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Clinically-relevant antibiotic-resistant bacteria in process waters and wastewater from poultry and pig slaughterhouses and assessment of the bacterial dissemination into surface waters

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Meinen Eltern und meinem Mann <3

Man merkt nie, was schon getan wurde, man sieht immer nur, was noch zu tun bleibt.

Marie Skłodowska Curie (1867-1934)

Abstract

Clinically-relevant antibiotic-resistant bacteria in process waters and wastewater from poultry and pig slaughterhouses and assessment of the bacterial dissemination into surface waters

The objective of this thesis was the investigation of clinically-relevant antibiotic-resistant bacteria in process waters and wastewater from poultry and pig slaughterhouses and the assessment of the bacterial dissemination into surface waters. Process waters were collected in the delivery and dirty areas of poultry and pig slaughterhouses. Their in-house wastewater treatment plants (WWTPs) were sampled as well. Furthermore, to assess the bacterial spread into surface waters, samples from the respective municipal WWTPs including the receiving water bodies were collected. The samples were screened for (1) ESKAPE-bacteria (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.), (2) ESBL (extended spectrum β-lactamase)-producing *Escherichia coli* and (3) colistin-resistant *Enterobacteriaceae* (i.e. *E. coli*, *Klebsiella* spp., *Enterobacter* spp.) by culture-dependent methods. Based on the results, the clinical relevance of the target bacteria and the efficacy of the wastewater treatment management were assessed. From 185 water samples, a total of 1,482 isolates of the target species were recovered, which were ubiquitous along the investigated slaughtering and wastewater chains as well as in the on-site preflooders. They exhibited highly heterogeneous antibiotic-resistance patterns. Extraintestinal-pathogenic ESBL-producing *E. coli* was isolated to a greater extent in the samples from poultry slaughterhouses and municipal WWTPs. Furthermore, isolates originating from poultry slaugterhouses, exhibited the highest abundance of *mcr-1* gene located on a variety of transferable plasmids. In the samples collected from the pig slaughterhouses, livestock-associated (LA)-MRSA of CC398 was dominant. A wide variety of clinically relevant clones among ESBL-producing, and colistin-resistant *K. pneumoniae* isolates was detected both in the slaughterhouses and municipal WWTPs. Of note, ESKAPE bacteria with the highest potential risk to humans, such as carbapenemase-producing *Enterobacteriaceae* (CPE), vancomycin-resistant enterococci (VRE) as well as healthcare-associated (HA)-MRSA of CC5 and CC22 were mainly detected in municipal wastewater.

Process waters and wastewater from slaughterhouses and especially from municipal WWTPs constitute an important reservoir of antibiotic-resistant bacteria with clinical relevance. They pose a risk to human health, since they may colonize and infect slaughterhouse and WWTPs' employees with occupational exposure to contaminated waters. The target bacteria were detected in the effluents from the in-house WWTPs of poultry slaughterhouses and municipal WWTPs, underlying their inefficacy in reducing the microbial loads. Thus, their broad dissemination into the environment can be expected. In order to reduce the input of antibiotic-resistant bacteria into the slaughterhouses and their subsequent discharge into the surface waters, the prescription and consumption patterns of antibiotics in livestock production need to be reconsidered. Furthermore, use of innovative state-of-the-art wastewater treatment technologies needs to be encouraged, especially for direct dischargers.

Kurzfassung

Klinisch relevante antibiotikaresistente Bakterien in Prozesswässern und Abwässern aus Geflügel- und Schweineschlachthöfen, sowie Bewertung der bakteriellen Verbreitung in die Oberflächengewässer

Ziel der vorliegenden Arbeit war die Untersuchung klinisch relevanter antibiotikaresistenter Bakterien in Prozesswässern und Abwässern von Geflügel- und Schweineschlachthöfen sowie die Bewertung der bakteriellen Verbreitung in die Oberflächengewässer. Dafür wurden in Anlieferungs- sowie in schwarzen Produktionsbereichen von Geflügel- und Schweineschlachthöfen Proben der Prozesswässer genommen. Die betriebseigenen Kläranlagen wurden ebenfalls beprobt. Darüber hinaus wurden zur Beurteilung der Verbreitung von Bakterien in die Oberflächengewässer Proben aus den jeweiligen kommunalen Kläranlagen einschließlich Vorfluter entnommen. Die Proben wurden auf (1) ESKAPE-Bakterien (*Enterococcus* spp., *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.), (2) ESBL (extended spectrum β-lactamase)-produzierende *Escherichia coli* und (3) Colistin-resistente *Enterobacteriaceae* (i.e. *E. coli*, *Klebsiella* spp., *Enterobacter* spp.) mittels kultureller Verfahren untersucht. Basierend auf den Ergebnissen wurden die klinische Relevanz der Zielbakterien sowie die Effizienz des Abwassermanagements bewertet.

