracellular matrix components like adhesins, amyloid-forming proteins and exopolysaccharides. In case of *E. coli*, the biofilm production depends on the assembly of curli fibers and the secretion of cellulose (Barnhart and Chapman, 2006; Serra et al., 2013).

Curli fimbriae mediate the attachment and adherence to surfaces but are also involved in invasion and host immune response (Barnhart and Chapman, 2006; Olsen et al., 1989). They are encoded by the *csgBAC* gene operon (structural subunits), the *csgEFG* operon (curli specific transport system) and their transcription is activated by CsgD (Figure 6) (Gerstel et al., 2003; Hammar et al., 1995). The CsgD protein is also involved in the regulation of the cellulose biosynthesis (Brombacher et al., 2006; Chirwa and Herrington, 2003; Ogasawara et al., 2011). The expression of curli fibers is temperature-dependent (below 32°C) and occurs usually under unfavorable growths condition (low osmolarity, starvation) (Brombacher et al., 2006).

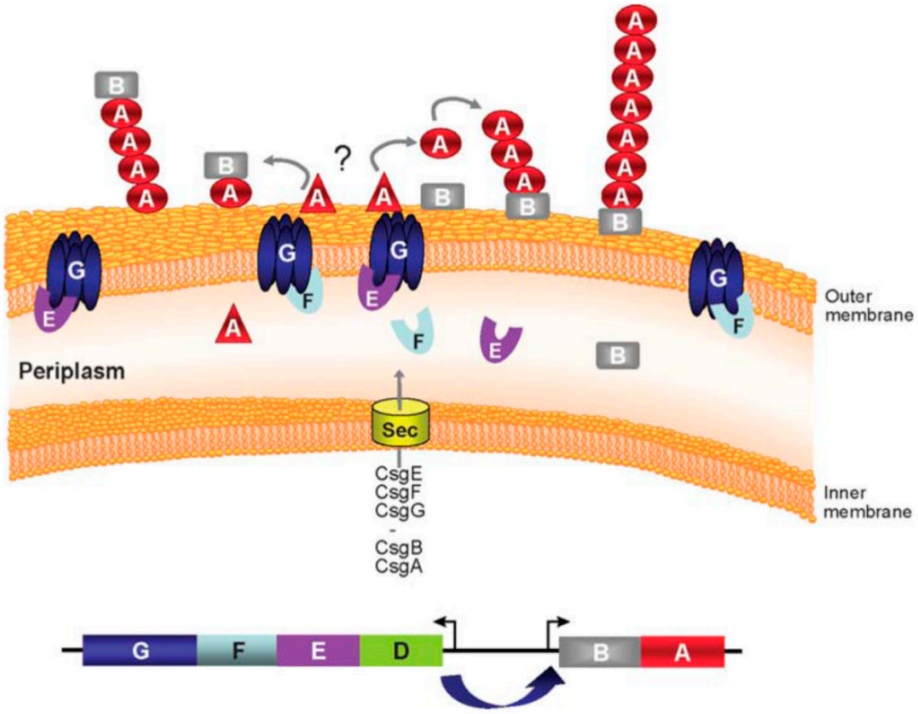

Figure 6: Schematic model of the assembly of curli fibers: CsgD (green) acts as regulator of CsgB (grey) and CsgA (red) which are the structual subunits of the curli fiber. CsgG (blue) is an outer membran protein which interacts with CsgF (light blue) and CsgE (violet) for the translocation of CsgA and CsgB (Barnhart and Chapman 2006)

Cellulose is a polysaccharide which is built up on β-(1→4)-D-glucose molecules. It is extremely stable and has high water retention capacities and besides its role in biofilm formation, it is involved in cell-cell interactions and adherence. For the synthesis of cellulose four enzymes were identified which are encoded by the so-called *bcsABCD* operon. However, different operons and gene arrangements have been determined in various cellulose producing bacteria (Coque et al., 2008; Omadjela et al., 2013; Romling and Galperin, 2015; Zogaj et al., 2001). For *E. coli* it was shown that the production of cellulose is also regulated by CsgD and usually has a negative effect on the expression of curli fibers (Bokranz et al., 2005; Gualdi et al., 2008; Gualdi et al., 2007; Hufnagel et al., 2014). However, the

cross-regulation of curli and cellulose is highly complex and can vary between commensal and pathogenic strains (Da Re and Ghigo, 2006; Lasaro et al., 2009; Mika and Hengge, 2014).

In this study, the ability of *E. coli* isolates to form biofilms was observed by a macrocolony assay (Serra et al., 2015; Serra et al., 2013). The dye Congo red was used as marker for the curli expression and the spatial architecture of the colony allowed drawing conclusions on the cellulose production (Publication II).

2. Outline of the study

The investigations, described here, were carried out between 2014 and 2017 within the framework of the RESETII consortium ("ESBL and Fluorchinolon-Resistance in Enterobacteriaceae", www.reset-verbund.de) funded by the Federal Ministry of Education and Research (BMBF, grant no.: 01KI1313C) as part of the sub-project "Transmission of ESBL-/AmpC-producing Enterobacteriaceae in the entire production chain of broilers: points of hazard and intervention". Data and results are based on samples which were taken and analyzed in cooperation with the doctoral thesis of Katrin Dähre (Institute for Animal Hygiene and Environmental Health, ITU) and Philine von Tippelskirch (Institute of Food Safety and Food Hygiene, ILH) from the Freie Universitiät Berlin (FUB).

2.1. Proceeding of samplings

The whole RESETII sub-project of the FUB comprised various samplings from different stages of the production chain of an integrated broiler production in Germany. To investigate possible vertical transmissions of ESBL-/AmpC-producing Enterobacteriaceae along this production chain, eggs from ESBL-/AmpC-positive broiler breeders (parent flocks, Table 8) were traced in batches through the respective production stages. This sampling concept resulted in longitudinal investigations of parent flocks (1st sampling), hatchery (2nd to 4th sampling), fattening farms (5th to 7th sampling) and slaughterhouses (8th sampling) (Figure 7). Trace samplings were carried out seven times along the broiler production chain (chain A to G). An initial screening sampling (0th sampling) was performed to determine the status of resistance of various broiler breeders and to define the seven respective parent flocks to be investigated during the study. The selection of parent flocks was done with respect to their ESBL-/AmpC status, age/production age as well as organizational time schedule.

Table 8: Metadata on production chain investigations

	Chain A	Chain B	Chain C	Chain D	Chain E	Chain F a	Chain G
Sampling period	11.08.14 - 06.10.14	16.09.14 - 11.11.14	15.12.14 - 10.02.15	26.01.15 - 28.04.15	17.06.15 - 24.08.15	29.07.15 - 29.09.15	15.01.16 - 02.03.16
Age of parent flock	29 weeks	57 weeks	43 weeks	50 weeks	51 weeks	50 weeks	58 weeks
Fattening flock no.	142	281	144	145	148	150	292
Farm no.	1	4	4	4	7	7	3
Fattening barn no.	11	41	43	43	74	74	34
Animals per barn	22,500	21,800	21,700	22,000	23,000	22,500	22,000
Antibiotics	Tylosin	-	-	-	-	-	-
Slaughterhouse	1	2	2	2	2	2	2

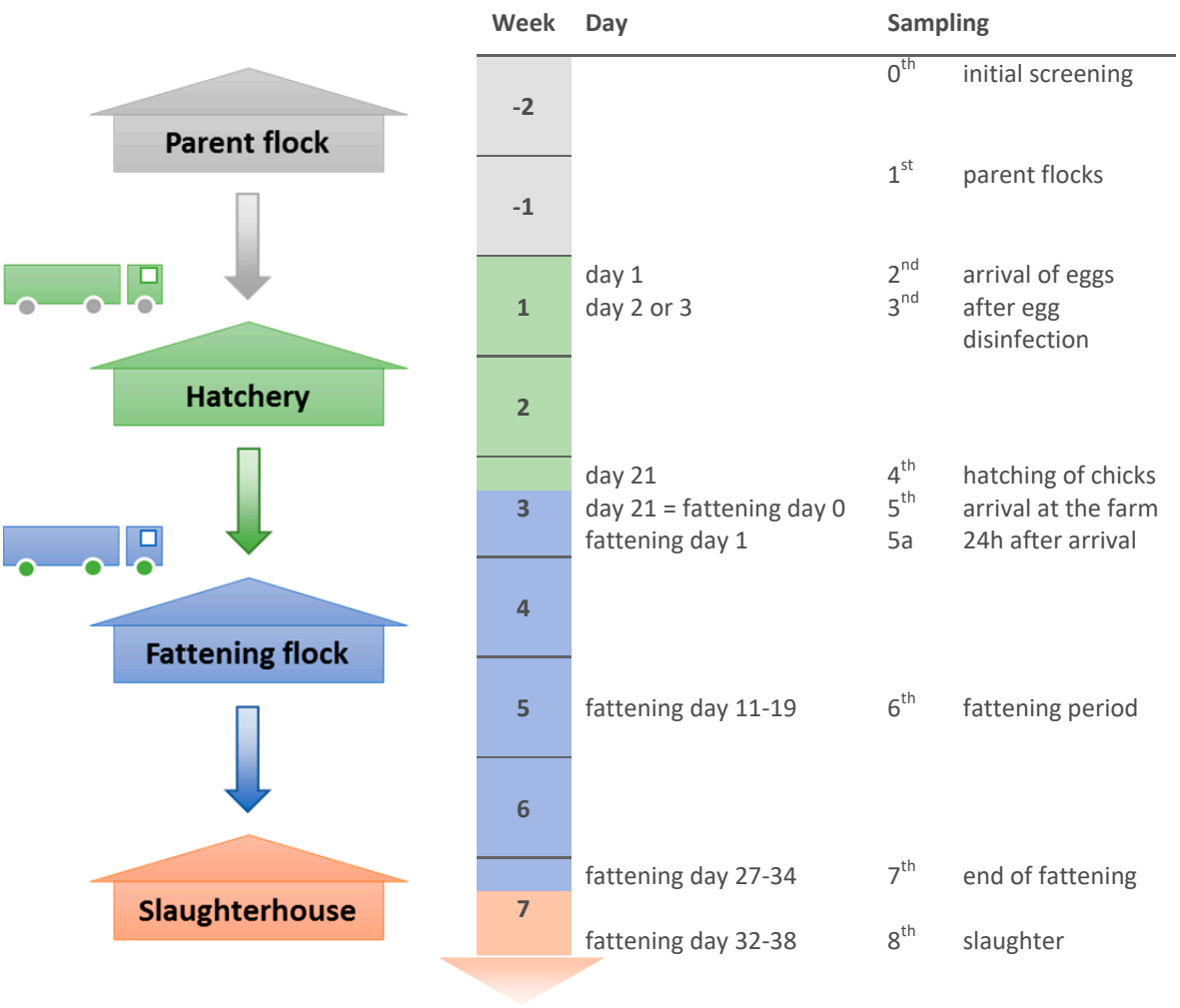

Figure 7: Time schedule of the samplings of one broiler production cycle

Investigations of one production chain cycle lasted approx. 10 weeks and were done by the ITU (0th to 7th sampling) and the ILH (8th sampling). During this time various samples were collected from eggs and chicken (individual animal samples) as well as from feces, carcasses, air and barn and slaughterhouse facilities (environmental samples). Additionally, swab samples from inside of the hatchery, transportation trucks, barns, and the slaughterhouses were taken (swabs: flock A-G; swabs and gauze swabs: flock E-G) (Table 9, Figure 8).

Table 9: Samples taken adapted from Dähre et al. 2017

Sampling time point[$]	Individual animal samples	Environmental samples	Swab samples
1st - Screening samples		Pooled faeces Boot swab	
2nd - Arrival of eggs	40x Egg surface, 40x Egg content, 40x Egg inner surface	2x Air samples (before and after arrival), Feathers*, Flies*	2x Truck, Wall, Ground, Drain
3rd - After disinfection of eggs	40x Egg surface	Air samples, Feathers*	Wall, Ground, Incubator racks, Ventilator
4th - Hatching of chicks	40x Cloacal swabs	Chicks dust, 2x Crushed eggshells	Chicks boxes, Discarder of eggshells, 2x Band-conveyors during chicks', inspection, Chicks° after vaccination, Transport boxes*
5th - Arrival at the farm	40x Cloacal swabs	Boot swab, Feed, Litter, Drinking Water, Dust, Air samples	2x Truck, 2-3x Chicks boxes, Feeding trough, Water trough, Barn wall, Hangers of barn equipment, Ventilator
5a - 24h after arrival[#]	40x Cloacal swabs	Pooled faeces, Boot swab	
6th - Middle of the fattening periode	40x Cloacal swabs	Pooled faeces, Boot swab, Feed, Litter, Drinking Water, Dust, Air samples	Feeding trough, Water trough, Barn wall, Hangers of barn equipment, Ventilator, Chicken°*
7th - End of fattening periode	40x Cloacal swabs	Pooled faeces, Boot swabs[§], Feed, Litter, Drinking Water, Dust, Air samples	Feeding trough, Water trough, Barn wall, Hangers of barn equipment, Ventilator, Chicken°*
8th - At slaughter	25x Caecum	25x Skin samples, 25x Filet samples, 2-3x Scalding water, Air samples*	White area: 2-3x Carcass hook, 2-3x Gut hook, 2x Washer Black area: 2-3x defeathering machine, 2x Chicken boxes , Chicken hook*

$ 1st – Parent flock, 2nd to 4th – Hatchery, 5th to 7th – Fattening flock, 8th - Slaughterhouse;# only flock D-G;
§ all barns of the farm were sampled (flock E-G); *samples not taken for all seven flocks; °plumage swab

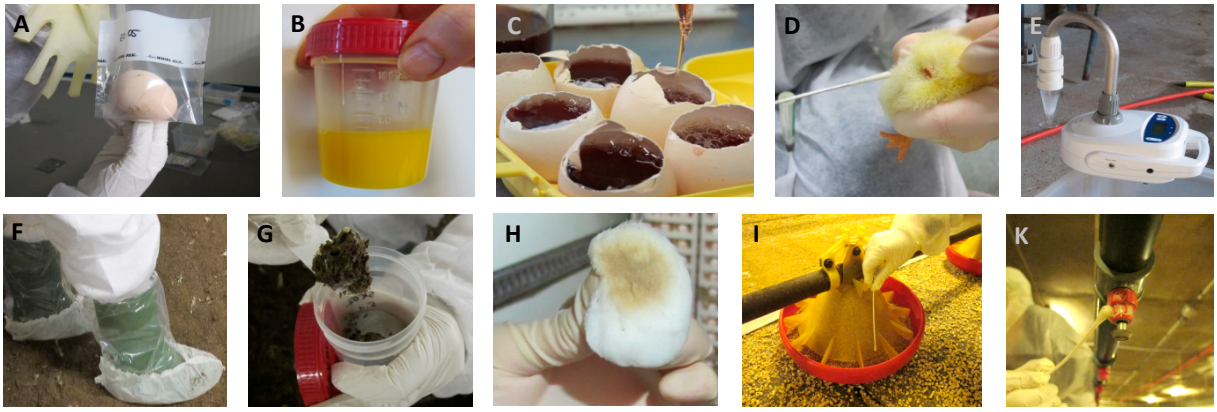

Figure 8: Selection of investigated samples; A – Eggshell outer surface, B – Egg content, C – Egg inner surface, D – Cloacal swab, E – Air sample (automated Coliolis µ), F – Boot swab, G – Pooled feces, H – Gauze swab, J – Feed trough, K – Water trough (Dähre, 2017)

Microbiological examinations of the samples concerning the presents of ESBL-/AmpC-producing Enterobacteriaceae (prevalence determination), antimicrobial resistance testing using disk diffusion tests (Karlsmose, 2010) and screening of the most prominent *bla* resistance genes using a multiplex real-time PCR (Roschanski et al., 2014) of putative resistant isolates, were performed by the ITU and the IHL for their respective samples. Further molecular analyses of resistant strains like sequencing of detected resistance genes, MLST, PFGE typing and WGS analyses were carried out at the ITU (Projahn et al., 2017). *In silico* determination of the presence of certain VAGs were performed by in-house bioinformatics tools of the Robert-Koch Institute Berlin (RKI).

2.2. Resistant Isolates

During the whole project ITU and ILH performed 56 samplings along the broiler production chain. In total 3766 samples (90 samples from parent flock screenings + 3676 samples along the production chain) were investigated concerning ESBL-/AmpC-producing Enterobacteriaceae. Numbers of positive samples detected at the respective broiler production stages during the different sampling time points were listed in table 10. From the 578 positive samples (25 parent screenings + 553 production chain) up to ten ESBL-/AmpC-producing Enterobacteriaceae were isolated and further investigated. Samples are collected within the RESETII FUB sub-project and are, therefore, also components of the doctoral thesis by Katrin Dähre (“Stufenübergreifender, vertikaler Transfer ESBL-/AmpC-produzierender Enterobakteriaceen in der Masthähnchenhaltung”, ITU) and Philine von Tippelskirch (“Prävalenz von Extended-Spectrum ß-Lactamase- und AmpC-ß-Lactamase-bildenden Enterobacteriaceae in Mastgeflügel”, ILH), however, with different analyses aspects.

Table 10: Numbers of ESBL-/AmpC-positive samples and numbers of total samples taken during the study. Results were partially published in Daehre et al., 2017 and von Tippelskirch et al., 2018

Sampling time point	Chain A	Chain B	Chain C	Chain D	Chain E	Chain F	Chain G
1st - Screening samples							
Boot swabs	1/1	1/1	1/1	1/1	3/3	0/3	1/4
Pooled feces	3/3	3/3	3/3	2/3	3/3	1/3	1/4
2nd - 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
3rd - 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
4th - Hatching of chicks							
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
5th - Arrival at the 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	0/28
5a - 24h after arrival							
Cloacal swabs	nd	nd	nd	0/40	1/40	2/40	2/40
Env. samples#	nd	nd	nd	0/2	0/2	2/2	0/2
6th - Middle of the fattening period							
Cloacal swabs	12/40	17/40	40/40	2/80*	6/40	2/40	0/40
Env. samples#	4/12	10/12	9/13	3/32*	3/16	3/16	2/18
7th - 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	7/25+	8/25+	4/28+
8th - At slaughter							
Caecum	21/25	1/25	22/25	5/25	21/25	13/25	0/25
Skin samples	6/25	0/25	17/25	9/25	24/25	20/25	21/25
Filet	8/25	0/25	21/25	4/25	11/25	0/25	5/25
Env. samples#	3/12	1/12	3/13	4/13	8/18	8/18	4/18

Nd - not determined; # Environmental samples incl. air and swab samples; *Two samplings at the middle of the fattening period; + Boot swabs were taken from all chicken barns

For the investigation of possible vertical transmissions of certain ESBL-/AmpC-producing Enterobacteriaceae along the stages of the broiler production chain a hierarchical analysis scheme was applied to the detected isolates. First of all, Genus and Species of the strains were determined. In case of *E. coli*, the next step was the analyses of the respective phylogroup. With the determination of the phylogroup it was possible to exclude non-related strains (strains belonging to different phylogroups) from the transmission investigations. Only strains showing the same phylogroup, harboring *bla* resistance genes encoding for the same beta-lactamase were then analyzed by PFGE or WGS. The detected types of resistant ESBL-/AmpC-producing *E. coli* were highly variable (Table 11); however, few examples of possible transmission routes could be identified.

Table 11: Detected variants of ESBL-AmpC-producing Enterobacteriaceae in the study partially published in Daehre et al., 2017 and von Tippelskirch et al., 2018. Isolate characteristics used for transmission investigations are highlighted in bold.

Chain	Sampling (No.)	No. of pos. samples	Species	*bla*-Genes	Phylogroup
A	Parent flock	4	*E. coli*	TEM-1*/ TEM-52	A
		1	*E. coli*	TEM-1*	A
	Fattening periode	61	*E. coli*	TEM-1*/ CMY-2	E, E/D, B1, F
		8	*E. coli*	CMY-2	A, E, E/D
	Slaughterhouse	6	*E. coli*	TEM-52	A, A/C, B1, E
		3	*E. coli*	CMY-2	E/D
		25	*E. coli*	TEM-1*/ CMY-2	A/C, E/D
		1	*E. coli*	TEM-1*/ TEM-52/ CMY-2	E/D
		1	*E. coli*	TEM-1*/ CTX-M-15/ CMY-2	E/D
		3	*E. fergusonii*	TEM-52	Neg.
		2	*P. mirabilis*	TEM-1*/ CTX-M-15	Neg.
B	Parent flock	2	*E. coli*	TEM-1*/ CTX-M-1	A, A/C
		6	***E. coli***	**CTX-M-1**	A, **B1**
		4	***E. fergusonii***	**TEM-52**	**neg.**
	Hatchery (2nd)	4	***E. coli***	**CTX-M-1**	**B1**
	Hatchery (3rd)	1	***E. fergusonii***	**TEM-52**	**neg.**
	Hatchery (4th)	1	*E. coli*	TEM-1*/ CMY-2	E/D
		2	*K. pneumoniae*	SHV-1*/ SHV-2	neg.
	Fattening (5th)	1	*E. coli*	TEM-1*/ CMY-2	F
		1	*E. coli*	CMY-2	F
		2	*K. pneumoniae*	SHV-1*/ SHV-2	neg.
	Fattening (6th+7th)	12	*E. coli*	CMY-2	E/D, A/C
		34	*K. pneumoniae*	SHV-1*/ SHV-2	neg.
		7	*K. pneumoniae*	SHV-2	neg.