Aus 185 Wasserproben wurden insgesamt 1.482 Isolate der verschiedenen Zielspezies isoliert, die entlang der untersuchten Schlacht- und Abwasserketten sowie in den Vorflutern ubiquitär vorhanden waren. Die untersuchten Isolate zeigten sehr heterogene Antibiotikaresistenzmuster. Extraintestinal-pathogene ESBL-produzierende *E. coli* wurden zum größten Teil in den Proben von Geflügelschlachthöfen und kommunalen Kläranlagen nachgewiesen. Darüber hinaus zeigten Isolate aus Geflügelschlachthöfen die höchste Abundanz des *mcr-1*-Gens, was auf einer Vielzahl übertragbarer Plasmide lokalisiert war. In den Proben aus Schweineschlachthöfen waren Nutztier-assoziierte (LA)-MRSA des CC398 predominant. Eine Vielzahl klinisch relevanter Klone unter ESBL-produzierenden, und Colistin-resistenten *K. pneumoniae*-Isolaten wurde sowohl in den Schlachthöfen als auch in den kommunalen Kläranlagen nachgewiesen. Bemerkenswerterweise wurden ESKAPE-Bakterien mit dem höchsten potenziellen Risiko für den Menschen, wie Carbapenemase-produzierende *Enterobacteriaceae* (CPE), Vancomycin-resistente Enterokokken (VRE) sowie Krankenhaus-assoziierte (HA)-MRSA der CC5 und CC22, hauptsächlich in kommunalem Abwasser nachgewiesen.

Nichtsdestotrotz bilden Prozesswässer und Abwasser aus Schlachthöfen und insbesondere aus kommunalen Kläranlagen ein wichtiges Reservoir für antibiotikaresistente Bakterien mit klinischer Relevanz. Sie stellen ein Risiko für die menschliche Gesundheit dar, weil sie die Mitarbeiter von Schlachthöfen und Kläranlagen, mit Exposition gegenüber kontaminiertem Wasser, kolonisieren und infizieren können. Die Zielbakterien wurden in den Abläufen der betriebseigenen Kläranlagen von Geflügelschlachthöfen und kommunalen Kläranlagen nachgewiesen. Dies deutet auf deren Ineffizienz bezüglich der Reduktion der mikrobiologischen Belastung hin. Somit kann eine breite Verbreitung der Zielbakterien in der Umwelt nicht ausgeschlossen werden. Um den Eintrag von antibiotikaresistenten Bakterien in die Schlachthöfe und deren anschließende Einleitung in die Oberflächengewässer zu verringern, müssen die Verschreibungs- und Verbrauchsmuster von Antibiotika in der Tierproduktion angepasst werden. Ein Ansatzpunkt um den Austrag von antibiotikaresistenten Bakterien zu vermindern, wäre die Förderug und Implementierung innovativer Abwasserbehandlungstechnologien, insbesondere bei Direkteinleitern.

Contents

1. Introduction

1.1. Antibiotic usage in poultry and pig production in Germany

An antibiotic is a substance produced by, or derived (chemically produced) from a microorganism that selectively destroys or inhibits the growth of other microorganisms (1). The action mechanisms of main classes of antibiotics fall into four categories: (1) inhibition of cell wall synthesis (e.g. β-lactams, vancomycin, bacitracin); (2) damage of cell membrane (e.g. polymyxins); (3) inhibition of DNA replication (e.g. quinolones, sulfonamides) and (4) inhibition of protein synthesis (e.g. macrolides, lincosamides, tetracyclines) (2). Antibiotics are approved preparations either with a single active substance or several different active substances for various medical indications, such as infectious diseases in humans and animals or chemotherapy treatment. They could be used for a topical treatment or applied by oral, parenteral (e.g. subcutaneous, intramuscular or intravenous) or other types of administration routes (2).

From the beginning of 1950s, antibiotics have been widely used in animal husbandry in particular to prevent infections (prophylaxis) and their further spread (metaphylaxis) as well as to promote feed efficiency in order to increase animals' growth rates and yield (3). In 2015 in the OIE region that included 91 countries worldwide, 104,779 tons of antibiotics were used in livestock production (4), whereas most of them (78%) were applied in cattle, swine and poultry. Since 2006, the use of antimicrobials in livestock feeds for promoting growth and increasing yield has been banned in the European Union (5). Furthermore, their prophylactic use in German farming is also prohibited and metaphylactic use, i.e. treating a group of animals when one has signs of infection, is heavily restricted (6, 7). However, globally, using antibiotics as growth promoters is still an established practice. As of 2017, 29% (45/155) of countries worldwide reported use of antibiotics for this purpose, including big livestock producing and exporting countries such as USA, China and Brazil (4).

The approved antibiotic classes for veterinary medicine differ between countries worldwide (8). Furthermore, there are differences between approved antibiotic classes for veterinary and human medicine (9, 10). The active substance classes approved in Germany in veterinary and human medicine are shown in Fig. 1.1.1.

Overall, 24 classes are available for treatment of animals and humans. All active substance classes allowed in veterinary medicine are also approved in human medicine. However, they possess different clinical relevance. Their significance for human and veterinary medicine is stated in the lists published by World Health Organization (WHO) and World Organisation for Animal Health (OIE), respectively (9, 10).

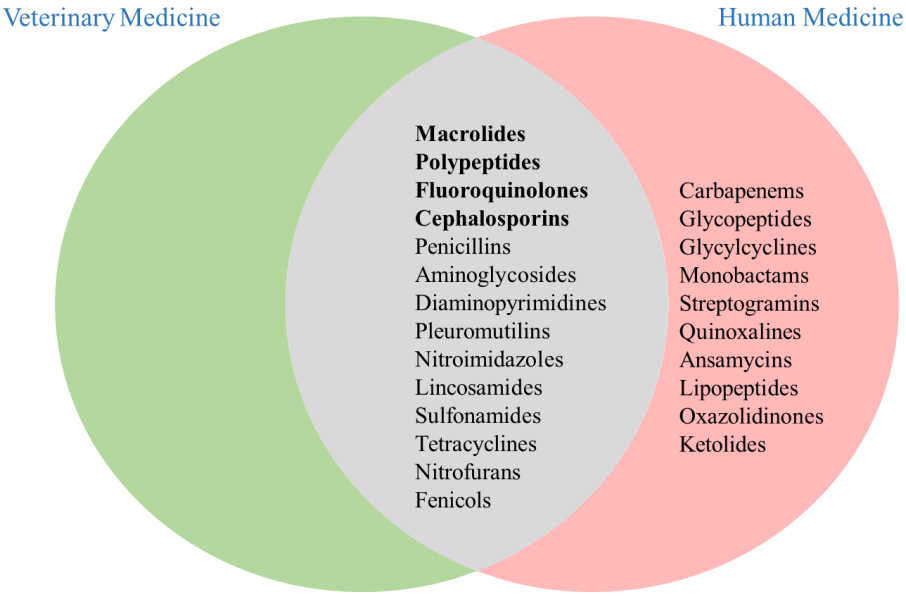


Figure 1.1.1 Availability of 24 classes of active substances in human and veterinary medicine in Germany (classes of active substances in bold: HPCIA for human medicine according to the WHO classification). Modified according to [9, 10]

Noteworthy, antimicrobial classes considered by WHO as Highest Priority Critically Important Antimicrobials for humans (HPCIA, i.e. macrolides, polymyxins, quinolones, cephalosporins of 3rd and higher generation) are also approved for the use in animals (10). They are crucial in combating specific infectious diseases in livestock and there is a lack of sufficient therapeutic alternatives for veterinary medicine. Therefore, they belong to the Veterinary Critically Important Antimicrobial Agents (VCIA) (Fig. 1.1.2).

Veterinary Critically Important Antimicrobial Agents (VCIA)	Veterinary Highly Important Antimicrobial Agents (VHIA)	Veterinary Important Antimicrobial Agents (VIA)
•**Aminoglycosides**	•Ansamycin	•Arsenical
•**Phenicols**	•**Cephalosporins (1st-2nd generation)**	•Bicyclomycin
•**Cephalosporins (3rd-4th generation)**	•Ionophores	•Fusidic acid
•**Macrolides**	•**Lincosamides**	•Orthosomycins
•**Penicillins**	•Phosphonic acid	•Quinoxalines
•**Fluoroquinolones**	•**Pleuromutilins**	•Streptogramins
•**Sulfonamides**	•**Polypeptides**	•Thiostrepton
•**Diaminopyrimidines**	•Quinolones (1st generation)	
•**Tetracyclines**		

Figure 1.1.2 Categorization of veterinary important antimicrobial agents for food producing animals according to the OIE classification (in bold: approved classes of active substances for treatment of fattening chickens and pigs in Germany). Modified according to [10]

Polypeptides, lincosamides, pleuromutilins and cephalosporins of 1st and 2nd generation are critical for veterinary medicine as well. However, in contrast to VCIA, less than 50% of the OIE Member Country contributions identified their clinical importance and categorized them as Veterinary Highly Important Antimicrobial Agents (VHIA) (10).