	Slaughterhouse	2	*E. fergusonii*	TEM-52	neg.
C	Parent flock	2	*Escherichia spp.*	TEM-1*/ CMY-2	neg./B1
		1	*E. fergusonii*	TEM-52	neg.
		3	***E. coli***	**CMY-2**	**F**, A/C
	Fattening (5th)	3	***E. coli***	**CMY-2**	**F**
	Fattening (6th+7th)	113	***E. coli***	**CMY-2**	A, B2, **F**
		1	*E. coli*	TEM-1*/ CMY-2	F
	Slaughterhouse	42	***E. coli***	**CMY-2**	A, **F**, B1, E
		25	*E. coli*	TEM-1*/ CMY-2	F, B1, E/D
		2	*E. coli*	TEM-1*/ CTX-M-1	B1
D	Parent flock	1	*E. coli*	TEM-1*/ CMY-2	A
		3	*E. coli*	CMY-2	A, F
		1	*E. coli*	CTX-M-1	F
	Fattening (6th+7th)	8	*E. coli*	CMY-2	B2, B1
	Slaughterhouse	2	*E. coli*	SHV-12	F
		2	*E. coli*	SHV-2a	E/D
		9	*E. coli*	CTX-M-1	F, B1
		12	*E. coli*	CMY-2	B2, E/D
		1	*E. coli*	TEM-1*/ CMY-2	E/D
		1	*E. coli*	TEM-52	A/C
		1	*P. mirabilis*	TEM-1*/ CMY-2	Neg.
E	Parent flock	6	*E. coli*	CTX-M-1	F
	Fattening (5a)	1	*C. freundii*	CMY	neg.
	Fattening (6th+7th)	10	*E. coli*	CMY-2	F, E/D
		17	*E. coli*	SHV-12	B1
	Slaughterhouse	3	*E. coli*	TEM-52	A, E/D
		13	*E. coli*	CMY-2	F, E/D
		53	*E. coli*	SHV-12	A/C, B1
		2	*E. coli*	TEM-1*/ SHV-12	B1
		1	*E. coli*	SHV-2	E/D
		1	*K. pneumoniae*	TEM-1*/ SHV-27	Neg.
F	Parent flock	1	*E. coli*	TEM-1*/ CTX-M-15	F
	Hatchery (2nd)	1	*C. freundii*	CMY	neg.
	Hatchery (4th)	1	***E. coli***	**SHV-12**	**F**
		1	*C. freundii*	CMY	neg.
	Fattening (5th)	3	*E. coli*	SHV-12	B1
	Fattening (5a)	3	*E. coli*	CTX-M-1	A
		1	*E. coli*	SHV-12	B1

	Fattening (6th+7th)	8	***E. coli***	**SHV-12**	B1, **F**
		1	*E. coli*	CTX-M-1	A
	Slaughterhouse	13	***E. coli***	**SHV-12**	B1, **F**
		1	*E. coli*	TEM-1*/ CTX-M-1	B1
		23	*E. coli*	CTX-M-1	F
		1	*E. coli*	CTX-M-1/ CMY-2	F
		1	*E. coli*	CMY-2	A/C
		18	*E. fergusonii*	CMY-2	Neg.
		6	*K. pneumoniae*	SHV-1*/ SHV-2	Neg.
		2	*K. pneumoniae*	SHV-2	Neg.
		1	*C. freundii*	CMY-133 L6I P185H	Neg.
G	Parent flock	1	*E. coli*	TEM-1*/ TEM-52	A/C
		3	*E. coli*	TEM-52	A/C, B1
		1	*E. coli*	CMY-2	B1
	Hatchery (4th)	1	*E. coli*	TEM-1*/ TEM-52	A
	Fattening (5th)	1	*E. fergusonii*	TEM-52	neg.
	Fattening (5a)	3	*C. freundii*	CMY	Neg.
	Fattening (6th+7th)	4	*C. freundii*	CMY	Neg.
	Slaughterhouse	2	*E. coli*	CTX-M-1	F
		23	*E. coli*	TEM-1*/ CTX-M-15	A/C
		6	*P. mirabilis*	CMY-2	Neg.
		1	*C. freundii*	CMY-133	Neg.

*broad-spectrum beta-lactamase (BSBL)

3. Publications

3.1. Publication I

Projahn, Michaela; Daehre, Katrin; Roesler, Uwe; Friese, Anika (2017): **Extended-Spectrum-Beta-Lactamase- and Plasmid-Encoded Cephamycinase-Producing Enterobacteria in the Broiler Hatchery as a Potential Mode of Pseudo-Vertical Transmission**. In: Applied and Environmental Microbiology 83 (1). DOI: 10.1128/AEM.02364-16.

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Extended-Spectrum-Beta-Lactamase- and Plasmid-Encoded Cephamycinase-Producing Enterobacteria in the Broiler Hatchery as a Potential Mode of Pseudo-Vertical Transmission

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ABSTRACT Antimicrobial resistance through extended-spectrum beta-lactamases (ESBLs) and transferable (plasmid-encoded) cephamycinases (pAmpCs) represents an increasing problem in human and veterinary medicine. The presence of ESBL-/pAmpC-producing commensal enterobacteria in farm animals, such as broiler chickens, is considered one possible source of food contamination and could therefore also be relevant for human colonization. Studies on transmission routes along the broiler production chain showed that 1-day-old hatchlings are already affected. In this study, ESBL-/pAmpC-positive broiler parent flocks and their corresponding eggs, as well as various environmental and air samples from the hatchery, were analyzed. The eggs were investigated concerning ESBL-/pAmpC-producing enterobacteria on the outer eggshell surface (before/after disinfection), the inner eggshell surface, and the egg content. Isolates were analyzed concerning their species, their phylogroup in the case of *Escherichia coli* strains, the respective resistance genes, and the phenotypical antibiotic resistance. Of the tested eggs, 0.9% ($n = 560$) were contaminated on their outer shell surface. Further analyses using pulsed-field gel electrophoresis showed a relationship of these strains to those isolated from the corresponding parent flocks, which demonstrates a pseudo-vertical transfer of ESBL-/pAmpC-producing enterobacteria into the hatchery. Resistant enterobacteria were also found in environmental samples from the hatchery, such as dust or surfaces which could pose as a possible contamination source for the hatchlings. All 1-day-old chicks tested negative directly after hatching. The results show a possible entry of ESBL-/pAmpC-producing enterobacteria from the parent flocks into the hatchery; however, the impact of the hatchery on colonization of the hatchlings seems to be low.

IMPORTANCE ESBL-/pAmpC-producing enterobacteria occur frequently in broiler-fattening farms. Recent studies investigated the prevalence and possible transmission route of these bacteria in the broiler production chain. It seemed very likely that the hatcheries play an important role in transmission and/or contamination events. There are only few data on transmission investigations from a grandparent or parent flock to their offspring. However, reliable data on direct or indirect vertical transmission events in the hatchery are not available. Therefore, we conducted our study and intensively investigated the broiler hatching eggs from ESBL-/pAmpC-positive broiler parent flocks as well as the hatchlings and the environment of the hatchery.

KEYWORDS antibiotic resistance, *Enterobacteriaceae*, broiler chicken, hatchery, ESBL, pAmpC, AmpC

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Antimicrobial resistance is a challenging problem in public health. Transferable resistances to extended-spectrum cephalosporins (ESCs) have a rising impact on treatment strategies in both human and veterinary medicine (1, 2). Especially, extended-spectrum beta-lactamases (ESBLs) and plasmid-encoded cephamycinases (pAmpCs) protect pathogens against a variety of beta-lactam antibiotics, including third- and fourth-generation antibiotics. The most widespread ESBL genes in Germany are CTX-M-1, CTX-M-15, and CTX-M-14, which occur in humans as well as in animals (3, 4). A frequently detected pAmpC beta-lactamase is the CIT-type CMY-2 enzyme (5, 6).

Pathogenic ESBL-/pAmpC-producing enterobacteria can cause severe problems; however, commensal enterobacteria harboring ESBL-/pAmpC resistance genes were also detected in companion animals as well as farm animals, including broiler chickens (7–14). Especially, those farm animals can act as reservoirs for resistant bacteria and introduce the ESBL-/pAmpC-producing enterobacteria into the food production process (4, 15–17). Various studies also demonstrated a high prevalence of ESBL/pAmpC-producing enterobacteria in broiler chicken farms and the respective environment in Germany (7, 8, 18–22). It turned out that the 1-day-old (parent) broiler chicks already seemed to be colonized by these resistant bacteria when arriving at the farms (19, 23).

However, there are different important levels within the broiler production chain that can in general contribute to the transfer of resistant bacteria. It starts with the grandparents of the broiler chicken and proceeds to the hatcheries, down to the broiler-fattening farms, and, subsequently, to the slaughterhouse and food production process. Previous studies showed possible transmissions of pathogenic as well as commensal resistant enterobacteria between the levels of broiler production (23–26). The transmission or spread of highly similar plasmids harboring the respective resistance genes was also discussed (27). All these data suggest that hatcheries responsible for either the grandparent chicks or the parent chicks seemed to be involved in possible transmission routes. A direct transmission from the grandparent or the parent chicken to their offspring through the eggs could not yet be confirmed. The objective of this study was therefore to analyze all potential transmission routes of ESBL-/pAmpC-producing enterobacteria within the first levels of the broiler production chain. For that, eggs from ESBL-/pAmpC-positive parent flocks were tracked through the various stations within the hatchery, and the corresponding hatchlings were finally analyzed. At every sampling, different environmental and air samples were also considered.

RESULTS

ESBL-/pAmpC-producing enterobacteria. ESBL-/pAmpC-producing enterobacteria were found in samples from all four sampling time points. Out of the 36 samples from seven parent flocks (time point one), 24 samples (66.7%) were positive for ESBL-/pAmpC-producing enterobacteria after preenrichment in LB medium (Table 1). Without preenrichment, ESBL-/pAmpC-producing enterobacteria were detected in only 41.7% of the specimens. The microbial counts of the resistant bacteria varied between 1.67E + 01 and 7.17E + 05 CFU per boot swab or g of feces (geometric mean, 6.91E + 04 CFU).

From the hatchery in total, 200 specimens from eggs and chicks as well as 22 to 27 environmental and air samples from each of the flocks (n = 1,571) were analyzed. Before disinfection, ESBL-/pAmpC-producing enterobacteria were found on the outer surface of five eggs (1.8%), with four eggs belonging to flock B and one egg belonging to flock F (Table 1). The resistant bacteria were detected by the enrichment method only. None of the samples from the egg content and the inner surface or from the air and the environment were positive before routine egg disinfection. After disinfection, one egg surface (0.4%) belonging to flock B was positive for ESBL-/pAmpC-producing enterobacteria using the preenrichment method (Table 1). Again, all environmental and air samples were negative at this time. At the hatching of the chicks, we detected ESBL-/pAmpC-producing enterobacteria in four out of 65 environmental samples (6.2%) (Table 1). The positive samples were dust collected inside the hatcher, a crushed egg shell sample, and an environmental swab collected from flock B and a swab taken from

TABLE 1 Occurrence of ESBL-/pAmpC-producing enterobacteria and of total enterobacteria in samples of broiler parent flocks and in the hatchery

Sampling	No. of positive samples/total no. of samples, prevalence (%)[a]						
	Flock A	Flock B	Flock C	Flock D	Flock E	Flock F	Flock G
Parent flock							
Boot swab							
ESBL-/pAmpC-producing enterobacteria	1/1	1/1	1/1	1/1	3/3	0/3	1/4
Total enterobacteria	1/1	1/1	1/1	1/1	3/3	3/3	4/4
Feces							
ESBL-/pAmpC-producing enterobacteria	3/3	3/3	3/3	2/3	3/3	1/3	1/4
Total enterobacteria	3/3	3/3	3/3	3/3	3/3	3/3	4/4
Before disinfection of eggs (hatchery)							
Egg surface							
ESBL-/pAmpC-producing enterobacteria	0/40	4/40, 10	0/40	0/40	0/40	1/40, 2.5	0/40
Total enterobacteria	12/40, 30	24/40, 60	12/40, 30	7/40, 17.5	16/40, 40	29/40, 72.5	21/40, 52.5
Egg inner surface							
ESBL-/pAmpC-producing enterobacteria	0/40	0/40	0/40	0/40	0/40	0/40	0/40
Total enterobacteria	0/40	5/40, 12.5	5/40, 12.5	2/40, 5	5/40, 12.5	0/40	2/40, 5
Egg content							
ESBL-/pAmpC-producing enterobacteria	0/40	0/40	0/40	0/40	0/40	0/40	0/40
Total enterobacteria	0/40	0/40	0/40	0/40	0/40	0/40	0/40
Environmental samples							
ESBL-/pAmpC-producing enterobacteria	0/3	0/5	0/4	0/6	0/7	0/3	0/6
Total enterobacteria	1/3, 33.3	0/5	2/4, 50	0/6	3/7, 42.9	0/3	0/6
Air samples							
ESBL-/pAmpC-producing enterobacteria	0/4	0/4	0/4	0/4	0/4	0/4	0/2
Total enterobacteria	0/4	0/4	0/4	0/4	0/4	0/4	0/2
After disinfection of eggs (hatchery)							
Egg surface							
ESBL-/pAmpC-producing enterobacteria	0/40	1/40, 2.5	0/40	0/40	0/40	0/40	0/40
Total enterobacteria	0/40	4/40, 10	1/40, 2.5	1/40, 2.5	0/40	1/40, 2.5	3/40, 7.5
Environmental samples							
ESBL-/pAmpC-producing enterobacteria	0/4	0/4	0/4	0/4	0/4	0/3	0/5
Total enterobacteria	0/4	0/4	1/4, 25	0/4	0/4	0/3	1/5, 20
Air samples							
ESBL-/pAmpC-producing enterobacteria	0/4	0/2	0/2	0/2	0/2	0/2	0/2
Total enterobacteria	0/4	0/2	0/2	0/2	0/2	0/2	0/2
Hatching of chicks (hatchery)							
Cloacal swabs							
ESBL-/pAmpC-producing enterobacteria	0/40	0/40	0/40	0/40	0/40	0/40	0/40
Total enterobacteria	0/40	0/40	2/40, 5	0/40	0/40	0/40	1/40, 2.5
Environmental samples							
ESBL-/pAmpC-producing enterobacteria	0/8	3/9, 33.3	0/8	0/11	0/9	0/9	1/11, 9.1
Total enterobacteria	0/8	5/9, 55.6	3/8, 37.5	10/11, 90.9	7/9, 77.8	5/9, 55.6	9/11, 81.8

[a]For the ESBL-/pAmpC-producing enterobacteria data, numbers of ESBL-/pAmpC-positive samples and total numbers of samples of each sample type are stated. For the total enterobacteria data, the prevalences of samples positive for ESBL-/pAmpC-producing enterobacteria and for total enterobacteria are given.

the station for the automatic separation of chicks and eggshells of flock G. All 280 cloacal swabs from the hatchlings were negative concerning the resistant bacteria.

Total enterobacteria. In all boot swabs ($n = 14$) and feces samples ($n = 22$) from the parent flocks, enterobacteria were detected on MacConkey agar no. 3 (Oxoid, Wesel, Germany) without cefotaxime (MC−) agar plates after enrichment (Table 1). In addition, enumeration of total enterobacteria was done for the feces samples. We counted up to 1.01E + 08 enterobacteria/g of feces. The geometric mean (1.54E + 07 CFU/g of feces) was higher than for the resistant enterobacteria and resulted in a mean proportion of 0.6% ESBL-/pAmpC-producing enterobacteria of the total enterobacteria.

At the hatchery, all 1,571 samples were also analyzed concerning total enterobacteria. After the preenrichment, 200 samples (12.7%) were positive on MC− agar plates (Table 1). Of these samples, 73% ($n = 146$) were collected during the arrival of the eggs. Outer surfaces of the eggs (43.2%) from all seven flocks as well as inner eggshell surfaces (6.8%) and environmental swabs (21.4%) were positive concerning enterobac-

TABLE 2 ESBL/pAmpC resistance genes and species plus phylogroups of enterobacteria isolated from samples of the parent flocks and the hatchery

Flock	Sampling	Sample(s)	Species	Phylogroup	Gene(s)
A	Parent flock	Boot swab, pooled feces	*E. coli*	A	TEM-1[a]/TEM-52
B	Parent flock	Boot swab, pooled feces	*E. fergusonii*	Negative[b]	TEM-52
			E. coli	A/C	TEM-1[a]/CTX-M1
				A	TEM-1[a]/CTX-M1
				A	CTX-M1
				B1[c]	CTX-M1
	Before disinfection	Outer egg shell surface	*E. coli*	B1[c]	CTX-M1
	After disinfection	Outer egg shell surface	*E. fergusonii*	Negative[b]	TEM-52
	Hatching	Hatchlings dust	*E. coli*	E	TEM-1[a]/CMY-2
		Crushed egg shell, Environmental sample	*K. pneumoniae*	Negative	SHV-1[a]/SHV-2
C	Parent flock	Boot swab, pooled feces	*E. fergusonii*	Negative	TEM-1[a]/CMY-2
				Negative	TEM-52
			E. coli	A/C	CMY-2
				B1	TEM-1[a]/CMY-2
				F	CMY-2
D	Parent flock	Boot swab, pooled feces	*E. coli*	A	TEM-1[a]/CMY-2
				A	CMY-2
				F	CTX-M1
				F	CMY-2
E	Parent flock	Boot swab, pooled feces	*E. coli*	F	CTX-M1
F	Parent flock	Boot swab, pooled feces	*E. coli*	A/C	TEM-1[a]/CTX-M15
	Before disinfection	Outer egg shell surface	*C. freundii*		CMY
G	Parent flock	Boot swab, pooled feces	*E. coli*	A/C	TEM-1[a]/TEM-52
				B1	TEM-52
				B1	CMY-2
	Hatching	Environmental sample	*E. coli*	A	TEM-1[a]/TEM-52

[a]Broad-spectrum beta-lactamase (BSBL) gene.
[b]PFGE cluster V (see Fig. 1).
[c]PFGE clusters I to III (see Fig. 1), ST1665.

teria. The egg contents and the air samples were all negative. For the 120 egg surfaces with enterobacterial growth on MC− agar plates, enumeration was possible for 42 eggs (35.0%). The geometric mean was calculated as 8.71E + 04 enterobacteria/egg surface (minimum, 2.67E + 01 enterobacteria/egg surface; maximum, 3.47E + 06 enterobacteria/egg surface). After the disinfection of eggs, we still found 10 out of 280 outer egg surfaces (3.6%) to be positive for total enterobacteria (Table 1). For two of them, the total amount could be counted as 8.36E + 07 CFU and 7.33E + 02 CFU. In addition, enterobacteria could be detected in two feather samples from the hatchery (flocks C and G). All collected air samples after disinfection of the eggs were negative. After hatching of the chicks, 39 of 65 environmental samples (60.0%), including hatchlings dust and crushed eggshells, were positive on MC− agar plates after preenrichment (Table 1). At the same time point, only three cloacal swabs (1.1%) from the hatchlings were positive for enterobacteria, two from flock C and one from flock G. For one cloacal swab from flock C, enumeration on MC− agar plates led to a total of 9.48E + 05 enterobacteria.

Characterization of beta-lactamase genes and phylotyping. Molecular analyses showed that all seven parent flocks were colonized with up to four different types of ESBL-/pAmpC-producing enterobacteria, as shown in Table 2.

Isolates of the four positive eggs of flock B were all determined to be *Escherichia coli* of the phylogroup B1 harboring a CTX-M-1 gene. These strains show molecular characteristics identical to those of strains of the respective parent flock (*E. coli*, phylogroup B1, CTX-M-1 resistance gene [Table 2]). Therefore, randomly chosen isolates from pooled feces and the boot swab of the parent flock B as well as from the four egg

surfaces were further analyzed by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The fifth ESBL-/pAmpC-positive outer eggshell before disinfection was determined from flock F. From this specimen, a *Citrobacter freundii* isolate which was positive for a CIT-type CMY gene was identified.

After disinfection of the eggs at the hatchery, one egg was still positive for ESBL-/pAmpC-producing enterobacteria on the outer surface. This egg originated also from flock B, and phylogroup-negative *Escherichia fergusonii* strains harboring the ESBL gene TEM-52 could be isolated. Again, comparable isolates were found in the respective parent flock B (Table 2), and therefore, PFGE and MLST analyses were applied also for those strains.

During hatching of the chicks of flock B, we found the dust of the hatching breeder, a crushed egg shell sample, and an environmental swab from the station for the automatic separation of chicks and eggshells to be positive for resistant enterobacteria. *E. coli* of phylogroup E/D harboring the broad-spectrum beta-lactamase (BSBL) gene TEM-1 and a CIT-type pAmpC gene (CMY-2) was isolated from the hatchling dust. Both other environmental samples were positive for *Klebsiella pneumoniae* harboring the resistance genes SHV-1 (BSBL) and SHV-2. From a swab from the station for the automatic separation of chicks and eggshells of flock G, *E. coli* strains of phylogroup A harboring the resistance genes TEM-1 (BSBL) and TEM-52 were isolated.

Antimicrobial resistance testing. The applied disk diffusion tests showed variation in the disk diffusion diameters according to the ESBL/pAmpC resistance genes verified by sequencing. Theses variations were independent from the respective phylogroups of the isolates. Strains with resistance against cefotaxime (CTX) showed a CTX-M-1 gene, whereas strains harboring a CTX-M-15 or TEM-52 gene showed resistance to CTX and ceftazidime (CAZ). The diameters of the inhibition zone of the CTX-M-15 isolates were up to 5 mm smaller for both antibiotics compared to those of the TEM-52 strains. All isolates with a sequenced ESBL gene showed an increase in the inhibition zone diameter of CTX in the presence of clavulanic acid of up to 20 mm. Isolates that are resistant against CTX, CAZ, and cefoxitin (FOX) and show an increase in the inhibition zone diameter of CTX in the presence of clavulanic acid of up to 5 mm on average harbor the CMY-2 gene. Isolates harboring both a BSBL TEM-1 gene and a TEM-52 gene or an SHV-1 (BSBL) and an SHV-2 gene showed only an intermediate response against CTX and CAZ.

PFGE and MLST analyses of flock B. From flock B, we isolated ESBL-producing *Escherichia* strains with comparable genotypes (phylogroup plus resistance gene) from specimens from the parent flock as well as from the hatchery. To determine the phylogenetic relationships of these strains, we did PFGE analyses of randomly chosen isolates from all specimens (Fig. 1).