The data on supplied quantities of antibiotics to veterinarians in Germany for certain fattening animals (pigs, cattle, turkeys and chicken) have been recorded since 2011 as required by the regulatory act DIMDI-Arzneimittelverordnung (11). Because of the nationwide Antibiotics Minimization Concept in animal husbandry, obligatory monitoring of antibiotic use in animals was introduced in 2014 in the 16th amendment of the German Pharmaceuticals Act ("Arzneimittelgesetz", AMG, sections 58a to 58d AMG (12). Based on this data, the therapy frequency for each animal population should be determined. Holdings in which animals were treated with antibiotics with an above-average frequency, are now obliged to cooperate with their veterinarians in order to determine the reason for the increased use of antibiotics and to develop and implement minimization strategies.
In 2017, a total of 733 tons of antibiotics were supplied to veterinarians in Germany (Tab. 1.1.1) (12).

Table 1.1.1 Supplied quantities of antibiotics by active substance class sold to veterinarians in Germany in the period 2011 to 2017 (classes of active substances in bold: HPCIA for human medicine according to the WHO classification). Modified according to [12]

	2011	2012	2013	2014	2015	2016	2017	Difference [%] 2011-2017
Folic acid antagonists	39.9	26.2	24.3	19.1	10.3	9.8	7.8	-73.9
Macrolides	173	145	126	109	52.5	54.7	54.7	-68.4
Tetracyclines	564	566	454	342	221	193	188	-66.7
Sulfonamides	185	162	152	121	72.6	68.8	62.4	-66.2
Penicillins	528	501	473	450	299	279	269	-49.0
Polypeptides	127	123	125	107	81.8	68.9	73.6	-42.2
Aminoglycosides	47.1	40.5	39.4	37.8	24.7	26.1	29.3	-37.8
Lincosamides	16.8	15.2	16.9	14.6	10.8	9.9	10.9	-35.4
4th gen. cephalosporins	1.4	1.4	1.4	1.4	1.3	1.1	1.1	-25.6
Fenicols	6.1	5.7	5.2	5.3	5.0	5.1	5.6	-8.9
Pleuromutilins	14.1	18.4	15.5	13.0	11.2	9.9	13.4	-5.2
1st gen. cephalosporins	2.0	2.1	2.1	2.1	1.9	2.0	2.0	-2.8
3rd gen. cephalosporins	2.1	2.3	2.3	2.3	2.3	2.3	2.3	+13.5

Table 1.1.1 (continued)

Fluoroquinolones	**8.2**	**10.4**	**12.1**	**12.3**	**10.6**	**9.3**	**9.9**	**+20.1**
Other*	0.12	0.11	1.89	2.47	0.31	2.80	3.41	+2669
Total	1.706	1.619	1.452	1.238	805	742	733	-57.0

* – Fusidic acid, ionophores, nitrofurans, nitroimidazoles.

In comparison to 2011 a decrease of 57% or around 973 tons was observed. As of 2017, only supplied quantities of 3rd generation cephalosporins, fluoroquinolones and other antibiotics (fusidic acid, ionophores, nitrofurans, nitroimidazoles) increased (Tab. 1.1.1). However, supplied quantities of antibiotics differ from the used ones. In 2017, 55.1% (404 tons) of the supplied amount of antibiotics were used for the treatment of infections in livestock. Of these, approximately 66.8% (270/404) were used to treat piglets, pigs and poultry for a wide range of applications including septicaemias, digestive, respiratory and urinary diseases. The differences between supplied and used amounts of individual active substance classes varied by 32% (e.g. macrolides) to 100% (e.g. 1st generation cephalosporins) (Fig. 1.1.3). In any case, in 2017 with a share of used quantities of 1.8% – 6.0% (0.042 tons – 0.064 tons), cephalosporins of 3rd/4th generation were barely used at all. Whereas the share accounted for by the used quantities of fluoroquinolones was 35.0% (3.47/9.90 tons). (12)

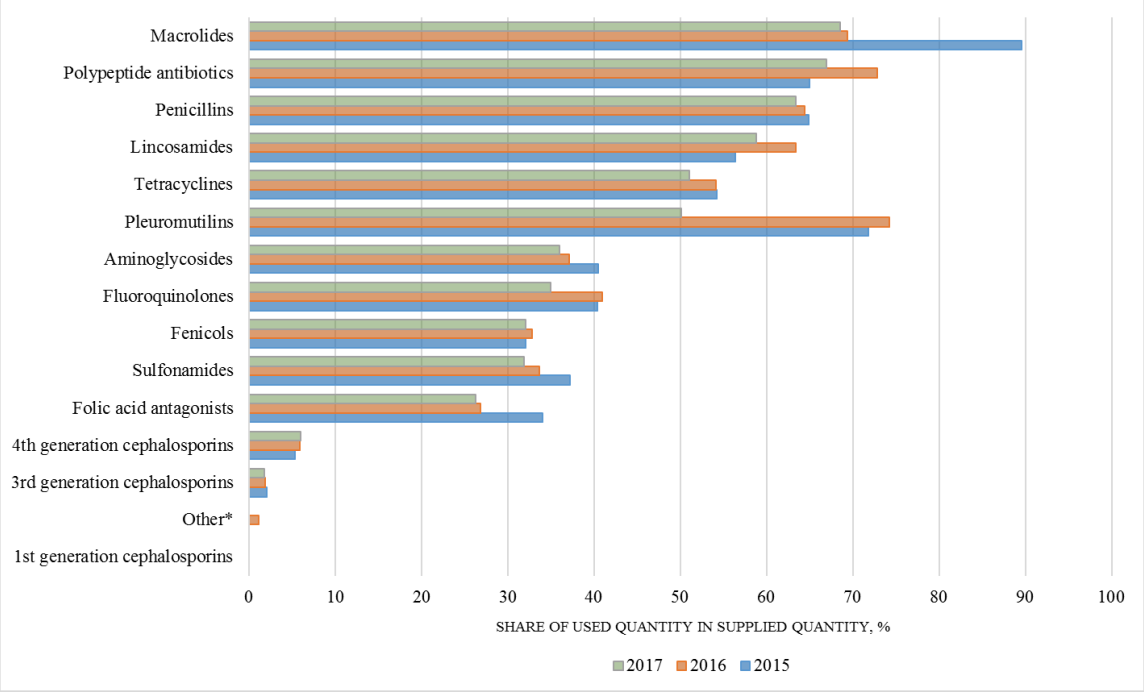


Figure 1.1.3 Comparison of used with the supplied quantities of antibiotics. Used quantities as % of the supplied quantity that is set as 100% for the individual active substance classes (2015 – 2017). * – fusidic acid, ionophores, nitrofurans and nitroimidazoles. Modified according to [12]

Interestingly, in the period 2011-2017, the supplied quantities of HPCIA developed in different ways. The supplied quantities of macrolides decreased by 118.3 tons (68.4%) to 54.7 tons, while the sales of polypeptides fell by 53.4 tons (42.2%) to 73.6 t. On the other hand, as of 2017 the sales of 3rd/4th generation cephalosporins fell insignificantly by 2.8% (0.1 tons) to 3.4 tons and the supplied quantities of fluoroquinolones even increased by 20.1% (1.7 tons) to 9.9 tons (Tab. 1.1.1). (12)

Overall, approximately 130 tons of antibiotics were used for fattening pigs in 2017. Penicillins (56 tons), tetracyclines (46 tons) and macrolides (16 tons) accounted for the largest amount of the used substances, whereas the used quantities of polypeptide antimicrobials, fluoroquinolones and 3rd/4th generation cephalosporins in this sector were low. This also mostly reflects the treatment frequency of fattening pigs with tetracyclines, penicillins and macrolides being the most frequently used classes of substances to treat swine infectious diseases. The treatment frequency for fattening pigs has decreased significantly since 2014 by approximately 56%. (12)

For the treatment of fattening chickens in 2017, around 63 tons of antibiotics were used. Polypeptide antibiotics (25 tons) accounted for the largest share of the total used quantity, followed by penicillins (18 tons), aminoglycosides (6 tons), macrolides (5 tons) and lincosamides (2 tons). Sulfonamides, tetracyclines and fluoroquinolones were only used in limited quantities. The use of aminoglycoside-lincosamide combinations, penicillins and polypeptide antibiotics on farms was predominant. Interestingly, out of all types of animal production the reduction in treatment frequency among fattening chickens was the lowest and has been continuously increasing since 2015. (12)