Evaluation of the PFGE gels showed four different band patterns, of which clusters I to III comprise the tested B1/CTX-M-1 isolates (Fig. 1A). The PFGE pattern of cluster I was found only once, in the boot swab from the parent flock. Cluster II included isolates from one pooled feces sample and all four egg surfaces before disinfection. Cluster III comprises identical band patterns from strains from the parent flock (boot swab and pooled feces) as well as from two of the eggs before disinfection. The band patterns of clusters I to III differ by only one band (I to II) or two bands (I to III). Identical band patterns in the PFGE gel imply a direct phylogenetic relationship. Therefore, we also checked the MLST type as a control for one isolate of each of the clusters. It turned out that the isolates were assigned to the same MLST type, sequence type 1665 (ST1665).

The PFGE cluster IV was restricted to the phylogroup-negative *E. fergusonii* isolates from all specimens collected from the parent flock and the outer surface of the disinfected egg (Fig. 1B). The tested isolates showed identical band patterns and were therefore determined to be phylogenetically related. For the phylogroup-negative strain, the MLST type could not be determined.

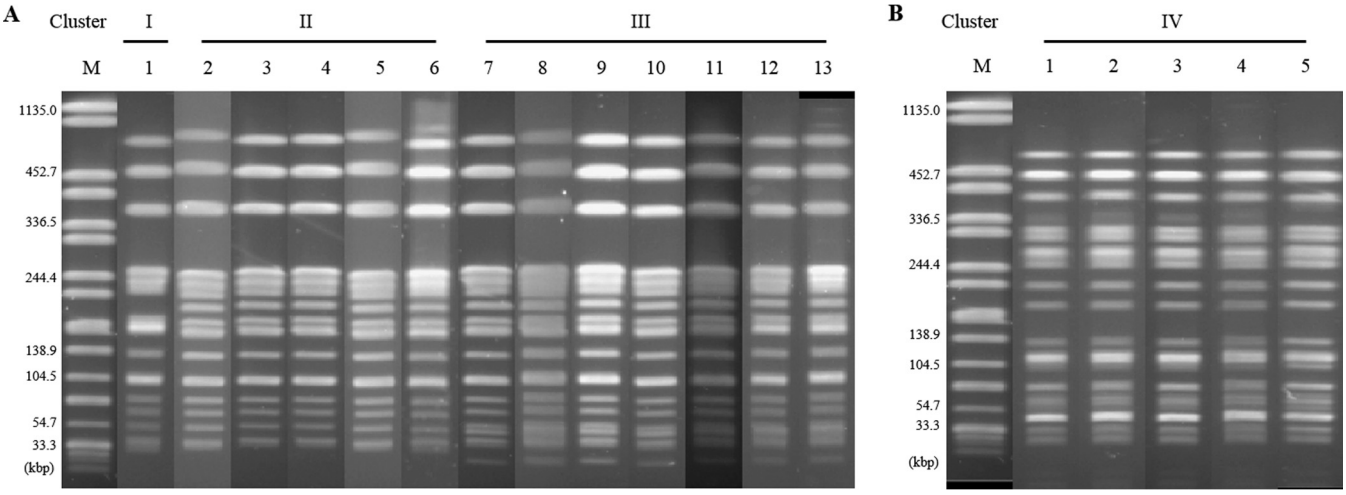

FIG 1 PFGE pattern of ESBL-positive isolates from flock B. (A) Comparison of *Escherichia coli* isolates from the parent flock and the outer surface of four eggs at the time of arrival at the hatchery. Isolates were of phylogroup B1 and harbor a CTX-M1 gene. Lane M, *Salmonella* Braenderup H9812; lanes 1 and 12, feces sample 2, parent flock; lanes 2 and 9, surface egg 4; lanes 3 and 7, feces sample 3, parent flock; lanes 4 and 13, boot swab, parent flock; lanes 5 and 10, surface egg 3; lane 6, surface egg 1; lane 8, feces sample 1, parent flock; lane 11, surface egg 2. (B) Comparison of phylogroup-negative *Escherichia fergusonii* isolates which were positive for the TEM-52 gene. Samples were taken from the parent flock and at the hatchery after disinfection of the eggs. Lane M, *Salmonella* Braenderup H9812; lane 1, feces sample 3, parent flock; lane 2, feces sample 2, parent flock; lane 3, feces sample 1, parent flock; lane 4, boot swap, parent flock; lane 5, surface egg 5.

DISCUSSION

It is already known that commensal ESBL-/pAmpC-producing *E. coli* strains occur in healthy chickens on broiler farms (7, 8, 28), and even 1-day-old chicks seemed to be affected (19). Recently published studies described the introduction and the circulation of ESBL-/pAmpC-producing *E. coli* strains in broiler farms and along the whole broiler production process (23, 25, 27). They showed that there is a clonal spread through grandparent flocks as well as parent flocks and broiler farms. However, there were missing links in the longitudinal follow up, especially in the hatcheries. Therefore, we conducted our study to investigate the transmission routes of ESBL-/pAmpC-producing enterobacteria from the parent flocks to the hatchery and, subsequently, the hatched chicks.

Parent flocks. The detected resistance genes as well as the respective prevalences were in concordance with previously published studies on broiler chickens in Germany (3, 17, 19). We found ESBL-/pAmpC-producing *E. coli* strains of different phylogroups comparable to those in a recent study on the genetic diversity of *E. coli* in poultry production (29). In addition, we isolated *E. fergusonii* strains harboring resistance genes against beta-lactam antibiotics from two parent flocks. Since a multidrug-resistant *E. fergusonii* strain was also found in chickens (30), studies on ESBL/pAmpC producers in poultry and veterinary public health agencies should also consider these bacteria.

For all phenotypically resistant enterobacteria except *Enterobacter* spp., a respective ESBL or pAmpC gene could be identified. *Enterobacter* spp. are known to show intrinsic resistance to beta-lactam antibiotics due to alterations of outer membrane proteins (31).

Interestingly, the results of the disk diffusion diameters showed variations according to the respective gene type, which have not, to our knowledge, been described before in detail.

Transmission to the hatchery via egg surfaces. From each of the seven parent flocks, we tracked the corresponding eggs to the hatchery and analyzed the outer surfaces of 40 eggs of the same batch before and after disinfection, respectively. In contrast to Mezhoud et al. (32), we used a modified shell rinse method (33) instead of crushing the whole eggshell to distinguish between the bacteria originating from the outer and inner shell surfaces. Using the shell rinse method, we could demonstrate the contamination of five eggs with ESBL-/pAmpC-producing enterobacteria prior to their

disinfection (flock B, *E. coli* B1 plus CTX-M-1; flock G, *Citrobacter freundii* plus CMY). In a recent study on egg contamination in Belgium, the Netherlands, and France, ESBL producers could be isolated from broiler chicken eggs as well (32). Using comparative analyses, we could show that the ESBL strains found on egg surfaces of flock B harboring a CTX-M-1 are highly related to those isolated from their parent flock (Fig. 1). This clearly demonstrates that the introduction of ESBL-producing *E. coli* strains occurs directly from the parent flock into the hatchery. Furthermore, we could show that up to 3.47E + 06 total enterobacteria were present on the outer surfaces of the eggs which were transported to the hatchery. This finding matches previously published data (34). The prevalence of total enterobacteria with 42.9% positive eggs demonstrates, in principle, a high input of bacteria into the hatchery.

The same batch of eggs was again sampled after their disinfection by formaldehyde fumigation, which is routinely done in hatcheries for egg sanitation (34). On one egg surface, a still-viable *E. fergusonii* isolate harboring a TEM-52 gene was found. Again, PFGE analyses verified the phylogenetically relationship to the respective isolates from its parent flock B. This emphasizes the hypothesis of a direct transmission via the eggs. In total, 280 egg surfaces were analyzed after disinfection, of which 3.6% were positive for enterobacterial growth on MC− agar plates. Therefore, even after the disinfection of the eggs, they could serve as a contamination source; however, it seems to have a rather low impact.

Transmission to hatchery via the environment. Before disinfection, all environmental and air samples from the hatchery were negative concerning ESBL-/pAmpC-producing enterobacteria. However, in three flocks, we found up to 50% of the environmental samples to be positive for non-ESBL/pAmpC enterobacteria. It seems that resistant strains in the environment of the hatchery where the eggs arrive are absent or their load is very low; therefore, they are under the detection limit of our sampling method.

After disinfection of the eggs, only two feather samples showed enterobacterial growth on MC− agar plates. As feathers are introduced into the hatchery usually stuck to the eggshells via feces, they have an impact on bacterial transmission into the hatchery similar to that of the eggs themselves. This could be avoided by removing the feathers in the parent flock farms. Also, none of the air samples taken in the particular rooms before and after disinfection showed enterobacterial growth. Taken together, a relevant transmission of ESBL-/pAmpC-producing enterobacteria via air or contaminated surfaces seems to be unlikely. A study on this transmission route was not done before.

Transmission to hatchlings via eggs. One possible transmission route of ESBL-/pAmpC-producing enterobacteria to hatchlings we investigated was the direct vertical transfer through reproductive organs of the hens. This was shown for layering hens in cases of experimental infections with *Salmonella enterica* serovar Enteritidis (35) but, to our knowledge, not for bacteria of the genus *Escherichia*. Therefore, we investigated the total egg content (egg white and albumin) of 40 eggs from each parent flock after their arrival in the hatchery. None of the tested eggs were positive for any enterobacterial growth; therefore, we would exclude this transmission route for ESBL-/pAmpC-producing enterobacteria.

Early contamination of eggs can also occur after the lay through penetration of the eggshells. Eggshell and eggshell membranes are natural defense barriers, but penetration through and multiplication within the egg were shown for salmonellae in various studies (35–39). To our knowledge, this was also not yet demonstrated for *E. coli*. Penetration of pathogens through the eggshell occurs more likely in cases of incomplete cuticle or with variations in pH, temperature, humidity, or vapor (35, 38). In the investigated hatchery, the conditions at our samplings were constant and, therefore, preventive for the penetration of bacteria into the eggs. However, from 6.8% of the inner surfaces of the eggs, enterobacteria could be isolated which were not resistant to a beta-lactam antibiotic. This demonstrates a potential growth of enterobacteria on the

inner surface of an egg and is comparable to the results of another study (37). Nevertheless, further validation of the method needs to be done. There is no suitable method to analyze the bacterial growth on the outer and inner surfaces of the same egg so far. We intensively tested and optimized our methods for the investigation of the outer and inner egg surfaces before starting the samplings to clearly show a penetration of the pathogens through the eggshell and avoid contamination. However, this might happen because of tiny breaks in the eggshell or cuticle occurring during the filling of the eggshells with the hot liquid MC− agar (56°C).

At the last sampling time point, we investigated the gut microbiota of the recently hatched chicks using cloacal swabs. In our study, none of the 280 recently hatched chicks were positive for ESBL-/pAmpC-producing enterobacteria. In fact, only three chicks (1.1%) were already colonized with enterobacteria.

Taken together, our results show that a strict vertical transmission of ESBL-/pAmpC-producing enterobacteria from broiler parent flocks to their hatchlings through the eggs could not be verified. We also demonstrated that a difference of 1 or 2 days in the sampling of hatchlings can change the outcome of a study. The colonization process of the hatchlings with the gut microbiota occurs within a few days and needs, therefore, to be further analyzed.

Transmission to hatchlings via the environment. Studies reported that 1-day-old broiler chicks are already and the grandparent chicks are colonized with ESBL-/pAmpC-producing *E. coli* (19, 40); they therefore contribute to the spread of these bacteria at the farm level. As discussed before, it is more likely that the chicks get colonized by the uptake of resistant bacteria from the environment of the hatchery. This pseudo-vertical transfer of ESBL-/pAmpC-producing enterobacteria might occur in the hatcheries or during the process of discarding eggshells, chicks' inspection, vaccination, counting, and loading them into the transportation boxes. We found four ESBL-/pAmpC-positive samples in the hatchery after hatching of the chicks in hatchling dust, crushed egg shells, and two swabs from the environment of the hatchery connected to the process of separation of eggshells from the chicks. This supports the hypothesis of a potential pseudo-vertical transfer from the hatchery environment to the hatchlings.

To our knowledge, the outer eggshell surface (before and after disinfection) as well as the inner shell surface and the egg content of broiler hatching eggs were not analyzed before in a published study. Microbiological analyses and the comparison of molecular data from isolates of parent flocks, eggs, and environmental samples showed a low level of transmission of ESBL-/pAmpC-producing enterobacteria from the parent flocks to the hatchery. We found resistant bacteria in the environment of the hatchery after hatching of chicks, but a strict vertical transfer for the ESBL-/pAmpC-producing enterobacteria could not be shown.

MATERIALS AND METHODS

Samplings. In our study, seven different broiler parent flocks, their corresponding eggs at the hatchery, their hatchlings, and various environmental samples were investigated concerning the occurrence of ESBL-/pAmpC-producing enterobacteria in Germany in the years 2014 and 2015.

(i) Parent flocks. Sixteen parent flocks of an integrated broiler production were initially investigated using at least three pooled feces and one boot swab. Out of these, only ESBL-/pAmpC-positive flocks were then included in the study. Seven positive parent flocks were selected, which were of different ages: 29 weeks (flock A), 57 weeks (flock B), 43 weeks (flock C), 50 weeks (flocks D and F), 51 weeks (flock E), and 58 weeks (flock G). Analyses of the parent flocks were done approximately 1 week before starting the samplings at the hatchery. The eggs of all studied parent flocks were transferred to the same hatchery.

(ii) Before egg disinfection. Samplings in the hatchery were performed at three different time points. The first sampling was done during the arrival of the eggs at the hatchery. Environmental samples were taken from the wall and the ground or the drain using sterile swabs (dry cultural swab with flexible polystyrene [PS] stick; Nerbe Plus GmbH, Winsen [Luhe], Germany), moistened with phosphate-buffered saline (PBS), before the eggs arrived. The sampled spots were approximately 100 cm^2 in size. In addition, two air samples were taken using two different sampling techniques analyzing 1,000 liters of air. The first instrument was the impactor Biotest Hycon RCS Plus air sampler (Biotest AG, Dreieich, Germany) used with an airflow of 50 liters/min. For the detection of resistant and nonresistant enterobacteria, Hycon blank strips (Millipore, Darmstadt, Germany) were filled with MacConkey agar no. 3 (Oxoid, Wesel, Germany) containing 1 mg/liter cefotaxime (AppliChem, Darmstadt, Germany) (MC+) and MacConkey

agar no. 3 without cefotaxime (MC−), respectively. As a second air sampling instrument, the automated impinger Coriolis μ (Bertin Technologies) was used, and air was collected in 10 ml of PBS with an airflow of 250 liters/min.

After the arrival of the eggs at the hatchery, two air samples were again taken, as well as environmental swabs from the transport trolleys of the eggs and the truck (except for flock A). From each of the parent flocks, eggs were collected for analyzing the outer surface of the shell, the egg contents, and the inner shell surface. Therefore, 40 eggs were directly put into sterile Whirl-Pak stand-up bags (540 ml volume) for analyses of the outer surface using the shell rinse method according to Musgrove et al. (33).

For analyses of the egg contents and the inner shell surface, an additional 40 eggs were collected.

(iii) After egg disinfection. The second sampling at the hatchery was performed after routine disinfection of the eggs by formaldehyde fumigation, which was usually done twice. Again, 40 eggs from the same batch were collected for outer surface analyses only. The collecting procedure was the same as described before. Two air samples were taken, as well as three environmental swabs from the wall, the ground, and the incubator racks. If possible, feathers or insects, like flies, were collected from the respective room in the hatchery directly into sterile sample bags.

(iv) Hatching. The third sampling at the hatchery was done after the hatching of the chicks originating from the previously sampled parent flock. Therefore, 40 cloacal swabs of the hatchlings (dry cultural swab with aluminum stick; Nerbe Plus GmbH, Winsen [Luhe], Germany), 8 to 10 g of hatchling dust directly collected from the inside the hatchers, and two pooled eggshell samples each containing five crushed eggshells were collected into sterile Whirl-Pak stand-up bags. Up to five swabs from the environment of the hatchery were taken during the process of discarding eggshells and chicks' inspection, vaccination, counting, and loading into transport boxes.

Laboratory analyses. (i) Pooled feces and boot swabs. Boot swabs and 20 g of feces were inoculated in stomacher bags in 200 ml and 180 ml of Luria-Bertani (LB) medium (Carl Roth, Karlsruhe, Germany), respectively, and were treated by stomaching for 2 min at 200 rpm (stomacher 400 circulator; Seward Limited, West Sussex, United Kingdom). After that, aliquots of the sample suspensions were taken for enumeration of ESBL-/pAmpC-producing enterobacteria on MC+ agar plates and an enumeration of all enterobacteria in feces samples on MC− agar plates. Therefore, 100 μl of an appropriate dilution was plated out onto three plates of the respective agar. Colonies were counted according to their morphology after aerobic incubation at 37°C for 24 h. For preenrichment, the stomacher bags with the LB sample mixtures were incubated aerobically 24 h at 37°C. After that, 10 μl of the incubated LB mixtures was streaked out with an inoculation loop on MC+ and MC− agar plates. Agar plates were aerobically incubated at 37°C for 24 h for qualitative detection of ESBL-/pAmpC-producing enterobacteria as well as for analysis of the total enterobacterial composition. Species identification was done using matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS; MALDI Microflex LT and Biotyper database, Bruker Daltonics, Bremen, Germany). Up to 10 suspicious ESBL-/pAmpC-producing enterobacteria of each sample with different colony morphology (two isolates each morphology) on MC+ agar plates were isolated and stored for further susceptibility testing and molecular analyses.

(ii) Outer surface of eggs. Analyses of the outer surface of the eggs before and after disinfection were done using a slightly modified shell rinse method (33). After adding 10 ml of LB medium to the Whirl-Pak stand-up bags, eggs were shaken for 10 min at 20°C and 200 rpm in a shaking incubator. Retained samples were taken for enumeration of ESBL-/pAmpC-producing enterobacteria on MC+ agar plates and of all enterobacteria on MC− agar plates, as described above. Bags containing eggs and LB medium were incubated aerobically for 24 h at 37°C without shaking, and 10 μl of the incubated LB mixtures was streaked out on MC+ and MC− agar plates, respectively. Colony counting and isolate identification were done as described above. Isolates from MC− agar plates were cross-checked for their growth on MC+ agar plates.

(iii) Inner surface of eggs and egg contents. Analyses of the inner surface and the egg content were done for the same egg. Therefore, the top of the egg was dipped for 2 s into 70% ethanol for disinfection, with a subsequent exposure time of at least 30 s and then opened sterile with the egg slicer Cregg (Brainstream, Oerlinghausen, Germany), which cuts the top of the egg and opens the egg at a diameter of approximately 35 mm. Egg yolk and albumin were put into a sterile 50-ml beaker with a screw cap (Sarstedt, Nümbrecht, Germany), gently mixed together with 20 ml of LB medium (10 ml for flock A), and incubated 24 h at 37°C. The empty eggshell was dried under a hood for at least 2 h and then filled with liquid MC− agar (56°C). The eggs were incubated at 37°C up to 48 h, and the shell was removed from the egg-shaped agar using sterile scalpels to analyze possible growth of enterobacteria originating from the inner surface of the eggs. If possible, colonies were picked. Additionally, the egg-shaped MacConkey agar was put into a Whirl-Pak stand-up bag, crushed and mixed manually with 30 ml of LB medium, and incubated for 24 h at 37°C. Enrichments of the egg content as well as the inner surface were streaked out on MC+ and MC− agar plates and incubated 24 h at 37°C. Plates were further analyzed concerning the growth of (resistant) enterobacteria, and isolation of colonies was done as previously mentioned.

(iv) Cloacal swabs. Swabs were cut off into a tube containing 1.5 ml of sterile PBS and were gently vortexed. Seven hundred fifty microliters of the sample was stored at 4°C as a retained sample. Seven hundred fifty microliters, including the swab, was used for a cultural enrichment in 9 ml of LB medium and a subsequent analysis for ESBL-/pAmpC-producing enterobacteria, as described before. In case of a positive enrichment of ESBL-/pAmpC-producing enterobacteria, the retained sample was used for enumeration of the (resistant) bacteria.

TABLE 3 Sequencing primers used in the study for determining the respective beta-lactamase genes

Primer	Sequence (5′ → 3′)	Product size (bp)	Reference
TEM-F	GCGGAACCCCTATTTG	963	27
TEM-R	ACCAATGCTTAATCAGTGAG		27
CTX-M1-SEQ_F	CCCATGGTTAAAAAATCACTGC	944	33
CTX-M1-SEQ_R	CAGCGCTTTTGCCGTCTAAG		33
SHV(−28)-F	GGCCCTCACTCAAGGATGTA	1,028	This study
SHV(+1000)-R	CCACGTTTATGGCGTTACCT		This study
CMY2(−80)-F	CAACACGGTGCAAATCAAAC	1,322	This study
CMY2(+1242)-R	CATGGGATTTTCCTTGCTGT		This study

(v) Environmental samples. Environmental swabs were investigated according to the cloacal swab protocol using 1 ml of PBS for enrichment in LB medium. The pooled crushed eggshells as well as the dust samples were processed as follows: 30 ml of LB medium was added to the Whirl-Pak stand-up bag, and the mixture was rigorously shaken by hand and incubated for 24 h following the enrichment procedure. The same procedure was applied for collected flies and feathers using 10 ml of LB medium. Environmental samples from flock A were not analyzed on MC− agar plates concerning the growth of total enterobacteria.

(vi) Air samples. The strips of the RCS Plus air sampler were aerobically incubated for 24 h at 37°C after air sampling. Colonies were counted according to their morphology and the respective species identified via MALDI-TOF MS. Up to 10 suspicious ESBL-/pAmpC-producing enterobacteria with two isolates of each colony morphology were isolated from the MC+ agar strip for further susceptibility testing and molecular analyses. Regarding the Coriolis, 3 ml of the PBS was added to 27 ml of LB medium and incubated aerobically at 37°C for 24 h for analyses of the ESBL-/pAmpC-producing enterobacteria as well as for analysis of the total enterobacterial composition.