1.2. Development of clinically-relevant antimicrobial resistances in animal husbandry

Antimicrobial resistance (AMR) is the ability of microorganisms, such as bacteria, to resist the effects of a defined concentration of antimicrobial agent (2). The antimicrobial resistance of bacteria is based on four main mechanisms: (1) modification of the target (loss or decrease in drug's affinity to its target); (2) production of enzymes which modify or inactivate the drug; (3) impermeability of the external membrane; and (4) efflux of antibiotics out of the cells (13). AMR could be either intrinsic (natural) or acquired during the treatment (13, 14). Intrinsic resistance could be considered as insensitivity and is characteristic for all the bacteria of a particular species or genus. Furthermore, a bacterial strain can acquire resistance spontaneously, e.g. by point mutations (13). The uptake of exogenous genes by horizontal transfer from other

bacteria could lead to drugs resistance as well (13). Acquired AMR is a consequence of antimicrobial use in both human and veterinary medicine and poses the "main undesirable side-effect" of antimicrobial treatment (14). This results in continuous selection of resistant bacterial clones without regards to their status as commensals, environmental bacteria or pathogens. Moreover, this natural selection process is exacerbated by anthropogenic factors such as indiscriminate and abusive use of antibiotics in human medicine and livestock (3, 15).

In contrast to veterinary medicine, more substances of one active class of antibiotics are approved for use in human medicine. However, most antibiotics used in animals are chemically related in structure to human therapeutics, share the range of efficacy and the same target in the cell (12, 16). Thus, they are mostly substrates for the same resistance mechanisms and the use of these antibiotics in livestock may select for cross-resistance to (critically) important antibiotics for human medicine. So even if a certain antibiotic is used in veterinary medicine, resistance to the veterinary drug can also confer resistance to the related antibiotic intended for use in humans (3, 14). Cross-resistance between HPCIA for human medicine (i.e. macrolides, polymyxins, quinolones, cephalosporins of 3rd and higher generation) as well as streptogramins and glycopeptides with their related substances for veterinary use is shown in Tab. 1.2.1.

In 2016, more than 700,000 humans died globally by infections with multidrug-resistant (MDR) bacteria. It was estimated that if no further action is taken, an annual death rate of 10 million humans may be reached by 2050 (17). Pathogens that are often associated with multiple antimicrobial resistances are attributed to ESKAPE bacteria (*Enterococcus spp.*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), which together with *Escherichia coli* cause the majority of life-threatening bacterial infections in health care facilities among critically ill and immunocompromised patients. For instance, 63.4% and 61.1% of the isolated microorganisms in healthcare-associated infections in Europe and the USA, respectively, are members of these species (18, 19).

Table 1.2.1 Cross-over between selected antibiotics used in veterinary and human medicine. Modified according to [3, 14]

Antibiotic class	**Antibiotic used for livestock**	**Related antibiotic used for humans**
Fluoroquinolones	Enrofloxacin, ofloxacin	Ciprofloxacin, moxifloxacin
Cephalosporins 3rd/4th generation	Ceftiofur, cefquinome	Cefotaxime, ceftriaxone

Table 1.2.1 (continued)

Polypeptides	**Colistin (polymyxin E)**	**Polymyxin B**
Macrolides	Spiramycin, tylosin, azithromycin	Erythromycin + lincosamides (e.g. clindamycin)
Streptogramins	Virginiamycin	Quinupristin-dalfopristin (Synercid)
Glycopeptides	Avoparcin*	Vancomycin

* – banned in EU from 1997

However, if the commensal relationship to the host is not disrupted, ESKAPE bacteria are generally not pathogenic (20). *Escherichia coli*, *K. pneumoniae*, *Enterobacter* spp. and *E. faecium* are natural inhabitants of the intestinal tract of humans and animals, whereas *S. aureus* primarily occurs on the skin and nasal passages (21). Livestock, especially pigs and poultry, are well-known reservoirs for these bacteria (22–24). *Acinetobacter* spp. and *P. aeruginosa* are ubiquitously present in almost all habitats, but especially in soil and aquatic environments (25, 26). Nevertheless, animals could be temporarily colonized by *P. aeruginosa* originating from the soil and bedding material as well as form their watering systems (26). However, to date only little information is available on their natural occurrence in livestock and wildlife or whether these animals are associated with an efficient transmission of the bacteria to humans (27–29).

ESKAPE bacteria can effectively adapt to inhibitory effects of antimicrobials. Besides their intrinsic resistances, they also efficiently acquire additional resistance determinants via horizontal gene transfer by exchanging mobile genetic elements (MGE) (30–32). This property allows an efficient adaptation of the bacteria to the prevailing conditions, which might be associated with a drastically increased frequency of treatment failures and severity of human infections (32). The consequences of antimicrobial resistance could be particularly serious when the activity of critically and highly important antimicrobials for human medicine is compromised (33). Important examples are *Enterobacteriaceae* in poultry and pig production chains that produce extended spectrum β-lactamases (ESBLs) and are resistant to fluoroquinolones, so called 3MDRO (multidrug-resistant Gram-negative organisms). Among livestock and products of animal origin, poultry and poultry products show the highest incidence of ESBL-producing bacteria with CTX-M-1, SHV-12 and TEM-52 being the most frequent ESBL-types, whereas the CTX-M-1 β-lactamase is the most prevalent type among pigs. Recent studies in Germany indicated a high prevalence of CTX-M-1 at 69.0% in isolates from chicken meat and at 18.0% in isolates form chickens (34, 35). ESBL genes encoding TEM-

52 and SHV-12 β-lactamases have been already frequently detected in poultry as well as in human isolates in Germany and other European countries (36–38). However, in human-associated ESBL-producing bacteria, CTX-M-15 is one of the most frequently encountered ESBL types worldwide (39). In chickens and poultry products the percentage of *bla*$_{\text{CTX-M-15}}$ producing *E. coli* is generally low at 0.0%-5.2% (40–43). ESBL/pAmpC-*E. coli* was recently also detected in 12.1% of pork meat samples in Germany (8).

A few studies reported on high contaminations rates of 10-13%, with ESBL-expressing *K. pneumoniae* of broilers in India (44, 45) and of local and imported meat in Ghana (17.5%) (46). Furthermore, ESBL-expressing *K. pneumoniae* were detected in pigs (21.5%) and exposed workers (11.3%) in Cameroon (47). However, ESBL-producing *K. pneumoniae* has been barely detected in healthy broilers and pigs in Europe (48, 49). Nevertheless, its sporadically occurrence in broilers during slaughter in Germany has been also recently described (50, 51). Not only cephalosporins but β-lactam antibiotics in general (e.g. penicillins) can be at the origin of resistance of ESBL-producing bacteria, as they possess the same mechanism of action, i.e. inhibition of cell wall synthesis (13).

Casella and colleagues (2017) reported on high rates of fluoroquinolone resistance (20.8%) among ESBL-producing *E. coli* isolated from retail chicken meat (52). Along with ESBL-encoding genes, Plasmid-Mediated Quinolone Resistance (PMQR) genes (e.g. *qnrBS*, *oqxAB*, *aac(60)Ib-cr*) are frequently co-located on a single plasmid causing reduced susceptibility or resistance to fluoroquinolones among livestock-associated bacteria (34). Thus, resistances against different antibiotics are often co-selected, which together with the phenomenon of cross-resistance narrows down the options of reducing the AMR and complicate the choice of appropriate antibiotic therapy for animals and humans.

Furthermore, since colistin has been extensively used in the European animal production for decades, bacteria from livestock, especially poultry, show a high incidence of plasmid-encoded mobilizable colistin resistance genes, primarily *mcr-1* (53). Furthermore, Elbediwi and colleagues (2019) emphasized the global spread and high incidence of *mcr-1* gene among colistin-resistant bacteria from animals and food products globally (54). *mcr-1* was detected with different prevalence in livestock-associated *Enterobacteriaceae* (0.04-20.3%) and in human clinical isolates (0.06-2%) (55, 56). This can compromise the activity of colistin as a last-resort antibiotic for treatment of human infections caused by MDR Gram-negative pathogens, especially *P. aeruginosa* and *A. baumannii* as well as carbapenemase-producing *Enterobacteriaceae* (CPE).

In addition, methicillin-resistant *Staphylococcus aureus* (MRSA) is widely disseminated in animal husbandry, predominately in pig production (57). According to Köck and colleagues (2014), livestock-associated (LA) MRSA are responsible for 10% of all human MRSA infections in German regions with intensive animal husbandry (58). Furthermore, in countries where glycopeptides are approved for use in animals and avoparcin is used in feed as growth promoter, vancomycin-resistant enterococci (VRE) carrying *vanA*/*vanB* are present in pigs and poultry as well. Pruksakorn and colleagues (2016) reported an overall prevalence rate for VRE of 24% in pigs in Thailand (59).

Moreover, first cases of emergence of carbapenemase-producing *E. coli* isolates in pigs and poultry as well as their meat exhibits a worrying trend. Since 2011 VIM-1-producing *Salmonella* Infantis and *E. coli* were occasionally detected in chicken and pig farms in Germany (60, 61).

Based on the remarkable genetic plasticity of some multi-resistance plasmids in human Gram-negative pathogens (62, 63), an acquisition of certain antimicrobial resistance determinants of livestock bacteria via interaction with polluted rural environments and food products (e.g. *mcr*-genes, ESBL) cannot be excluded. Moreover, there is an evidence that bacteria of different compartments (e.g. livestock, environment, humans, food) share the same antimicrobial resistance genes.