(vii) Antimicrobial susceptibility testing. For all suspicious isolates, disk diffusion tests with 30 μg of cefotaxime, 30 μg of cefotaxime plus 10 μg of clavulanic acid, 30 μg of ceftazidime, and 30 μg of cefoxitin (Liofilchem s.r.l., Roseto degli Abruzzi, Italy) were applied. Isolates were grown on MC+ agar plates, and a suspension in 0.85% NaCl solution with a McFarland standard of 0.5 was prepared from this culture. The following procedure was done according to the EUCAST guidelines (41). Disk diameters were analyzed using the EUCAST breakpoint tables (42). All enterobacteria suspicious for resistance concerning the tested beta-lactams were further investigated.

(viii) Real-time PCR and sequencing. Subsequently to the antimicrobial susceptibility testing, isolates were further analyzed using real-time PCR for the detection of the predominant beta-lactamase genes bla_{CTX-M}, bla_{SHV}, bla_{TEM}, as well as CIT-type pAmpC genes (43). Therefore, DNA was extracted by a simple boiling method, and the supernatant was used for all PCR analyses (43). Positive controls and no-template controls were used as published previously (43). Detected ESBL/pAmpC genes for at least one isolate of each sample were verified by sequencing, each with the primers shown in Table 3. The novel primers SHV(−28)-F, SHV(+1000)-R, CMY2(−80)-F, CMY2(+1242)-R were designed with Oligo-Perfect designer (Thermo Fisher Scientific, Inc., St. Leon Roth, Germany) using sequences with GenBank accession no. X53433 (SHV) and FR719923 (CIT-type pAmpCs) as templates. For both PCRs, 25 μl of the respective reaction mixture contained 12.5 μl of DreamTaq Green PCR Mastermix (Thermo Fisher Scientific, Inc.), 1 μl of each primer (10 μM), and 7.5 μl of PCR water. The PCR conditions for both genes were as follows: 5 min of initial denaturation at 94°C, 35 cycles of 30 s of denaturation at 94°C, 30 s of primer binding at 57°C, 1 min of elongation at 72°C, and a final elongation step at 72°C for 5 min. Nucleotide sequences were analyzed with BioNumerics version 6.6 (Applied Maths, Sint-Martens-Latem, Belgium) and compared with reference sequences from GenBank to determine the assigned gene number according to www.lahey.org/studies/.

(ix) Phylogenetic typing. Isolates which were determined to be *Escherichia coli* or *Escherichia fergusonii* by MALDI-TOF MS were further analyzed for their phylogenetic group, as described previously (44). The multiplex PCR was performed using primers as published previously, and PCR conditions were adjusted as follows: 3 min of initial denaturation at 94°C, 33 cycles of a 30-s denaturation at 94°C, primer binding for 30 s at 57°C, and a 1-min elongation at 72°C, with a final elongation step of 5 min at 72°C. The total reaction mixture of 15 μl contained 0.15 μl of each primer (0.3 μl of TspE4C2.1b and TspE4C2.2b), 7.5 μl of DreamTaq Green PCR Mastermix, and 5 μl of PCR water. Isolates showing band patterns in the agarose gel electrophoresis which were not specific for just one phylogroup were assigned as a combined phylogroup, for example, A/C.

(x) Pulsed-field gel electrophoresis. Isolates from parent flock B and from the corresponding egg surfaces of the same phylogroup harboring an identical ESBL gene were investigated by pulsed-field gel electrophoresis (PFGE) to further analyze their phylogenetic relationship. At least one isolate of each sample from the parent flock (feces and boot swab) and the egg surfaces at arrival at the hatchery and after disinfection of the eggs was analyzed. Isolates were grown aerobically in LB medium for 24 h at 37°C. From that, a bacterial suspension in PBS (Oxoid, Wesel, Germany) was made with an optical density at 600 nm (OD_{600}) of about 0.33. Plugs were prepared by mixing 250 μl of bacterial suspension and 375 μl of 1.5% LE GP agarose (Biozym Scientific GmbH, Hessisch Oldendorf, Germany), incubated at 56°C for 24 h in cell lysis buffer (500 mM EDTA [pH 9.5], 1% sarcosine, 0.9 mg of proteinase K/ml), and washed

six times in Tris-EDTA buffer. Whole-genomic DNA within agarose plug slices was enzymatically restricted for 4 h at 37°C using 15 U of XbaI (Thermo Fisher Scientific, Inc., St. Leon Roth, Germany) for each slice. Running conditions for the gel electrophoresis were used as published by Schaufler et al. (45). *Salmonella enterica* serovar Braenderup H9812 was used as a size standard, according to Hunter et al. (46).

(xi) Multilocus sequence typing. From each PFGE cluster, one isolate was analyzed with multilocus sequence typing (MLST) to determine their sequence type (ST). Primers were used as published previously (47). Sequences were analyzed with BioNumerics version 6.6, and the respective STs were assigned according to http://mlst.warwick.ac.uk/mlst/dbs/Ecoli.

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3.2. Publication II

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Environmental adaptation and vertical dissemination of ESBL-/pAmpC-producing *Escherichia coli* in an integrated broiler production chain in the absence of an antibiotic treatment

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Summary

High prevalence numbers of extended-spectrum beta-lactamase- (ESBL-)/plasmid-mediated AmpC beta-lactamase- (pAmpC-) producing *Escherichia coli* in broiler chicken and their distribution along the broiler production chain is an ongoing problem in food production. We, therefore, investigated resistant isolates along the broiler production chain to determine whether there is a constantly occurring direct vertical transmission of the ESBL-/pAmpC-producing *E. coli* from the parent flocks to their offspring or not. We, furthermore, analysed the isolates concerning the occurrence of virulence factors and their ability to form biofilms to estimate their potential to effectively colonize broiler chickens and/or persist and survive in the environment of the broiler production facilities. Using whole genome sequencing, we could show that ESBL-/pAmpC-producing *E. coli* were likely transferred in a step-wise process along the broiler production chain but not directly from the parent flock to the fattening flock with every single batch of offspring chickens. Additionally, resistant *E. coli* strains showing an extraintestinal pathogenic genotype as well as high numbers of virulence-associated genes including the production of curli fibres and cellulose have high capabilities to persist and spread in the broiler production chain.

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Introduction

Extended-spectrum beta-lactamase- (ESBL-) and plasmid-mediated AmpC beta-lactamase- (pAmpC-) producing Enterobacteriaceae are an increasing problem in public health and veterinary medicine (Pitout and Laupland, 2008; Ewers *et al.*, 2012; Kaesbohrer *et al.*, 2012). A major concern is the high prevalence of these resistant bacteria in the broiler production chain. ESBL-/pAmpC-producing Enterobacteriaceae were frequently isolated from broiler fattening farms worldwide (Bortolaia *et al.*, 2010; Randall *et al.*, 2011; Kameyama *et al.*, 2013; Maciuca *et al.*, 2015; Trongjit *et al.*, 2016) but were also found in broiler (grand) parent flocks (Dierikx *et al.*, 2013; Agersø *et al.*, 2014; Mo *et al.*, 2014; Zurfluh *et al.*, 2014). Therefore, different transmission scenarios of the resistant bacteria in the broiler production pyramid are discussed: A transmission from prior stages into the fattening farms (Giovanardi *et al.*, 2005; Dierikx *et al.*, 2013; Nilsson *et al.*, 2014; Huijbers *et al.*, 2016) as well as an insufficient cleaning and disinfection procedure in the chicken barns (Hiroi *et al.*, 2012; Luyckx *et al.*, 2015b). We recently showed a pseudo-vertical transfer of resistant bacteria from the parent flocks into the hatchery via contaminated eggshells (Projahn *et al.*, 2017). In addition, it was proved by whole genome analyses that there is in fact a horizontal transfer of ESBL producers between consecutively fattened flocks regardless of cleaning and disinfection procedures (Daehre *et al.*, 2017). However, it still remained unclear whether the resistant bacteria are permanently and repeatedly transferred along the whole broiler production chain with each production cycle, or if this transfer only occurs occasionally and certain strains, once introduced on the farm, are circulating constantly.

Escherichia coli naturally inhabits the gastrointestinal tract of mammals and birds and can be classified into commensal and pathogenic strains. Intestinal pathogenic

E. coli (IPEC) are causing diarrhoea syndromes and seldom colonize healthy humans whereas extraintestinal pathogenic *E. coli* (ExPEC) often innocuously colonize the gut but have the ability to cause severe infections such as meningitis or blood stream infections (Vila *et al.*, 2016). Recent studies compared the occurrence of certain virulence-associated genes (VAGs) in avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC) and commensal *E. coli* from humans and broiler chickens (Kemmett *et al.*, 2013; de Carli *et al.*, 2015; van Hoek *et al.*, 2016; Paixao *et al.*, 2016; Silveira *et al.*, 2016). It turned out that certain VAGs (e.g. *fimC, iha, tsh, ireA, neuC, astA, irp2, vat, iucD, chuA, iss)* could not be statistically significant linked to a particular type of pathogenicity, but were also detected in strains from healthy hosts. Therefore, some of these factors are increasingly termed as 'fitness factors' because they contribute to a successful colonization and enhanced survival in the gut and the environment but not necessarily cause diseases (Smith *et al.*, 2007; Frommel *et al.*, 2013; Wigley, 2015; Vila *et al.*, 2016). ESBL-/pAmpC-producing *E. coli* from healthy hosts were usually classified as commensal strains but recent investigations found out that these resistant strains also show characteristics of ExPEC or ExPEC-like strains and, therefore, have the ability for an enhanced colonization of the gut.

The presented study aimed three questions. First, is there a direct top-down transmission of ESBL-/pAmpC-producing *E. coli* from the parent flock via the hatchery to the respective fattening flock in the absence of an antibiotic treatment? Second, is the prevalence of ESBL-/pAmpC-producing *E. coli* in healthy broiler flocks influenced by their status as an ExPEC? Third, have the isolates found in the broiler production chain a higher ability to persist and survive in the environment?

To investigate the top-down transmissions across all stages of the broiler production chain (parent flock, hatchery, fattening flock/barn), we analysed 44 ESBL-/pAmpC-producing *E. coli* from two different broiler production chains (chain C and F) which were not treated with antibiotics by whole genome analyses (Daehre *et al.*, 2017; Projahn *et al.*, 2017). Ten ESBL-/pAmpC-positive strains from parent screening samples (S) and other parent or fattening flocks (A/11, B/41, D, E/73) showing the same phylogroup and resistance genes that were found in chain C or F, respectively, were also included to show a potential circulation of certain strains within the broiler production chain (please see Fig. 4 and Table S1 for assignment/glossary of samples and strains). Secondly, to address the question whether there is a difference in the VAGs related to ExPEC between high and low prevalent strains, 89 selected ESBL-/pAmpC-positive strains from three healthy broiler flocks (C/41, E/74, F/74) with different prevalence were analysed for their VAGs. In addition, to determine the potential to persist and survive in the environment, the expression of two major biofilm-associated extracellular matrix components (cellulose, curli fimbriae) was investigated for 13 strains from these three flocks.

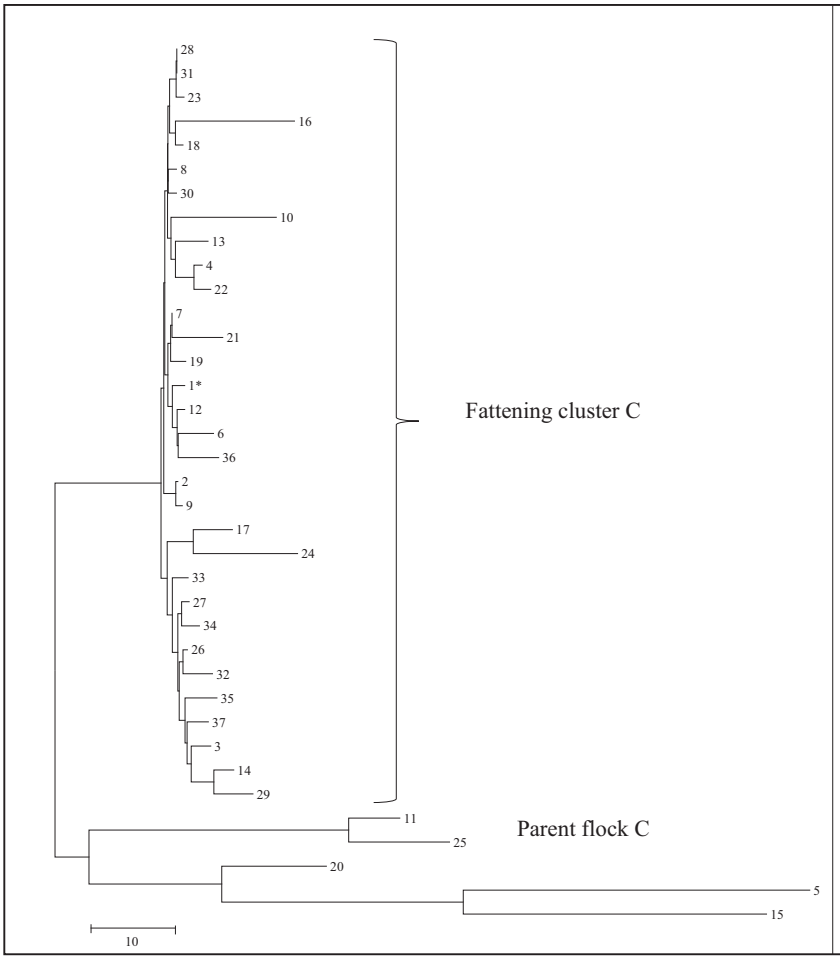
Results

Phylogenetic analyses

In silico multilocus sequence typing (MLST) using whole genome sequences revealed three different sequence types (STs). Isolates, previously determined as phylogroup F harbouring a $bla_{\text{CMY-2}}$ gene and sampled from the broiler chain C were assigned as ST-354. Samples from parent flocks D and screening parent flock S were also of ST-354 whereas isolates of the fattening flocks A/11 and B/41 turned out to be of ST-38. All isolates of phylogroup F harbouring a $bla_{\text{SHV-12}}$ gene of broiler chain F as well as from the additional samples belonged to the ST-117.

Comparative analyses of single nucleotide polymorphisms (SNPs) were carried out for isolates of the MLST types ST-354 ($n = 37$) and ST-117 ($n = 14$) respectively (Figs 1 and 2). The calculation of the number of SNP differences between the ST-354 strains ($bla_{\text{CMY-2}}$) resulted in one large cluster (fattening cluster: 0-32 SNPs difference, max. 6.4 SNPs Mbp^{-1}), which included all the isolates from the fattening flock C/41 ($n = 31$) as well as the isolate 5146 from the transportation truck of the same flock (Fig. 1). The SNP differences between this fattening cluster and the two strains (5008, 4989) from the respective parent flock C were calculated as 53 and 78 SNPs (10.5 and 15.6 SNPs Mbp^{-1}) respectively. The SNP differences between the fattening cluster and the samples from parent flock D and screening parent flock S were calculated as up to 118 SNPs (23.6 SNPs Mbp^{-1}).

The SNP difference calculation of the ST-117 strains revealed two clusters (Fig. 2). The first one includes only four isolates of which two were collected from samples of fattening flock F/74 (chain F) as well as two screening isolates of fattening flocks E/73 and F/79. The second cluster comprises the isolate 7553 from the hatchery environment (chain F), seven isolates of the fattening period of flock F/74 (chain F) as well as two isolates from other flocks fattened at the same farm at the same time as fattening flock F (barn 74) but in different barns (F/71, F/75). SNP differences among the strains of the clusters varied between 23 and 68 SNPs (max. 13.6 SNPs Mbp^{-1}) and 4 to 51 (max. 10.2 SNPs Mbp^{-1}) SNPs, respectively, whereas the differences between both clusters were determined as 3114 to 3147 SNPs (max. 629.4 SNPs Mbp^{-1}).



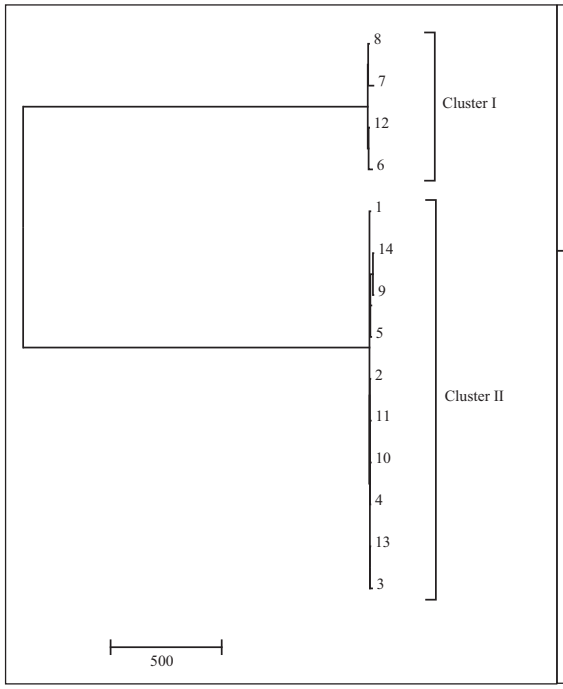
ID	Strain	Chain/Barn	Sample	MLST
28	5796	C/43	C.3-CloacalSwab3	ST-354
31	5794	C/43	C.3-CloacalSwab3	ST-354
23	5795	C/43	C.3-CloacalSwab3	ST-354
16	5367	C/43	C.2-CloacalSwab2	ST-354
18	5390	C/43	C.2-CloacalSwab3	ST-354
8	5332	C/43	C.2-EnvSwab3	ST-354
30	5323	C/43	C.2-EnvSwab1	ST-354
10	5685	C/43	C.3-Dust	ST-354
13	5664	C/43	C.3-Litter	ST-354
4	5350	C/43	C.2-CloacalSwab1	ST-354
22	5351	C/43	C.2-CloacalSwab1	ST-354
7	5337	C/43	C.2-Dust	ST-354
21	5679	C/43	C.3-PooledFaeces	ST-354
19	5272	C/43	C.2-Litter	ST-354
1*	5146	C/43	C.1-Truck	ST-354
12	5150	C/43	C.1-Litter	ST-354
6	5673	C/43	C.3-BootSwab	ST-354
36	5286	C/43	C.2-PooledFaeces	ST-354
2	5705	C/43	C.3-CloacalSwab1	ST-354
9	5706	C/43	C.3-CloacalSwab1	ST-354
17	5369	C/43	C.2-CloacalSwab2	ST-354
24	5876	C/43	C.3-CloacalSwab4	ST-354
33	5392	C/43	C.2-CloacalSwab3	ST-354
27	5274	C/43	C.2-Feed	ST-354
34	5284	C/43	C.2-BootSwab	ST-354
26	5159	C/43	C.1-BootSwab	ST-354
32	5354	C/43	C.2-CloacalSwab1	ST-354
35	5344	C/43	C.2-EnvSwab2	ST-354
37	5368	C/43	C.2-CloacalSwab2	ST-354
3	5257	C/43	C.2-Water	ST-354
14	5687	C/43	C.3-EnvSwab	ST-354
29	5394	C/43	C.2-CloacalSwab3	ST-354
11	5008	C	C-BootSwab	ST-354
25	4989	C	C-PooledFaeces	ST-354
20	6312	S	S-PooledFaeces	ST-354
5	5312	D	D-PooledFaeces2	ST-354
15	5306	D	D-PooledFaeces1	ST-354

*Isolate from truck sample

Fig. 1. Phylogenetic tree of ST-354 strains. A neighbour-joining tree was calculated (with 1000 bootstraps) using a SNP distance matrix calculated by MEGA7 and the Harvest suite. Isolates are indicated by the respective strain number and the sample from which they originate as well as the chain and barn number of the respective sampling. The sample name presents further information like the sampling time points (C.1 – arrival of the chicks at the farm, C.2 – middle of the fattening period, C.3 – end of the fattening period) and the sample matrix.

Virulence-associated genes

Whole genome contigs of the 89 strains were also investigated for their virulence profiles analysing 89 VAGs (Table S3) of which 19 genes were typically associated with IPEC strains (InVAGs) and 70 with ExPEC strains (ExVAGs). All strains were negative concerning the occurrence of InVAGs. In contrast, of the 70 ExVAGs, 37 genes were detected in ST-354 strains (53%; chain C, screening samples D+S), up to 25 genes in ST-117 strains (36%; chain F, screening samples E/73, F/71, F/75, F/79) and in the isolates of ST-38 (36%; samples A/11, B/41), respectively, and 19 ExPEC-related VAGs in the ST-2307 isolates (27%). All isolates of the different MLST types share 14 ExVAGs which were as follows: *crl* (curli fibre gene), *csgA* (curli fibre-encoding gene), *feoA/B* (major bacterial ferrous iron transporter, iron (II) transport system), *fimC* and *fimH* (type 1 fimbriae), *matA* (meningitis-associated and temperature-regulated fimbriae), *iroN* (catecholate salmochelin receptor), *iss* (increased serum survival), *sitA* and *sitC* (*Salmonella* iron transport system gene), *cvi* (structural genes of colicin V operon), *ompA* (outer membrane protein), *traT* (transfer protein), *astA* (heat stable cytotoxin associated with enteroaggregative *E. coli*) and *malX* (pathogenicity-associated island marker CFT073). Most variations between the isolates of the different MLST types were observed in the numbers of genes which contribute to adhesion and iron uptake (Table 1). Moreover, certain traits were exclusively found in isolates of a respective ST. *bfpM* (bundle-forming pilus), *ibeA* (invasion of brain endothelium), the *pap* operon (Pap pili adhesin), *tia* (toxigenic invasion locus in ETEC isolates) and *neuC* (K1 capsular polysaccharide) were detected in isolates of the ST-354



ID	Strain	Chain/Barn	Sample	MLST
8	10042	F/74	F.3-Dust	ST-117
7	10043	F/74	F.3-Water	ST-117
12	7242	E/73	E.3-BootSwab	ST-117
6	7908	F/79	F.3-BootSwab	ST-117
1*	7553	F	F.H-EnvSwab	ST-117
14	7812	F/74	F.2-CloacalSwab1	ST-117
9	7811	F/74	F.2-CloacalSwab1	ST-117
5	7892	F/75	F.3-BootSwab	ST-117
2	7810	F/74	F.2-CloacalSwab1	ST-117
11	7790	F/74	F.2-EnvSwab	ST-117
10	7768	F/74	F.2-PooledFaeces	ST-117
4	7864	F/74	F.3-BootSwab	ST-117
13	7865	F/74	F.3-BootSwab	ST-117
3	7873	F/71	F.3-BootSwab	ST-117

*Isolate from hatchery environment sample

Fig. 2. Phylogenetic tree of ST-117 strains. A neighbour-joining tree was calculated (with 1000 bootstraps) using a SNP distance matrix calculated by MEGA7 and the Harvest suite. Isolates are indicated by the respective strain number and the sample from which they originate as well as the chain and barn number of the respective sampling (E/73, F/71, F/74, F/75, F/79). The sample name presents further information like the sampling time points (F.H – Hatchery, F.2 – middle of the fattening period of chain F, F.3 – end of the fattening period of chain F, E.3 – end of the fattening period of chain E which was fattened at the same farm previously to the chickens of chain F) and the sample matrix.

whereas *fyuA* (yersiniabactin receptor), *ireA* (iron-responsive element), *irp2* (yersiniabactin synthesis) and *pic* (serine protease autotransporter) occurred only in ST-117 strains. *hek/hra* (heat-resistant agglutinin) could be detected in the three ST-38 isolates from the parent flocks A and B, only. Even though all analysed isolates showed very high numbers of detectable VAGs, according to the definition of ExPEC strains by Johnson *et al.* (2003)(≥2 of *papA/C*, *afa/dra*, *sfa/foc*, *iutA* and *kpsMTII*), only the ST-354 strains and two of three ST-38 strains (3565, 10026) could be assigned as an ExPEC. Interestingly, ExPEC-related invasion genes also occurred in ST-354 and ST-38 strains, only.

Table 1. Mean values of detected virulence-associated genes (VAGs). Highly variable categories are highlighted in bold.

MLST	ST-354	ST-117 (cluster 1)	ST-117 (cluster 2)	ST-2307	ST-38
Total no. of ExVAGs (70;14[a])	36.7	21	24.3	19	23.7
Adhesion (34;5[a])	**15.9**	**4**	**5**	**5**	**7**
Invasion (4;0[a])	2	0	0	0	1
Iron uptake (12;4[a])	**8.8**	**8**	**11**	**6**	**8**
Protection (6;4[a])	6	3	4	4	4.7
Toxins (12;1[a])	3	3	3.3	3	2
Miscellaneous (2;1[a])	1	2	2	1	1

a. Number of genes which were detected in all isolates.

Macrocolony assay

Two sets of isolates were analysed by the macrocolony assay. The first one comprised thirteen isolates from the whole genome approach, at least three isolates of each MLST type (chain C: ST-354, chain F: ST-117, screening samples from A/11+B/41: ST-38, Daehre *et al.* chain E: ST-2307). Isolates of fattening flocks C/41, F/74 and E/74 occurred with different prevalence in the respective fattening flocks (flock C/41: 73.1%, flock E/74: 26.4%, flock F/74: 5.7%). The second strain set included 42 isolates from investigations of seven different broiler production chains covering all detected genetic profiles (phylogroup + ESBL-/pAmpC gene) to get an overall impression on cellulose and curli production in different resistant isolates from the whole broiler production chain (Daehre *et al.*, 2017; Projahn *et al.*, 2017) (Tables S1 and S2). Isolates of the first set showed both, production of cellulose and curli, regardless of the flock prevalence, whereas in the second one, various phenotypes were determined (Fig. 3). In total, 28.6% (12/42) of the second strain set showed neither a production of curli nor cellulose, 7.1% (3/42) showed only a cellulose-positive phenotype and 9.5% (4/42) of the isolates were positive for

a curli production, only. Most of the strains (52.4%, 22/42) were phenotypically positive for both, curli and cellulose production. Only 16.7% (7/42) could not be assigned to a distinct phenotype in the macrocolony assay.

Discussion

We performed our study addressing the questions of a direct top-down transmission of ESBL-/pAmpC-producing *E. coli* from the parent flock via the hatchery to the respective fattening flock in the absence of an antibiotic treatment as well as the role of the ExPEC status and the ability to form biofilms on the prevalence of ESBL-/pAmpC-producing *E. coli* in healthy broiler flocks. Therefore, we investigated isolates (ST-354) from a parent flock and the corresponding fattening flock (chain C) using whole genome analyses. It turned out that the isolates of the parent flock and the fattening flock are closely related but the grouping into different clusters and the analyses of the SNP differences showed that there was no direct vertical spread of these resistant bacteria. ESBL strains from the fattening period, collected during a time frame of five weeks, differed in not more than 6.4 SNPs Mbp^{-1}. In contrast, isolates of the parent flock were collected only 17 days earlier but the SNP differences between isolates of the parent flock and the fattening flock were about 10.5 SNPs Mbp^{-1}. The number of SNP differences between the strains of the fattening period is higher than the numbers published for clonal outbreaks of enterohemorrhagic *E. coli* (EHEC) (de Been *et al*., 2014; Berenger *et al*., 2015; Rusconi *et al*., 2016) but overall lower than those described for interspecies transmissions of clonal ST-410 *E. coli* strains (Schaufler *et al*., 2016b). These findings suggest that ESBL-/pAmpC-resistant *E. coli* were more likely transferred from the parent flock into the production chain via an earlier event but not directly along the investigated batch of broiler chicken.

In addition, we isolated an ESBL-*E. coli* strain from the transportation truck and from the litter inside of the barn from the first day of the fattening period which clustered together with all the other strains from the whole fattening period of chain C. This indicates that a transmission of the resistant bacteria can also occur via the transportation process and that the resistant bacteria must have been already present in the hatchery than directly transmitted from the parent flock. This is in concordance with our findings that the resistant bacteria were introduced into the hatchery via contaminated eggshells and the hatchery acts as a bottleneck for the spread of these strains (Projahn *et al*., 2017).

Investigation of the ST-117 strains of chain F underlines the fact that there were ESBL *E. coli* already present in the hatchery which lead to the colonization of the recently hatched chicks. Whole genome analyses revealed two clusters. The first one comprised strains from the environment of the hatchery and the fattening period confirming the hatchery as a contamination source. The second ST-117 cluster comprised strains of the fattening flock F as well as strains of two other barns from the same farm. Due to the small number of SNP differences between the isolates, a transmission or exchange of these ESBL *E. coli* between the barns of the same farm is very likely. It was previously shown that (ESBL-) *E. coli* can survive the cleaning and disinfection procedures in the chicken barns which finally leads to a spread and the colonization of broiler chickens on the same farms (Luyckx *et al*., 2015a,b; Daehre *et al*., 2017). Interestingly, the two clusters differ in up to 629.4 SNPs Mbp^{-1} indicating that two different clonal lines of ST-117 strains were introduced into the same flock.

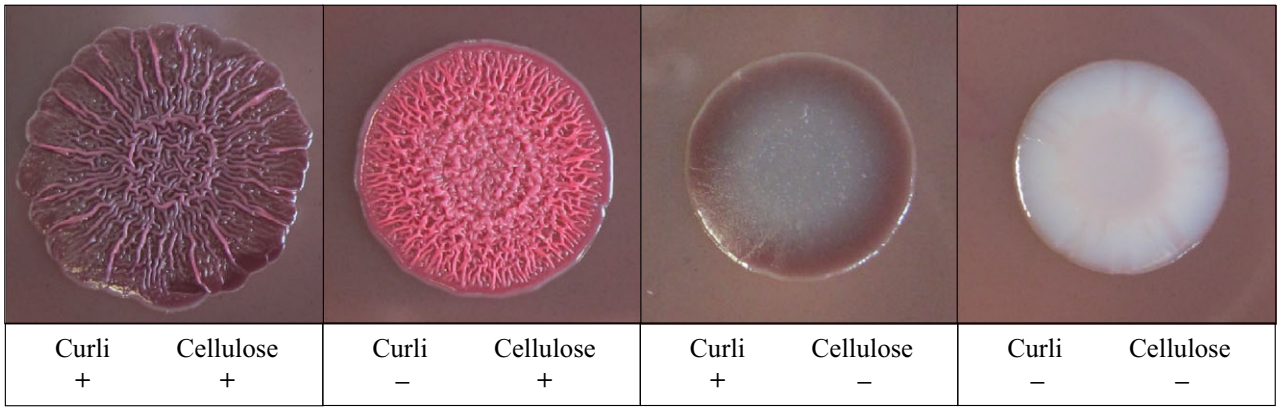


Fig. 3. Macrocolony assay. Categories of detected phenotypes in the investigated isolates. The figure shows exemplary macrocolonies of isolates 6818 (+/+), 6922 (–/+), 3233 (+/–), 6985 (–/–).

We previously investigated up to 150 samples from the fattening period of seven flocks each showing that there is an overall highly diverse *E. coli* population on these fattening farms (Daehre *et al*., 2017). Together with the results from this study, we could show that not only one prominent vertical or horizontal transmission route of ESBL producers exists. More precisely, certain ESBL-/pAmpC-producing *E. coli* seemed to be more likely introduced into the broiler production chain decades ago and now circulate constantly in different broiler production-related environments and evolve even without antibiotic pressure (Nilsson *et al*., 2014; Huijbers *et al*., 2016; Mo *et al*., 2016).

Investigations on the phenotypic characteristics and virulence patterns of ESBL-/pAmpC-producing *E. coli* indicate that certain traits usually associated with virulence factors correlate with the ability to adhere, persist and adapt to certain environmental circumstances (Guenther *et al*., 2012; Rodiger *et al*., 2015; Mo *et al*., 2016; Paixao *et al*., 2016; Schaufler *et al*., 2016a). In our study, high prevalence strains of ST-354 could be assigned as ExPEC and overall showed the highest number of ExVAGs, especially genes referring to adhesion factors, compared to low prevalent strains of ST-117 and ST-2307. ST-354 isolates harboured the *pap* operon which was shown to be responsible for the *in vitro* adhesion to not only human but also animal cell lines (Stromberg *et al*., 1990; Frommel *et al*., 2013). But intestinal colonization is also depending on the iron uptake ability of *E. coli* (Nowrouzian *et al*., 2006; Tenaillon *et al*., 2010). On the one hand, ST-117 strains of our study had the highest number of genes referring to iron uptake, on the other hand, these isolates had low numbers of genes which contribute to adherence factors. Furthermore, these strains were found with low prevalence in the investigated fattening flock F (5.7%). Interestingly, ST-2307 isolates had overall the lowest numbers of ExVAGs including adherence traits but occurred with higher prevalence in the fattening flock (23.5%) than ST-117 strains assuming that not only high numbers of VAGs but a certain combination or set of virulence traits can promote the colonization of the avian gut. However, specific factors for the binding to avian host cells are poorly understood and should be therefore further investigated.

The ability to form bacterial biofilms is a benefit in the survival against host defence factors, antibiotics, physical and chemical stress as well as disinfectants (Hall-Stoodley *et al*., 2004; Flemming and Wingender, 2010). Using a macrocolony assay, we could show that more than half of our tested strains from both investigated sets were able to produce curli fibres and the exopolysaccharide cellulose which are necessary to form stable and strong biofilm matrices (Olsen *et al*., 1989; Barnhart and Chapman, 2006; Uhlich *et al*., 2006; Serra *et al*., 2013, 2015). These positive strains were isolated from all the seven investigated flocks by Daehre *et al.* and included also the 14 selected isolates from the whole genome analyses of ST-354, ST-117, ST-38 and ST-2307 from our study. Here, the production of curli fibres and cellulose was not linked to a certain phylogroup or ESBL/pAmpC resistance gene and was also independent from the prevalence of these strains in the respective flocks (flock C: 73.1%, flock E: 26.4%, flock F: 5.7%) indicating that resistant *E. coli* occurring on broiler farms in general show good abilities to survive on the farms and in the environment. Cellulose also has high water holding capacities which protects the bacteria against dehydration (O'Sullivan, 1997). This could be a big advantage in surviving, for example, the cleaning and disinfection procedure in the hatchery and the broiler fattening farms. Daehre *et al.* already showed that the ST-2307 strains were transmitted from one broiler flock to the consecutively fattened flock in the same barn. The results of the present study suggest that biofilm production as well as high numbers of gene conferring to adherence might support the persistence of resistant bacteria and the stable colonization of broiler chickens.

Addressing our first question, we demonstrated that the transmission of ESBL-/pAmpC-producing Enterobacteriaceae in the broiler production chain is more or less a summary of different transmission routes including persistence and circulation of different clonal lineages independently from antibiotic usage instead of a direct vertical top-down transmission along the broiler production chain. Second, the occurrence of high numbers of ExPEC VAGs seemed to contribute the colonization process. However, regarding the avian host, not only high numbers of VAGs but also a certain set of virulence traits seemed to contribute this colonization. This needs to be further investigated. Finally, high numbers of isolates producing curli fibres and cellulose were detected which support the survival of resistant strains in the environment of the broiler production chain.

Experimental procedures

Bacterial isolates

ESBL-/pAmpC-producing *E. coli* originate from individual animal samples (cloacal swabs) and environmental samples (swabs and housing environment) of different stages of the broiler production chain (parent flock, hatchery, fattening farm) collected in Germany during the years 2014 and 2015 (Daehre *et al*., 2017; Projahn *et al*., 2017).

In total, 54 isolates were selected on the basis of their phylogroup and the *bla* resistance gene for detailed genomic analyses using WGS to determine possible

transmission events along the broiler production chain (Fig. 4; Table S1).

Escherichia coli of phylogroup F encoding a CMY-2 cephamycinase were collected from samples of the parent flock C ($n = 2$) as well as from the respective fattening flock C (barn 43; $n = 31$) and the transportation truck ($n = 1$) of fattening flock C from the hatchery to the fattening farm. Additionally, strains showing the same characteristics were isolated from screening samples (faeces or boot swab), collected at different time points within the study, of parent flock D ($n = 2$) and parent screening flock S ($n = 1$) and the fattening flocks A (barn 11; $n = 1$) and B (barn 41; $n = 2$).

Isolates of phylogroup F coding for a SHV-12 beta-lactamase were determined from the investigation of the broiler chain F. Here, an environmental gauze swab from the hatchery (hatching of the flock F chicks; $n = 1$) as well as different samples from the fattening period of flock F (barn 74; cloacal swabs, gauze swabs, environmental swabs, housing environment; $n = 9$) were analysed. Additionally, isolates of phylogroup F encoding a SHV-12 beta-lactamase from boot swabs of fattening barns E/71, F/73, F/75 and F/79 were included in the comparative genome analyses. These samples originate from other flocks fattened at the same time at the same farm as flock F or E, respectively, but in different barns.

Escherichia coli isolates of phylogroup B1 harbouring a bla_{SHV-12} gene ($n = 35$) were included in the study for the investigation of VAGs. These isolates were derived from two broiler flocks subsequently fattened in the same barn (flock E/74 and F/74) and from an additional gauze swab from the transportation truck of flock F (Daehre *et al.*, 2017).

Whole genome sequencing

Genomic DNA preparation was performed using the MasterPure™ DNA purification kit (Epicentre, Illumina) according to the manufacturer's instructions. Whole genome sequencing was performed using the Nextera XT Kit for library preparation and the Illumina MiSeq Reagent Kit v3 (300-bp paired-end sequencing with 50–100× coverage). The NGS tool kit (Patel and Jain, 2012) was used for quality control of the sequence read data. High-quality reads were *de novo* assembled into contiguous sequences using CLC Genomics Workbench 8.0 (CLC bio, Aarhus, Denmark), and annotation of draft genomes was performed with RAST server (Aziz *et al.*, 2008).

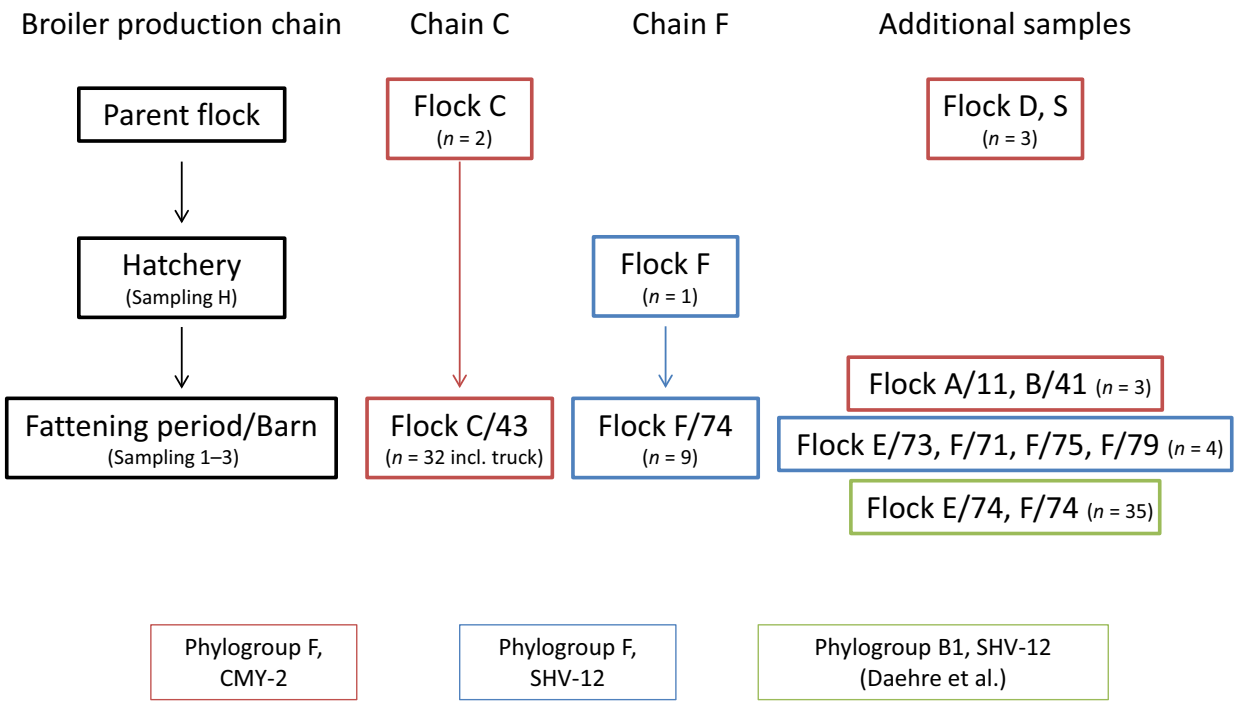


Fig. 4. Origin and number of *E. coli* isolates used in the study for transmission investigations using WGS. Isolates of the same genetic characteristics (phylogroup + ESBL/pAmpC gene) are coloured in red, blue and green, respectively. Additional samples from the fattening period of flock F (F/71, F/75, F/79) were collected at the same time from other barns within the same farm as F/74. Isolates from flock E/74 and F/74 were only investigated concerning VAGs and Biofilm production.

Phylogenetic analyses

Whole genome data were used for *in silico* determination of MLST types using the CGE Bacterial Analysis Pipeline (Thomsen *et al*., 2016).

The bacterial core genome and the number of SNP differences were calculated between isolates of the same MLST type using Harvest suite 1.0 (Treangen *et al*., 2014) and MEGA 7.0 (Kumar *et al*., 2016). Phylogenetic trees were constructed using a pairwise distance matrix and the neighbour-joining algorithm (MEGA 7.0, 1000 bootstraps) based on the number of calculated SNP differences between the isolates.

Virulence-associated genes

The presence of 89 virulence-associated genes (VAGs) in the genomes of all 54 investigated isolates (Fig. 4) was checked using an automated in-house BLAST search. VAG analyses were also performed for additional 35 isolates of the ST-2307 (fattening flock E), which originate from samples collected from the same barn as fattening flock F but from the previous fattening round (Table S3), to overall investigate samples from fattening flocks with differing ESBL/pAmpC prevalence (flock C: 73.1%, flock E: 26.4%, flock F: 5.7%). Strains were previously investigated concerning a possible horizontal transmission scenario (Daehre *et al*., 2017).

Phenotypic characterization

To determine the expression of biofilm-associated extracellular matrix components (cellulose, curli fimbriae), a macrocolony assay (Schaufler *et al*., 2016a) was applied to thirteen selected isolates from the whole genome approach (at least three isolates of each determined MLST type) as well as to 38 *E. coli* and four *E. fergusonii* isolates of the studies of Projahn *et al.* and Daehre *et al.* representing all the different detected isolate profiles (phylogroup + *bla* resistance gene) determined during the investigation of seven broiler production chains (Tables S1 and S2).

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1. Isolates used in the study for investigation of vertical transmissions, occurence of virulence associated genes and production onf curli.

Table S2. Isolates of the second set investigated by a macrocolony assay. Isolates comprise all detected genotypes (phylogroup+bla resistance).

Table S3. Numbers and occurrence of the 80 investigated virulence associated genes.

4. Unpublished data

The presented publications focus on the transmission from the parent flocks to the hatchery (Publication I, chain B) and on the vertical transmission to the fattening flock (Publication II, chains C and F), respectively. For these purpose, different methods were used to determine the phylogenetic relationship of the selected isolates. PFGE analyses were applied to isolates of phylogroup B1 harboring a $bla_{CTX\text{-}M\text{-}1}$ gene as well as *E. fergusonii* strains harboring a $bla_{TEM\text{-}52}$ gene isolated from the parent flock and the hatchery (outer eggshell surfaces) of chain B, respectively. WGS was done for selected *E. coli* strains of phylogroup F positive for either a CMY-2 or SHV-12 beta-lactamase, respectively, isolated from samples of chain C or chain F, respectively. However, further analyzes on the topic were done, which were not part of the respective publications.

4.1. S1-PFGE plasmid analyses

For the transmission investigations of ESBL-/pAmpC-producing Enterobacteriaceae from the parent flocks to the hatchery XbaI-PFGE analyses of the respective isolates were used as shown in Publication I.

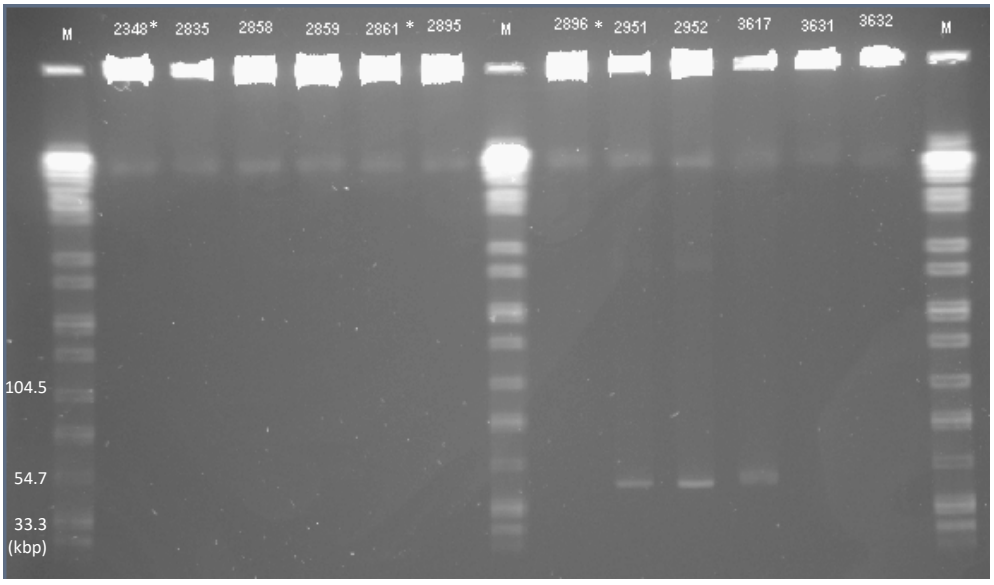

Figure 9:S1-PFGE of selected isolates from chain B. M: *Salmonella* Braenderup H9812; Isolates 2348 to 2896: *E. coli* phylogroup B1 + CTX-M-1 (eggshell surfaces); Isolates 2951 to 3617: *E. fergusonii* + TEM-52 (Eggshell surfaces, parent flock); Isolate 3631: *E. coli* phylogroup A/C + TEM-1 + CTX-M-1 (parent flock); Isolate 3632: *E. coli* phylogroup A + CTX-M-1 (parent flock). *Isolates of Lanes 11, 10, and 9 of Publication I, respectively.

In addition, for some of the investigated samples also a S1-PFGE was applied (Figure 9) to determine whether the $bla_{CTX\text{-}M\text{-}1}$ or $bla_{TEM\text{-}52}$ genes where chromosomally encoded or located on a resistance plasmid, respectively. Further *E. coli* isolates from the parent flock were included as comparison. The S1-PFGE was performed according to Rodriguez et al. (2009) (Rodriguez et al., 2009) with following

differing running parameters: 1s-25s, 17 h, 6 V/cm, 120 V. The S1-PFGE analyses of 12 selected isolates concerning the harboring of certain resistance plasmids revealed that *E. coli* strains expressing either a CTX-M-1 beta-lactamase or a CTX-M-1 beta-lactamase in combination with a TEM-1 beta-lactamase were negative concerning a plasmid band pattern in the agarose gel. Only for the three investigated *E. fergusonii* strains harboring a $bla_{\text{TEM-52}}$ gene a plasmid was detected. These plasmids had a size of more than 33.3 kbp but less than 54.7 kbp. This observation leads to the suggestion that neither the $bla_{\text{CTX-M-1}}$ nor the $bla_{\text{TEM-1}}$ genes are located on plasmids. In contrast, the TEM-52 beta-lactamase seemed to be plasmid-encoded; however, a distinct proof by WGS or DNA hybridization analyses is needed as the detected plasmid do not automatically has to harbor the respective resistance gene.

4.2. Transmission to the slaughterhouse

In Publication II, two scenarios for a vertical transmission from the parent flock (chain C) or the hatchery (chain F) to the fattening flocks were investigated using WGS data of selected isolates, however, the broiler production chain includes also the slaughterhouse. For the investigation of the transmission of ESBL-/pAmpC-producing *E. coli* from earlier stages to the processing step of the broiler chickens further selected isolates where included into the two WGS approaches investigated in Publication II, using similar analyses methods. Isolates from the slaughterhouse samples were also collected during the RESETII study and kindly provided by Philine von Tippelskirch (ILH, FUB). WGS analyses, VAG determination and Macrocolony assays were done as describes in Publication II.

4.2.1. Chain C - E. coli, phylogroup F, CMY-2 beta-lactamase

In the first transmission scenario of Publication II, the direct top-down transmission of ESBL-/pAmpC-producing *E. coli* from the parent flock C to the fattening flock C/43 was investigated. A large cluster was observed, including all the isolates from the fattening period but not the isolates collected from the investigated parent flocks. The extended investigation of the vertical transmission of *E. coli* isolates of phylogroup F expressing the CMY-2 beta-lactamase (chain C, Publication II) to the slaughterhouse (Figure 10) shows, that the three isolates from the caecum samples (8636, 8637, 8638) as well as the isolate collected from the plugging environment of the chickens (8634) cluster together with the strains from the fattening period as well as the truck sample (5146). The determined SNP differences of these samples to the respective nearest sample from the fattening period on the tree were calculated as 1 to 5 SNPs (0.2 to 1 SNPs Mbp^{-1}). The overall number of SNP differences in the fattening cluster was calculated as 1 to 32 SNPs (max. 6.4 Mbp^{-1}) which correlates which the previously calculated data in Publication II. Also the maximum SNP difference between the

fattening cluster and the parent flocks is comparable to those calculated earlier in the Publication II (117 SNPs, 23.6 SNPs Mbp^{-1}).

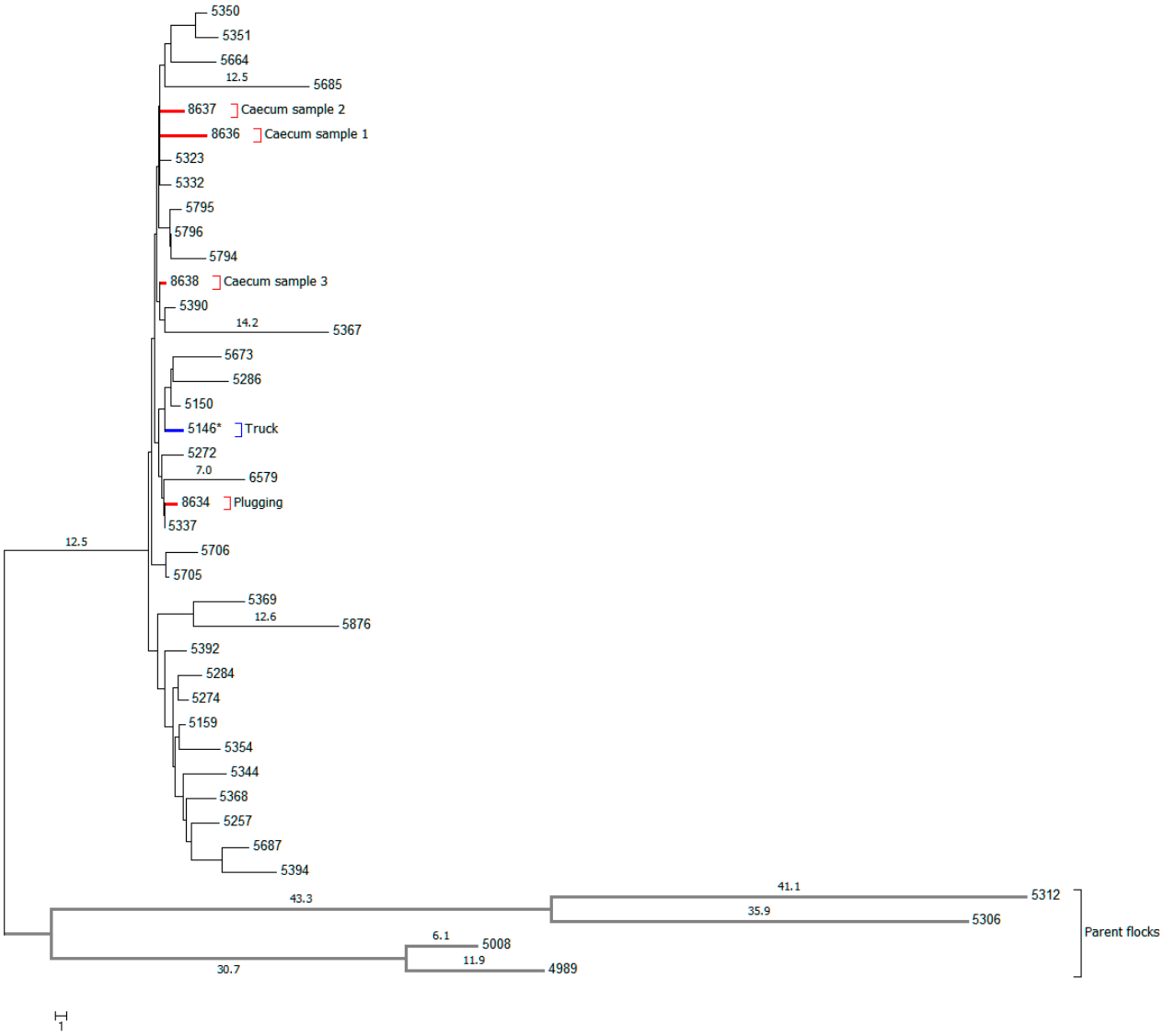

Figure 10: Investigation of the vertical transmission in chain C. A Neighbor joining tree was constructed of selected *E. coli* isolates of phylogroup F harboring a bla_{CMY-2} gene. The presented tree was calculated according to Publication II and includes all the previously analyzed isolates as well as four additional strains from the slaughterhouse sampling. Isolates from the parent flocks are displayed in dark grey, the truck sample is marked in blue and the additional isolates from the slaughterhouse samples are marked in red. Numbers on the branches indicate the SNP distance. *Isolate derived from a swab sample from the inside of the chicken truck from the hatchery to the farm

VAG analyses of the four slaughterhouse samples revealed the existence of 37 of the 89 investigated genes in the respective whole genome contigs. These 37 VAGs are associated with a potential ExPEC phenotype and are classified into the following categories adhesion (n=16), invasion (n=9), iron

uptake (n=2), protection (n=6), toxins (n=3) and miscellaneous (n=1), respectively. The detected genes and the occurrence of respective VAGs is in concordance with the results previously determined for the fattening cluster isolates (Publication II).

4.2.2. Chain F - E. coli, phylogroup F, SHV-12 beta-lactamase

The second transmission scenario investigated in Publication II, deals with the vertical transmission from the hatchery to the fattening flock. Therefore, selected *E. coli* isolates of phylogroup F positive for an SHV-12 beta-lactamase were further analyzed again using a WGS approach. The calculation of a Neighbor joining tree based on the numbers of SNP differences revealed two different clusters of which one (cluster II) includes the hatchery sample (7553) as well as isolates from the fattening farm and additional isolates collected at the same day from other barns of the same farm (7873 - F/71, 7892 - F/75), indicating on the one hand a transfer of resistant bacteria from the hatchery to the fattening farm and on the other hand shows up also possible contamination events on the farm level as the respective chicken derived from other parent flock (Publication II). From the chain F investigations additional data for ten isolates determined from the slaughterhouse by the ILH were available.

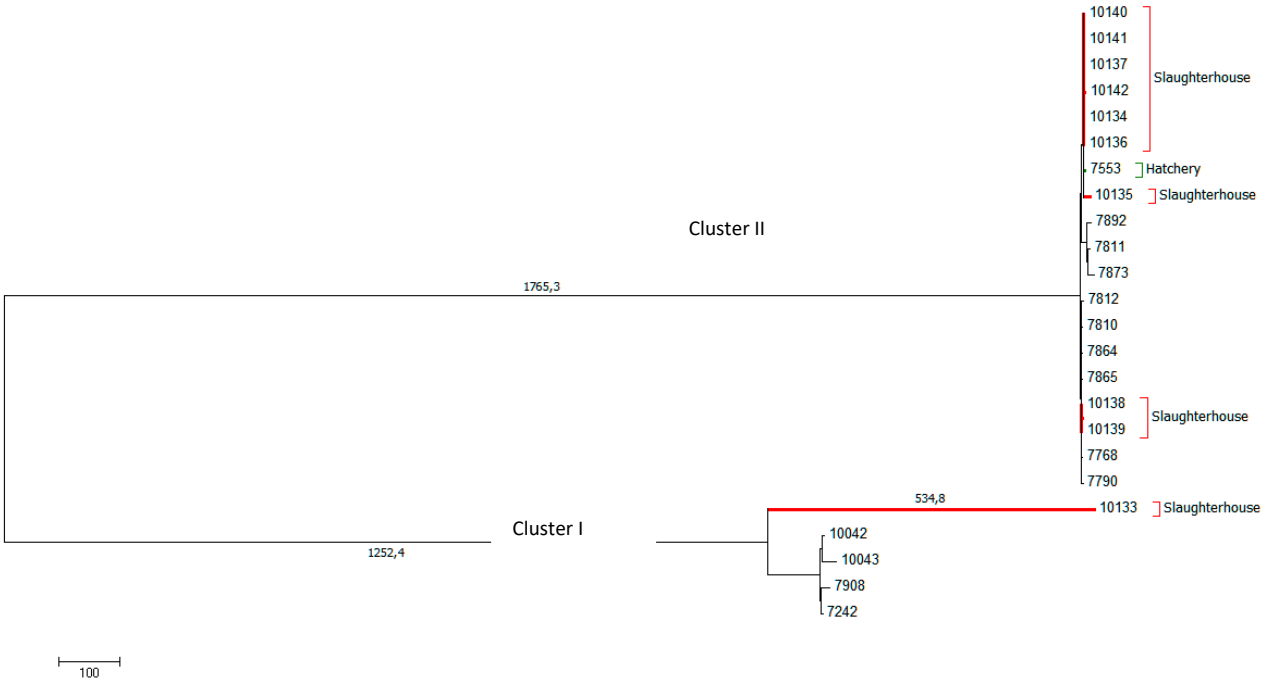

Figure 11: Investigation of the vertical transmission of chain F. A Neighbor joining tree was constructed of selected *E. coli* isolates of phylogroup F harboring a blaSHV-12 gene. The presented tree was calculated according to Publication II and includes all the previously analyzed isolates as well as 10 additional strains from the slaughterhouse sampling. The environmental isolate from the hatchery is highlighted in green and the additional isolates from the slaughterhouse samples are marked in red. Numbers on the branches indicate the SNP distance.

The recalculation of the phylogenetic tree containing also ten additional isolates from samples of the slaughterhouse revealed the same two clusters observed before (Figure 11). All slaughterhouse isolates except for one, are grouped together with the hatchery isolate in cluster II (Figure 12). These isolates were derived from caecum samples and skin samples of fattening flock F as well as from the scalding water which was sampled before the processing of flock F. The slaughterhouse isolate 10133 is found to be an outlier from cluster I as the calculated SNP differences from this isolate to the four isolates of the cluster I ranges between 624 to 641 SNPs (124.8 to 128.2 SNPs Mbp^{-1}). The calculated numbers of SNP differences in the enlarged cluster II ranges between 0 and 36 SNPs (max. 7.2 SNPs Mbp^{-1}) and in the cluster I 8 to 41 SNPs (1.6 to 8.2 SNPs Mbp^{-1}).

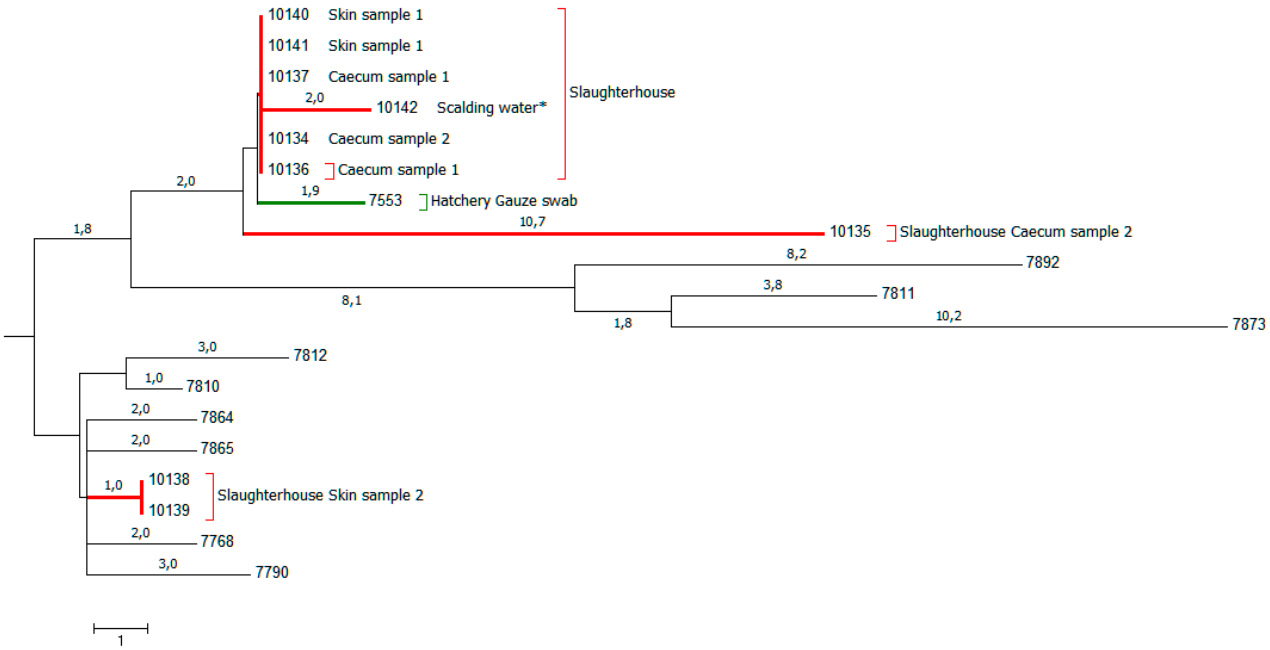

Figure 12: Close up of the larger subtree of Figure 13 (Cluster II). The environmental isolate from the hatchery is highlighted in green and the additional isolates from the slaughterhouse samples are marked in red. Numbers on the branches indicate the SNP distance. *The scalding water sample was taken prior to the processing of fattening flock F.

The SNP differences differ from those calculated previously in Publication II. This is due to the including of new isolates into the analyses and the calculation algorithm on which the tool parsnp of the Harvest suite is based on (Treangen et al., 2014). Parsnp uses the sequences segments which are found in all isolates, the so-called core-alignment, for the phylogenetic analyses. With increasing numbers of isolates the amount of similar nucleotide sequences which are found in all these isolates is decreasing and therefore also the number of detectable SNP differences can decrease. This phenomenon occurs especially when a large amount of samples is added to an originally small group

of investigated specimen. In case of the analyses of the phylogroup F, SHV-12 beta-lactamase isolates of chain F, ten further isolates were added to the isolate set (n=14) used in Publication II. In contrast, for the recalculation of the transmission scenario of chain C only four slaughterhouse samples were added to the original set of 37 isolates. Therefore, the changes in the calculation of the number of SNP differences differ more in the chain F analyses than in the chain C investigation.

The ten slaughterhouse samples were again also investigated concerning the occurrence of certain VAGs as previously described in Publication II (Table 12). For the isolate 10142, determined from the scalding water, also a Macrocolony assay was applied to examine the ability to produce curli fibers and cellulose (Figure 13).

Table 12: Mean distribution of VAGs in the isolates from the two clusters of the chain F phylogenetic analyses.

MLST	ST-117 (cluster 1, fattening periode)*	ST-117 (cluster 1, 10133 - slaughterhouse)	ST-117 (cluster 2, fattening periode)*	ST-117 (cluster 2, slaughterhouse)
Total no. of ExVAGs	21	23	24,3	22,8
Adhesion	4	5	5	5
Invasion	0	0	0	0
Iron uptake	8	9	11	9,8
Protection	3	4	4	4
Toxins	3	3	3,3	2
Miscellaneous	2	2	2	2

* Data from Publication II

Analyses of the VAG distribution in the ten slaughterhouse isolates from the chain F investigation showed slight variances in the occurrence of the two genes *sitA* (iron uptake) and *vat* (toxins) in these isolates. In addition, compared to the strains investigated from the fattening period in Publication II, the slaughterhouse strains which were clustered to the respective phylogenetic cluster I and II (unpublished data), showed also minor variations in the occurrence of certain VAGs, however, the overall numbers are comparable.

The performed macrocolony assay of the slaughterhouse strain 10142 showed a characteristic colony phenotype (Figure 13). Based on the color and the shape of the colony the production of curli fibers as well as the polysaccharide cellulose can be stated for this isolate. This was previously shown also for other selected isolates from the chain F investigation in Publication II.

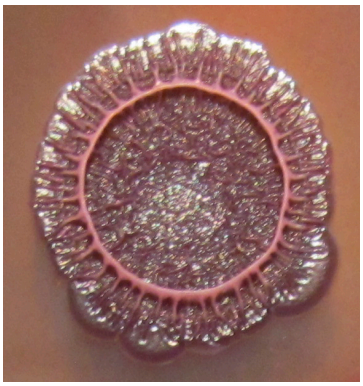

Figure 13: macrocolony of the isolate 10142

4.3. Intra-sample strain variability

For the chain C transmission investigations (*E. coli*, phylogroup F, CMY-2 beta-lactamase) isolates of various sample types, determined from the different stages of the broiler production chain, were selected (Publication II). One isolate per selected sample was chosen for WGS analyses except for the five cloacal swabs (KTs) from the fattening period of flock C/43, where up to three isolates of each cloacal swab were included into the phylogenetic calculations. These isolates were used for further investigation on the diversity of related isolates from one respective broiler chicken. Therefore, the numbers of SNP differences between isolates, derived from one cloacal swab of one chicken, were calculated according to Publication II using parsnp and MEGA7, and compared to the overall SNP differences between the isolates from all five cloacal swabs (Figure 14).

	KT01			KT02			KT03			KT04		KT05		
Isolate	5350	5351	5354	5367	5368	5369	5390	5392	5394	5705	5706	5794	5795	5796
5350		189	257											
5351	39		198											
5354	56	39												
5367	53	34	47		104	284								
5368	56	37	18	45		280								
5369	58	39	28	47	14									
5390	38	21	24	25	28	30		103	124					
5392	47	28	13	36	11	19	19		136					
5394	48	29	28	33	28	38	20	21						
5705	35	16	27	22	25	29	9	16	15		467			
5706	44	25	30	31	34	38	12	25	22	9				
5794	49	28	19	36	21	23	19	12	27	18	23		87	138
5795	46	27	36	31	22	24	20	25	24	15	24	23		95
5796	50	29	28	37	14	16	24	19	32	19	28	17	12	

Figure 14: Numbers of calculated SNP differences between all isolates from five cloacal swabs and between the isolates from one respective cloacal swab (KT). The intra-sample calculations are highlighted in different shades.

The numbers of calculated SNP differences between the isolates of the respective cloacal swab samples vary between 87 and 467 SNPs (17.4 to 93.4 SNPs Mbp^{-1}), the mean value is about 189.4 SNPs (37.9 SNPs Mbp^{-1}). The SNP differences between all isolates from the selected five cloacal swabs from the fattening period were determined as 9 to 58 SNPs (1.8 to 11.6 SNPs Mbp^{-1}) with an overall mean value of about 28.3 SNPs (5.66 SNPs Mbp^{-1}). The numbers of calculated SNP differences between the isolates of one respective swab sample are much higher than the SNP differences between all isolates from the five cloacal swabs. This is due to the parsnp algorithm for the determination of the core-alignment of the whole genome contigs. However, the values are good indications to estimate the clonality of the investigated *E. coli* strains.

5. Discussion

Within the FUB sub-project of the RESETII consortium various analyses concerning ESBL-/pAmpC-producing Enterobacteriaceae in the broiler production chain where done with emphasis on the identification of critical hazard points and the evaluation of possible intervention strategies against these resistant bacteria. The present thesis focuses on the molecular epidemiology of ESBL-/pAmpC-producing Enterobacteriaceae in the broiler production chain and possible vertical top-down transmission routes. However, further analyses on resistant isolates where done to estimate the phylogenetic relationship between certain isolates and to identify possible bacterial factors which might contribute to a vertical transmission and a preferential persistence in the environment of the broiler production chain.

5.1. *Dissemination of* bla *resistance genes*

ESBL-/pAmpC-producing Enterobacteriaceae are wide spread found all over the world. They could be isolated from clinical specimen as well as from healthy hosts including humans and various wild, companion and farm animals. However, it was found that certain ESBL-/pAmpC-resistance genes where predominantly found in certain geographical regions or a special host (Ewers et al., 2012). In Europe $bla_{CTX\text{-}M\text{-}14}$ and $bla_{CTX\text{-}M\text{-}15}$ as well as $bla_{SHV\text{-}12}$ positive *E. coli* strains were mainly isolated from humans whereas $bla_{CTX\text{-}M\text{-}1}$ and to a lesser content $bla_{CTX\text{-}M\text{-}15}$-harboring strains where found in companion animals. In contrast, in poultry especially broiler chicken the predominant detected *bla* genes encode for the CTX-M-1 beta-lactamase, the TEM-52 beta-lactamase and the CMY-2 beta-lactamase (Ewers et al., 2012). Besides the study presented here, many studies focusing on the prevalence of ESBL-/pAmpC-producing Enterobacteriaceae in broiler chickens and retail meat from poultry were published during the last years.

In this study, *E. coli* strains harboring the $bla_{CMY\text{-}2}$ gene were found to be the most prominent resistant strains. They were detected in 63% of the positive samples. As $bla_{CMY\text{-}2}$ is frequently found in isolates from broiler chicken, this finding was not surprising (Blanc et al., 2006; Dierikx et al., 2010). Smet et al., for example, detected the CMY-2 beta-lactamase in 49% of their samples (Smet et al., 2008), however, the overall high number of CMY-2 positive *E. coli* isolates in this study is biased, as these strains were associated with in general high prevalence of positive samples (up to 100%) in the respective broiler flocks. Nevertheless, also in samples from chicken retail meat pAmpC-producing *E. coli* are frequently detected. In this study, CMY-2 positive *E. coli* strains were found in samples from five out of seven samplings in the slaughterhouse which is in full concordance with findings from other recent studies (Borjesson et al., 2013; Pehlivanlar Onen et al., 2015). The second most detected *E. coli* population harbored $bla_{SHV\text{-}12}$ genes. These resistant strains were isolated from 19%

of the positive samples. In addition, in 11% of the positive samples CTX-M-1-producing *E. coli* were found. Both beta-lactamases are also widely spread in poultry isolates and found in retail meat from chickens (Bortolaia et al., 2010; Dierikx et al., 2010; Kola et al., 2012; Smet et al., 2008). *E. coli* isolates encoding a TEM-52, CTX-M-15, SHV-2, SHV-2a or SHV-12 beta-lactamase, respectively, were rarely detected. Interestingly, strains harboring a $bla_{\text{CTX-M-15}}$ gene were only found in one parent flock and two samplings from the slaughterhouse but not in samples from the hatchery or the fattening period. Overall, the highest diversity of *bla* resistance genes was detected in samples from the slaughterhouses. Up to five different genes encoding for beta-lactamases of the types TEM, SHV, CTX-M and CMY were found to be present in the different samples, indicating high frequencies of cross-contamination and transmission events during the processing of the broiler chickens. This correlates with a study, where also various types of ESBL-/pAmpC-genes were detected in chicken meat from supermarkets in Germany (Kola et al., 2012). In addition to the ESBL-/pAmpC-producing *E. coli*, in three chain investigations *E. fergusonii* strains harboring $bla_{\text{TEM-52}}$ genes and *K. pneumoniae* harboring $bla_{\text{SHV-2}}$ genes, respectively, were detected. These isolates were derived from samples of the investigation of chains B and F. Multi-drug resistant *E. fergusonii* were already detected in broiler chickens (Forgetta et al., 2012; Oh et al., 2012; Simmons et al., 2016). Nevertheless, almost all studies on ESBL-/pAmpC-producers in the broiler production focus on *E. coli* due to their high prevalence numbers. As also *E. fergusonii* can cause highly severe infections in humans and animals (Gaastra et al., 2014; Lagace-Wiens et al., 2010; Lai et al., 2011; Savini et al., 2008), future investigations should not be limited to a single pathogen. In addition, detailed investigations of further ESBL-/pAmpC-producing pathogens might contribute to new insights into transmission dynamics and colonization factors especially in the broiler production. *K. pneumoniae* is usually associated with foodborne diseases and clinical outbreaks (Cella et al., 2017; Davis and Price, 2016). Already in 1983, it was shown that resistance to cefotaxime could be transferred from *K. pneumoniae* strains to *E. coli* K12 (Knothe et al., 1983). Therefore, the study presented here, raises additional questions concerning the interplay of ESBL-producing *K. pneumoniae* and *E. coli* in broiler chickens. Furthermore, especially at the hatchery and at the slaughterhouse level AmpC-producing *C. freundii* and *P. mirabilis* were detected, although to low numbers. There is only limited information on the impact of these resistant bacteria from broiler chickens or other animals on humans and the public health (Barbour et al., 2012; van Hoek et al., 2015; Liao et al., 2015; Wong et al., 2013). It was already shown that certain clonal lineages are spreading, however, further investigations need to be done (D'Andrea et al., 2011; Literacka et al., 2004). Investigations of selected isolates from the hatchery also revealed, that in these cases the respective *bla* resistance genes seemed not to be located on plasmids (unpublished data). It was previously shown, that especially CTX-M beta-lactamase genes are also found to be located in the chromosome (Falgenhauer et al., 2016; Guenther et al., 2017; Rodriguez et

al., 2014). These chromosomal resistances genes seemed to be predominately associates with certain *E. coli* lineages like ST38 and ST131 (Guenther et al., 2017; Rodriguez et al., 2014). However, further investigations on the respective insertion events as well as the genetic stability and the transmission potential of these chromosomally encoded *bla* resistance genes are needed to estimate their impact on the transmission of resistance properties.

5.2. Phylogeny of E. coli strains

Due to the increasing amount of detected ESBL-/pAmpC-producing *E. coli* in samples from the broiler production (broiler chicken, retail meat) since 2003, studies were conducted to also investigate the chromosomal background of the resistant *E. coli* strains. Unfortunately, in the earlier studies different molecular methods were used to investigate the genetic lineages of the strains, so that is not easy to compare these data with each other (Bortolaia et al., 2010; Costa et al., 2009; Randall et al., 2011). However, it was found out, that the detected ESBL-/pAmpC-producing *E. coli* were highly diverse and that it is likely, that the *bla* resistance genes can easily spread via horizontal gene transfer (Canton et al., 2008; Dierikx et al., 2010). In this study, the determination of the phylogroup according to Clermont et al. was used to have a rough estimation of the overall diversity of the resistant *E. coli* strains (Clermont et al., 2013). Strains assigned to different phylogroups are determined as not related whereas strains of the same phylogroup are more likely to be phylogenetically related. It turned out, that during this study *E. coli* isolates of all phylogroups (A, A/C, B1, B2, E, E/D, F) could be detected, indicating that various types of ESBL-/pAmpC-producing *E. coli* occur in the broiler production and that respective transmission pathways of the resistant strains are highly complex. However, in each of the fattening flocks, colonized with ESBL-/pAmpC-producing *E. coli*, a predominant resistant *E. coli* lineage was identified. At the end of the fattening period of flock A, isolates with the molecular characteristics phylogroup E/D, *bla* resistance genes CMY-2 and TEM-1 where detected in 41 of the 52 investigated samples (78.8%). Broiler chickens of fattening flock B were mainly colonized with ESBL-positive *K. pneumoniae* strains. However, at the end of the fattening period *E. coli* strains of phylogroup E/D harboring a bla_{CMY-2} gene were still found in 18% of the collected samples. During the investigations of fattening flock C, AmpC-producing *E. coli* assigned to phylogroup F were frequently isolated (58.5% of the samples) and in flock E the predominant ESBL-producer was identified as phylogroup B1 *E. coli* harboring a bla_{SHV-12} gene in 17.9% of the samples collected from the end of the fattening period. Fattening flocks D and F were only colonized to a less amount and at the end of the fattening period of flock G none of the investigated cloacal swabs were positive for ESBL-/pAmpC-producers. These data show on the one hand, that neither a certain phylogroup nor a distinct *bla* resistance gene were associated with a high or low occurrence of ESBL-/pAmpC-producing *E. coli* in the investigated broiler fattening flocks of this

study, indicating that there might be additional factors, that support the colonization of broiler chickens with a respective ESBL-/pAmpC-positive strain. In addition, *E. coli* isolates of phylogroup B2, which are mainly associated with higher pathogenicity in humans, were rarely detected in samples of only two broiler chains (chain C and D). This correlates with findings from other studies on APEC and commensal strains in chickens (Ewers et al., 2007; Logue et al., 2017; Rodriguez-Siek et al., 2005), indicating that a zoonotic transmission of these strains might be possible, however, with a rather low frequency. On the other hand, due to the high diversity of detected ESBL-/pAmpC-producing strains, it was a difficult task to specify possible vertical transmission pathways of the resistant bacteria along the broiler production chain. However, three scenarios, based on the occurrence of isolates showing identical phylogroups and *bla* resistance genes, could be identified. These were further investigated using WGS data of selected isolates from the respective chain investigations.

Recently published studies on (clinical) ESBL-/pAmpC-producing *E. coli* in outbreak and transmission investigations also applied WGS on the respective isolates to determine the phylogenetic relationship of the isolated strains (Mo et al., 2016; Roderova et al., 2016; Ronco et al., 2017; Schaufler et al., 2016b). Respective conclusions are usually based on the determination of the number of SNP differences between the investigated isolates. *E. coli* is a highly diverse bacterial species, however, there is also a certain clonal evolution stated for this pathogen (Bletz et al., 2013a; Chaudhuri and Henderson, 2012; Nicolas-Chanoine et al., 2014; Whittam et al., 1998; Wirth et al., 2006; Zhang et al., 2006). In our study, the number of SNP differences in a determined phylogenetic cluster varied between 0 and 41 SNPs (Publication II, unpublished data). Other studies found partially lower numbers of SNP differences even between isolates from different species (Mo et al., 2016; Schaufler et al., 2016b). However, when determining the number of SNP differences between certain *E. coli* isolates, the respective reference sequences have to be taken into account as these have also an impact on the specific core-genome calculated for the study specific strain set. The more variable strains are that where compared to each other, the more shrinks the overall core-genome resulting also in a decreased number of detectable SNP differences. Mo at al. included sequence data from highly variable strains from the NCBI collection into their analyses and found a maximum of 14 SNP differences in their main phylogenetic cluster. Even strains of different MLVA types varied in not more than 63 SNPs. Schaufler et al. determined max. 75 SNPs in difference between *E. coli* isolates of different hosts (Schaufler et al., 2016b). In our study, no reference strains were included into the phylogenetic analyses to not further reduce the sensitivity of the SNP detection. Furthermore, we could show that *E. coli* strains isolated from the same cloacal swab of one respective chicken can vary in up to 467 SNPs (unpublished data) underlining the indeed high variability of *E. coli* isolates. However, the numbers of SNP differences between isolates are a powerful tool for phylogenetic

analyses when applying a sound set of (reference) strains into the calculation and keeping in mind certain variations between the respective studies.

5.3. Vertical transmission scenarios

During the RESETII study seven parent broiler flocks, their corresponding eggs and hatchlings were traced until the slaughterhouse and were investigated concerning ESBL-/pAmpC-producing Enterobacteriaceae to determine possible vertical top-down transmission routes of these resistant bacteria along the whole broiler production chain. These investigations demonstrate that there was not one prominent transmission route which occurred repeatedly and constantly from one production stage to the subsequently following stage. Already the high variability of detected ESBL-/pAmpC-producing Enterobacteriaceae lead to the assumption, that the transmission, spread and also persistence of these bacteria is much more complex.

With increasing detection rates of beta-lactamase positive Enterobacteriaceae, it turned out, that, instead of only detecting the type of ESBL/AmpC beta-lactamases, further information on the diversity of the bacteria are needed to understand the origin and spread of these resistant Enterobacteriaceae. Since it was found out that also parent flocks are already colonized with commensal ESBL-/AmpC-producing Enterobacteriaceae, further studies were carried out to investigate the distribution of these resistant bacteria in the broiler production pyramid and to examine whether the ESBL-producers found in the parent or grandparent flocks are transmitted to their offspring (Huijbers et al., 2016a; Mo et al., 2014; Mo et al., 2016; Poulsen et al., 2017). This was of great interest especially to countries which are importing the respective parent flock chicks from farms in other countries, assuming that they also import the "resistance problem" together with the chicks. Dierikx et al. investigated samples from grandparent chicks imported to the Netherlands, from parent chicks and the respective broiler chickens. They found high prevalence of ESBL-/AmpC-producing *E. coli* already in the grand parent chickens and positive environmental samples from the chicken houses after cleaning and disinfection (Dierikx et al., 2013b). This study pointed out, that there is a need of respective intervention strategies to prevent the contamination with the resistant bacteria. However, in this study the *bla* resistance genes were investigated, only, and further information on certain *E. coli* strains or lineages spreading are missing. Therefore, we used in our study in addition to the detection of the *bla* resistance genes also PFGE and WGS analyses, respectively, to determine the phylogenetic relationship of certain *E. coli* isolates as WGS data provide high resolution insights into the evolution and diversity of bacterial strains.

It was previously shown, that colibacillosis-causing *E. coli* which were found in young chickens were also detected in the corresponding parent flocks (Giovanardi et al., 2005; Petersen et al., 2006).

However, further studies were needed to examine whether this might be possible also for non-APEC, ExPEC(-like) or commensal resistant *E. coli* strains. In our study, we therefore investigated ESBL-/pAmpC-positive parent flocks and their corresponding hatching eggs, the recently hatched chicks as well as a variety of environmental samples and swabs. Using a modified shell-rinse method and PFGE analyses, we could now show that ESBL-producing *E. coli* as well as ESBL-producing *E. fergusonii* can be introduced from the parent flock to the hatchery via the contaminated outer surface of the hatching eggs and that the resistant bacteria can also survive the disinfection of the eggs with formaldehyde (Publication I), however, to a low amount. Further studies on ESBL-producers on chicken eggs, which were published after the start of the RESETII project, found also a low prevalence of positive samples concerning ESBL-producing Enterobacteriaceae (Mezhoud et al., 2016; Mitchell et al., 2015). Another working task was also to investigate whether the resistant Enterobacteriaceae might be also transmitted via the inside of the hatching egg to the respective hatchlings or if they can cross the egg shell barrier thru the egg shell pores. Therefore, we also investigated the egg content of hatching eggs from ESBL-/pAmpC-positive parent flocks, but in this study we found no indication for this transmission pathway. It is more likely that the recently hatched chicks get colonized via the airborne route or direct contact with the contaminated hatchery environment during sorting, chicks´ inspection and vaccination as shown in Publication I and II. The study by Petersen et al. already pointed out that the hatchery might play an important role as respective *E. coli* types from the parent chickens were also found in the hatchery fluff (dust, crushed egg shells) (Petersen et al., 2006). In our study, we detected positive environmental samples in the hatchery (hatchlings dust and swabs from the hatchery equipment) and could show, that there was a direct phylogenetic relationship between the isolates of the hatchlings dust and the broiler chickens investigated in the production chain F (Publication II). This supports the statement of Petersen et al. and also indicates that even a low amount of these resistant bacteria in the hatchery can lead to a constant dissemination of the respective strains.

In the study of Nilsson et al. they used MLST and MLVA data to follow positive parent flocks longitudinally through the broiler production. They stated that there is one clonal lineage which is transmitted through the production pyramid. However, their detected clones varied in up to two of the investigated MLVA loci and there were missing links in the vertical transmission investigation as the respective MLVA types were not detected directly after the import of the grandparent chickens and the samples from the parent hatchery were negative concerning pAmpC-producing *E. coli* (Nilsson et al., 2014). In Publication II it is shown that even if highly related ESBL-producing *E. coli* strains are found in the parent flock and the respective fattening flock this must not have been a direct vertical transmission. The detection of strains of two different MLVA types in the hatchery by Nilsson et al. supports our hypothesis of a stepwise introduction of resistant bacteria into the

hatchery and the constant evolution of certain strains. Until now, there are no sufficient data on the colonization dynamics of broiler chickens with commensal or ExPEC(-like) ESBL-/pAmpC-producing *E. coli* available. Current chicken infection models concentrated on APEC strains and used infection doses of at minimum 10^6 CFU (Antao et al., 2009; Oosterik et al., 2016; Stromberg et al., 2017). Although these studies investigate APEC infections in the lung, a respective colonization dose for the gut was not determined. Our data from the hatchery investigation assume that the colonization dose for the resistant bacteria is far below 10^6 CFU (Publication I+II). Therefore, investigations on the colonization dynamics and the minimum colonization doses of ESBL-/pAmpC-producing commensal or ExPEC(-like) *E. coli* need to be carried out to estimate the impact of the low amount of detected resistant bacteria in the hatchery on the transmission dynamics of respective strains.

In our study, none of the broiler fattening flocks where treated with a beta-lactam antibiotic. However, in six out of seven investigated fattening flocks we detected ESBL-/pAmpC-positive broiler chickens indicating that the carriage of these resistant bacteria was not linked to an antibiotic treatment but that the chicken can get colonized during their life just by simply up taking these ESBL-/AmpC-producing Enterobacteriaceae from the environment (Daehre et al., 2017). During our investigations we found a large amount of different ESBL-/pAmpC-producing Enterobacteriaceae, however, each of our positive fattening flocks was mainly colonized by a certain resistant strain. This indicates that some of the ESBL-/pAmpC-producing Enterobacteriaceae, especially *E. coli* strains, seemed to dominate and might had certain advantages in colonization and persistence processes. Recent studies underline our findings that there is on the one hand a highly diverse population of ESBL-/pAmpC-producing *E. coli*, but that on the other hand there are certain lineages that seemed to preferentially circulate and be transmitted even without the usage of antibiotics (Huijbers et al., 2016a; Mo et al., 2014; Mo et al., 2016; Poulsen et al., 2017). Therefore, we hypothesize that there might be certain bacterial characteristics which support on the one hand the survival and transmission in the broiler production chain but also the colonization of the chicken gut.

The occurrence of ESBL-/pAmpC-producing Enterobacteriaceae in broiler chicken has also an impact on the production and processing of chicken retail meat. It was already shown in the late 1970s, that fecal contamination of broiler carcasses during the processing is a challenging problem (Notermans et al., 1977; Thomas and McMeekin, 1980). Cross-contamination between carcasses is occurring most likely via aerosols, process water and contact between carcasses and the equipment. Therefore, the scalding, defeathering and evisceration processes were discussed as sources for these cross-contaminations (Allen et al., 2003; Ho et al., 2004; Jimenez et al., 2003; Mead, 1993; Mead et al., 1993). Although, studies described an decrease of the amount of resistant Enterobacteriaceae detected on the broiler carcasses during processing, these bacteria are still found on the processed

and packaged retail meat and are therefore considered to be of a potential human health risk (Doi et al., 2010; Dolejska et al., 2011; Egea et al., 2012; Evers et al., 2017; Lyhs et al., 2012; Mitchell et al., 2015; Pacholewicz et al., 2015c; Rouger et al., 2017; Zweifel et al., 2015). In the RESETII study, we further investigated samples from the seven fattening flock also on slaughterhouse level (von Tippelskirch et al., 2018). We applied WGS on selected isolates from ESBL-/pAmpC-positive caecum samples and from the slaughterhouse environment of chains C and F to investigate the introduction of the resistant *E. coli* strains from the fattening farm into the slaughterhouse (unpublished data). The analyses of chain C isolates revealed that the slaughterhouse strains cluster together with the ones determined from the fattening period. This indicates clearly the introduction of the resistant strains into the processing steps. As a highly related strain was found on the plugging equipment after the defeathering of flock C, our investigation also demonstrate that the defeathering process is indeed a source for cross-contamination during processing. This is especially problematic when dealing with highly resistant Enterobacteriaceae. It was already discussed, that certain surfaces of the processing equipment has a certain susceptibility to bacterial attachment and support the formation of biofilms (Arnold and Silvers, 2000; Ho et al., 2004). In Publication II, we also showed that selected ESBL-/pAmpC-producing *E. coli* of chain C have the ability to produce curli fibers and cellulose which support a possible attachment on the processing equipment. WGS analyses of the slaughterhouse isolates from flock F underline the fact, that ESBL-/pAmpC-producing *E. coli* are frequently transmitted to the slaughterhouse facilities via contaminated fattening flocks. In addition, we could show that also the scalding water acts as contamination source of broiler carcasses. The SNP cluster II comprises isolates of the fattening flock F/74 as well as of fattening flocks F/71 and F/75. These flocks were fattened at the same farm during the same fattening period and flock F/71 was likely processed before F/74 at the slaughterhouse assuming, that the contamination of the scalding water occurred during this time and the respective strains were therefore already found in the scalding water before the processing of flock F/74. These findings underline the assumptions that the scalding water leads as contamination source during processing of broiler chickens. These data indicate that the processing of ESBL-/pAmpC-contaminated broiler chickens in a slaughterhouse very likely lead to the contamination of previously not colonized broiler chickens with these resistant bacteria. Therefore, interventions at the slaughterhouse level should be applied but also strategies are needed to prevent the broiler chickens from the colonization at the farm level.

Taken together, the data determined during this thesis work show, that there are vertical transmissions of ESBL-/pAmpC-producing Enterobacteriaceae occurring in the broiler production chain. However, the transmission between the parent flock chickens and their respective offspring are not direct. During this study we showed, that the resistant bacteria were introduced from the parent flocks into the hatchery via the outer surface of contaminated hatching eggs. This might be

possible also for other parent flocks assuming that there is a constant introduction happening. Once introduced into the hatchery, the ESBL-/pAmpC-producing Enterobacteriaceae can persist even to a low detectable amount. By a certain chance, recently hatched chicks can get infected and colonized with these resistant strains. In contrast, ESBL-/pAmpC-producing Enterobacteriaceae occurring in broiler fattening chicks are constantly and direct introduced into the slaughterhouse and the contaminates broiler carcasses can lead as sources for cross-contamination of the processing environment and equipment as well as other broiler carcasses.

5.4. Distribution of VAGs and biofilm production

Commensal *E. coli* and, ExPEC and APEC strains, respectively, differ in their genetic composition concerning genes that refer to host invasion, colonization, toxins and other virulence associated functions like biofilm production (Hall-Stoodley et al., 2004; Pitout, 2012; Smith et al., 2007; Wigley, 2015). Usually investigated factors belonged to different types of pili or fimbriae, secretion systems or toxins as well as their association with mobile elements which support horizontal gene transfer (Ewers et al., 2007; Holden and Gally, 2004; Nowrouzian et al., 2006). However, it turned out, that there is no single distinct factor that leads to a certain type of disease in humans or poultry. A current classification of ExPEC strains is based on the occurrence of two or more of the following virulence markers: *papA* (P fimbriae structural subunit) and/or *papC* (P fimbriae assembly), *sfa/foc* (S and F1C fimbriae subunits), *afa/dra* (Dr-antigen-binding adhesins), *kpsMT* II (group 2 capsular polysaccharide units), and *iutA* (aerobactin receptor) (Johnson et al., 2003). A diagnostic method for the detection of APEC strains seemed to be also challenging. Various studies described different strategies and gene sets for the determination of theses *E. coli* strains. Genes described as potential markers for the detection of APEC strains were *iutA* (aerobactin receptor), *hlyF* (hemolysin), *iss* (increased serum survival), *iroN* (iron outer membrane receptor), *ompT* (outermembrane protein), *tsh* (temperature-sensitive hemagglutinin), *iucC/D* (aerobactin synthesis), *cva-cvi* (colicin V synthesis), *astA* (heat stable cytotoxin associated with enteroaggregative *E. coli*), *vat* (vacuolating autotransporter toxin), *irp2* (iron repressible protein) and *papC* (fimbrial major pilin protein) (Ewers et al., 2005; Johnson et al., 2008; Skyberg et al., 2003). However, recent studies found that especially *iss, tsh, iucD, astA, vat* and *irp2* are increasingly detected also in commensal *E. coli* strains from healthy broiler and humans (Carli et al., 2015; van Hoek et al., 2016; Kemmett et al., 2013; Paixao et al., 2016; Silveira et al., 2016). This led to the overall assumption that certain virulence factors are contributing to a better survival in the host and are, therefore, considered as survival factors (Frommel et al., 2013; Smith et al., 2007; Vila et al., 2016; Wigley, 2015). However, pathogenic *E. coli* strains usually seemed to harbor higher numbers of VAGs or survival factors. In our study, we analyzed isolates from three broiler chain investigations (C, E, F) also concerning an expanded set of VAGs (Publication II) and

found, that numbers of VAGs differed in up to 18 genes between the chain isolates. All isolates showed overall high numbers of VAGs but only isolates with highest numbers of VAGs could be also classified as ExPEC according to Johnson et al., 2003. This contributes the findings, mentioned above, that it is more likely that certain combinations of virulence or survival contributing factors refer to enhanced viability, persistence and colonization attributes especially in broiler chickens. Most of the genes/gene products and/or operons that might have an impact on the bacterial fitness or virulence are not jet investigated concerning their impact on the colonization of humans or broiler. It might be also possible that a certain factor that is of minor concern in human colonization plays an important role in the colonization of boiler chickens. Therefore, further investigations on certain virulence and survival factors are needed especially in combination with the ESBL-/AmpC-resistance phenotype of the *E. coli* strains. Another important topic is the possible impact of certain virulence factors on the cross-contamination events during the slaughterhouse processing of the broiler chicken. It can be hypothesized that especially certain adhesion factors might contribute an increased attachment of resistant *E. coli* isolates on the broiler skin on the one hand or on the equipment surfaces in the slaughterhouse during processing on the other hand. Therefore, further investigations on certain VAGs and their functions are needed.

Besides the occurrence of VAGs for the colonization of and the survival in a respective host, also the ability to form biofilms plays an important factor in the survival and the pathogenicity of *E. coli* strains. Biofilms protect the bacterial cells from certain environmental factors as well as the host defense system and antibiotic treatment (Flemming and Wingender, 2010; Hall-Stoodley et al., 2004; McCrate et al., 2013). Major components of *E. coli* biofilms are curli fibers and the polysaccharide cellulose (Barnhart and Chapman, 2006; Serra et al., 2013). The macrocolony assay is a simple and useful method for the determination of the ability of *E. coli* strains to produce these components, however the molecular regulation of these structures is much more complicated and yet not fully understood (Da Re and Ghigo, 2006; Lasaro et al., 2009; Mika and Hengge, 2014). Concerning the biofilm production, it was already shown that the carriage of ESBL-plasmids might also lead to an enhanced virulence or could have an impact on chromosomally encoded genes like *csgD* which is involved in the regulation of curli fimbriae and the cellulose production (Schaufler et al., 2016a). In the study it turned out, that more than 50% of the tested isolates were able to produce both curli fibers and cellulose (Publication II). As already discussed in Publication II, the ability to form biofilms might be a special advantage in surviving in the single steps of the broiler production chain. Furthermore, it was previously found that certain materials support the formation of biofilms on the slaughterhouse equipment (Arnold and Silvers, 2000; Ho et al., 2004). This is especially problematic in the context of antibiotic resistant bacteria as biofilms have also higher resistances against disinfection. Usually the scalding and defeathering processes are prone to support bacterial cross-

contamination events. Detailed investigations on the biofilm formation of ESBL-/pAmpC-producing *E. coli* and their survival in the slaughterhouses and during the broiler processing are not done. In the RESETII study also the *E. coli* strains determined from the slaughterhouse were found to be positive concerning the production of curli fibers and cellulose. Therefore, further investigations should be carried out to get further insights into the contamination of broiler carcasses with resistant *E. coli* during slaughter and to examine possible intervention strategies against these bacteria.

6. Conclusion

ESBL-/pAmpC-producing *E. coli* occur in high frequencies in broiler chickens and were also detected in chicken meat (Idelevich et al., 2016; Kola et al., 2012). Broiler chickens are therefore considered as potential source for the infection of humans. However, many studies determined differences in the *E. coli* types and the *bla* resistance genes between humans and chickens and found out, that chicken meat is not necessarily the main source of exposure of ESBL-/pAmpC-resistant Enterobacteriaceae for humans (de Been et al., 2014; Belmar Campos et al., 2014; Carmo et al., 2014; Evers et al., 2017; Ewers et al., 2012; Valentin et al., 2014). It was found, that there is low similarity between ESBL-/AmpC-genes from livestock or food-associated reservoirs to those found in humans (Dorado-Garcia et al., 2017). pAmpC-producing *E. coli* are frequently detected in broiler chickens but occur only rarely in clinical human samples (Alonso et al., 2016; Ewers et al., 2012; Izdebski et al., 2013; Rodriguez-Bano et al., 2012). In contrast, pathogenic *E. coli* of phylogroup B2 and MLST type ST131 are only found in low numbers in samples from healthy broiler chickens but often occur in humans or other animals like wild birds and dogs (Dautzenberg et al., 2016; Ewers et al., 2010; Nicolas-Chanoine et al., 2014; Peirano et al., 2013; Pitout and DeVinney, 2017; Pomba et al., 2014; Totsika et al., 2011). Nevertheless, as broiler fattening flocks get also colonized by these resistant bacteria even without the use of antibiotics, they represent a source for the dissemination and spread of ESBL-/pAmpC-resistant Enterobacteriaceae into the environment (Agersø et al., 2014; Daehre et al., 2017; Huijbers et al., 2016b; Mo et al., 2014; Päivärinta et al., 2016). Therefore, effective intervention strategies are needed on all stages of the broiler production chain. In this study, it could be shown, that there is a stepwise pseudo-vertical transmission from the parent flocks into the respective hatchery (Publication I+II). The recently hatched chicks can get infected with the resistant bacteria either in the hatchery or later on at the fattening farms (Publication II, Daehre et al., 2017). Once colonized, they introduce the ESBL-/pAmpC-resistant *E. coli* into the slaughterhouse and the respective chicken meat gets contaminated very likely during processing and via cross-contamination (unpublished data). The impact of certain steps especially in the slaughterhouse is not well investigated as many studies examine only phylogroups or MLST types and *bla* resistance genes of the respective isolates (Blaak et al., 2015; Borjesson et al., 2016; Dierikx et al., 2013b; Skjot-Rasmussen et al., 2012). Therefore, detailed studies using WGS data are necessary to determine all cross-contamination sources also for other bacterial species like *E. fergusonii* and *K. pneumoniae*. There is very limited information on the dissemination of ESBL-/pAmpC-producing *E. coli* during the processing of broiler chickens (Pacholewicz et al., 2015a; von Tippelskirch et al., 2018). Furthermore, when developing intervention strategies against the ESBL-/pAmpC-resistant Enterobacteriaceae, certain bacterial factors providing an enhanced colonization of chickens and survival in the environment of the single stages of the broiler production chain should be considered as well.

7. Summary

Enterobacteriaceae which demonstrate resistance against beta-lactam antibiotics are of major concern in public health. The occurrence of extended-spectrum beta-lactamase (ESBL)-/AmpC beta-lactamase-producing *E. coli* in humans and animals is a challenging problem in antibiotic treatment in human and veterinary medicine. Especially in broiler chickens these resistant bacteria were frequently detected and a certain relevance of the exposure of contaminated chicken meat to humans is controversially discussed during the last decades. However, is was found, that ESBL-/pAmpC-producing Enterobacteriaceae were detectable on all stages of the broiler production and that already one-day old (grand-/parent)chicks can be affected. During the RESETII-project we therefore investigated seven ESBL-/pAmpC-positive parent broiler flocks, their respective hatching eggs as well as the subsequent broiler fattening flocks at the fattening farms as well as on slaughterhouse level concerning the occurrence of the resistant bacteria (chain investigations A-G). This project was performed by the Institute for Animal Hygiene and Environmental Health as well as the Institute of Food Safety and Food Hygiene from the Freie Universität Berlin. The task of the presented PhD thesis was to investigate the collected samples and resistant strains with regard to possible vertical transmission events in the whole broiler production chain. Therefore, high resolution molecular methods like pulsed-field gel-electrophoresis (PFGE) as well as whole genome sequencing (WGS) was applied to the respective isolates to determine their phylogenetic relationship. Using these methods, it could be shown that the introduction of the ESBL-/pAmpC-producing Enterobacteriaceae from the parent flocks into the hatchery occurred via contaminated egg shells (pseudo-vertical transmission) and that these bacteria can also survive the disinfection procedure. The hatchery acts as a reservoir for the resistant bacteria and certain strains are stepwise transmitted to the recently hatched chicks via the hatchery environment. However, the colonization of the chicks can also occur later on at the fattening farms. This indicates that the transmission routes of the ESBL-/pAmpC-producing Enterobacteriaceae in the broiler production are highly complex. Once the broiler chickens are infected with the resistant bacteria they directly introduce them into the slaughterhouse and the processing steps of the chicken meat. Especially the scalding and the defeathering steps are critical for cross-contamination events as shown in this study. Furthermore, selected isolates from all stages of the broiler production where investigated concerning the occurrence of virulence associated genes (VAGs) and the ability to produce curli fibers and cellulose. These factors support the colonization of a respective host as well as the survival in unfavorable environmental conditions. Using a macrocolony assay it could be shown that most of the tested *E. coli* isolates were able to produce curli fibers and cellulose which are the major components of biofilms. Therefore, it is very likely that these ESBL-/pAmpC-producing *E. coli* strains are also highly resistant against environmental stress and for example might also better survive certain cleaning and

disinfection procedures. In addition, the investigated *E. coli* strains also showed up to harbor high numbers of VAGs. However, the numbers and the distribution of certain genes and virulence gene classes varied among the strains. Therefore, no specific VAG could be identified for the enhanced survival in broiler chickens. Furthermore, only isolates of one chain transmission investigation could be assigned as extra-intestinal pathogenic *E. coli* strains (ExPEC). This indicates that further investigations on the impact of certain bacterial factors on the survival, spread and transmission of ESBL-/pAmpC-producing *E. coli* in the broiler production chain are needed. For the successful development and application of intervention strategies against these resistant bacteria in the whole broiler production chain the complex network of transmission routes as well as supporting bacterial factors should be considered. Therefore, further detailed studies using an appropriate high number of samples and isolates as well as respective high resolution molecular methods need to be carried out.

8. Zusammenfassung

Molekulare Epidemiologie und vertikale Transmission von ESBL-/AmpC-produzierenden Enterobacteriaceae entlang der Masthähnchenproduktionskette

Enterobacteriaceae, welche eine Resistenz gegenüber Beta-Laktam Antibiotika aufzeigen, sind ein großes Problem für die öffentliche Gesundheit. Das Vorkommen von Extended-Spektrum Beta-Laktamase (ESBL)-/AmpC Beta-Laktamase-produzierenden *E. coli* in Mensch und Tier ist eine Herausforderung für die Behandlungsstrategien in der Human- und Veterinärmedizin. Besonders in Masthähnchen finden sich diese resistenten Bakterien und daher wird auch dem kontaminierten Hühnchenfleisch eine große Bedeutung zugemessen bei einer möglichen Übertragung auf den Menschen. Dieses Thema wird in den letzten Jahren jedoch auch sehr kontrovers diskutiert. Es konnte gezeigt werden, dass ESBL-/AmpC-produzierende Enterobacteriaceae sich in allen Stufen der Masthähnchenproduktionskette nachweisen lassen und dass auch bereits Eintagsküken kolonisiert sein können. Innerhalb des RESETII-Projektes wurden daher sieben ESBL-/pAmpC-positive Elterntierherden, deren Bruteier und die zugehörigen Masttierherden auf den Farmen und im Schlachthof bezüglich des Vorkommens dieser resistenten Bakterien untersucht (Kettenuntersuchungen A-G). Das Projekt wurde in den Instituten für Tier- und Umwelthygiene sowie für Lebensmittelsicherheit und –hygiene der Freien Universität Berlin durchgeführt. Im Rahmen dieser Doktorarbeit wurden die gesammelten Proben und resistenten Stämme insbesondere hinsichtlich möglicher vertikaler Übertragungswege innerhalb der Masthähnchenproduktionskette untersucht. Dabei wurden hochauflösende molekulare Methoden wie pulsed-field Gelelektrophorese (PFGE) und Ganzgenomsequenzierung (WGS) verwendet um die phylogenetische Verwandtschaft der untersuchten Isolate zu bestimmen. Es konnte gezeigt werden, dass der Eintrag der ESBL-/AmpC-

produzierende Enterobacteriaceae von den Elterntierherden in die Brüterei über kontaminierte Bruteioberflächen erfolgt (pseudovertikale Übertragung) und dass diese Bakterien auch die Desinfektionsmaßnahmen in der Brüterei überleben können. Die Brüterei fungiert dann als Reservoir für die resistenten Bakterien und einzelne Stämme werden nach und nach durch die Umgebung auf die geschlüpften Küken übertragen. Die Kolonisierung der frisch geschlüpften Küken kann aber auch erst im späteren Verlauf direkt auf der Mastfarm erfolgen. Diese Ergebnisse zeigen, dass die Möglichkeiten der Übertragungen von ESBL-/AmpC-produzierenden Enterobacteriaceae in der Masthähnchenproduktionskette sehr komplex sind. Sind die Masthähnchen jedoch einmal mit den resistenten Keimen kolonisiert, erfolgt ein direkter Eintrag in den Schlachthof und in die einzelnen Verarbeitungsschritte von Hähnchenfleisch. Insbesondere der Brühprozess und der Rupfvorgang sind kritische Schritte in Bezug auf mögliche Kreuzkontaminationen, wie in der vorliegenden Studie gezeigt werden konnte. Zusätzlich wurden ausgewählte Isolate aus allen Produktionsstufen der Masthähnchenkette auf das Vorkommen von Virulenz-assoziierten Genen (VAGs) hin untersucht sowie auf die Fähigkeiten Curli-Fimbrien beziehungsweise Zellulose zu produzieren. Diese Faktoren unterstützen die Kolonisierung eines entsprechenden Wirts und das Überleben unter ungünstigen Umweltbedingungen. Mit Hilfe eines Makrokolonieassays konnte gezeigt werden, dass die Mehrheit der untersuchten Isolate die Fähigkeit zur Bildung von Curli-Fimbrien und Zellulose besitzen, welche die Hauptkomponenten von bakteriellen Biofilmen darstellen. Daher ist es sehr wahrscheinlich, dass diese ESBL-/AmpC-produzierenden Enterobacteriaceae eine hohe Widerstandskraft gegen Umweltstress und beispielsweise auch gegen die Reinigungs- und Desinfektionsvorgänge aufweisen. Zusätzlich besaßen die untersuchten Isolate eine hohe Anzahl an VAGs in ihren Genomen. Die Gesamtzahl dieser VAGs sowie die Verteilung der VAGs unterschiedlicher Genkategorien variierten jedoch zum Teil zwischen den Stämmen. Es konnte daher kein spezifischer Faktor identifiziert werden für die Kolonisierung von Masthähnchen. Außerdem konnten nur Isolate aus einer einzigen Kettenuntersuchung als sogenannte extra-intestinal pathogene *E. coli* (ExPEC) ausgemacht werden. Das zeigt, dass weitere Untersuchungen benötigt werden, bezüglich der Überlebensmöglichkeiten, der Verbreitung und der Übertragung von ESBL-/AmpC-produzierenden *E. coli* innerhalb der Masthähnchenproduktionskette. Für die Entwicklung und erfolgreiche Anwendung von Interventionsmaßnahmen gegen diese resistenten Bakterien in der Masthähnchenproduktionskette sollten das komplexe Übertragungsnetzwerk sowie bestimmte bakterielle Faktoren mit in Betracht gezogen werden. Dazu bedarf es weiterer detaillierter Studien mit einer entsprechenden Anzahl an Proben und Isolaten und die Anwendung von hochauflösenden molekularen Methoden.

9. References

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V. List of publications

Publications

Projahn M., Daehre K., Roesler U., Friese A. (2017) Extended-Spectrum-Beta-Lactamase- and Plasmid-Encoded Cephamycinase-Producing Enterobacteria in the Broiler Hatchery as a Potential Mode of Pseudo-Vertical Transmission. Applied and Environmental Microbiology 83 (1) 10.1128/AEM.02364-16.

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Presentations

Projahn M., Dähre K., von Tippelskirch P., Orquera S., Alter T., Friese A., Rösler U. Epidemiological relationship of ESBL-/ AmpC-producing Enterobacteriaceae in the broiler production chain; National Symposium on Zoonoses Research : 15 - 16 October 2015 ; Program and Abstracts, Berlin

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Poster

Dähre K., Projahn M., von Tippelskirch P., Orquera S., Alter T., Roesler U., Friese A. Occurrence of ESBL-/ AmpC-producing Enterobacteriaceae along the broiler production chain National Symposium on Zoonoses Research : 15 - 16 October 2015, Berlin

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Dähre K., Projahn M., Rösler U., Friese A. ESBL-/AmpC producing Enterobacteria along the broiler production chain; Dahlem Research School (DRS) doctoral Symposium 2016, Berlin

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VII. Selbständigkeitserklärung

Hiermit bestätige ich, dass ich die vorliegende Arbeit selbständig angefertigt habe. Ich versichere, dass ich ausschließlich die angegebenen Quellen und Hilfen Anspruch genommen habe.

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Michaela Projahn

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