Taking the above mentioned into account, livestock can represent carrier of bacteria with clinically relevant antimicrobial resistances (i.e. ESKAPE bacteria). Thus, poultry and pigs from different fattening farms, may represent a hotspot for introducing such bacteria into slaughterhouses. As a consequence, their in-house wastewater treatment plants (WWTPs) may provide a conduit for the entry of bacterial pathogens and resistance genes into the environment and community.

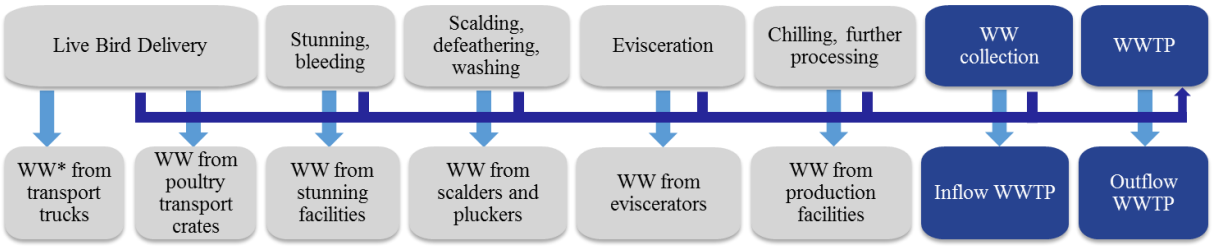
1.3. Process waters and wastewater accruing in poultry and pig slaughterhouses

In the last 30 years the meat production has doubled worldwide and it is expected to double until 2050 (64). In 2018 the production volume of meat was 330.5 million tons and the global meat sector was valued at 945.7 billion US dollars (64). At present, pork and poultry are the two most produced types of meat worldwide. Also in Europe pigs and poultry are accounted to the main livestock types processed along with cattle (3). In Germany in the 1st half of 2020, 2.6 million tons of pork and 800,000 tons of poultry were produced (65).

The meat industry is one of the biggest consumers of freshwater used in agriculture for producing animal feed and livestock (66). Animal production consumes 29% of the water used

in agricultural production which accounts for 92% of the global freshwater footprint (66). Furthermore, meat processing plants and slaughterhouses consume high amounts of water as well. Depending on the organization of the slaughtering process, 5,000-21,000 L of water per ton of poultry meat are consumed (67, 68), whereas 9,000-17,500 L of water are needed for one ton of pork (69). Thus, slaughtering as well as cleaning and sanitizing of production facilities are water consuming processes. Noteworthy, in the EU only potable water is allowed for food processing (70).

The poultry slaughter flow chart is shown in Fig 1.3.1. In general, broilers are slaughtered at the age of 30 to 40 days. Mostly, they are transported for slaughtering as an entire flock, whereas the transport is often carried out using different kind of crates. After unloading of broilers in waiting areas and their transfer to the shackle line, they are normally stunned by controlled atmosphere systems (CAS) using carbon dioxide.



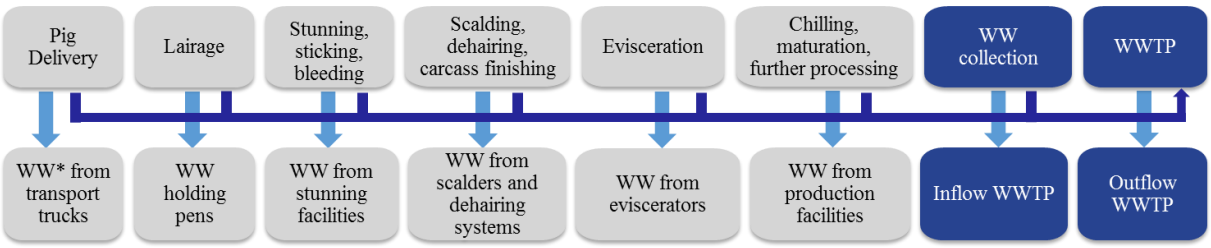
*- wastewater

Figure 1.3.1 Flow scheme of poultry slaughtering and accruing of wastewater. Modified according to [68, 69]

Further, the broilers are bled out through double-sided neck cut or throat cut before they enter the scalding process. Scalding is used to loosen the feathers and facilitate their removal during the plucking process and is done using hot water (50-63 °C) or steam that decreases the risk of cross-contamination and reduces water consumption by up to 75%. Subsequently, the birds are defeathered using pluckers with rubber fingers and prior to evisceration the carcasses are rinsed using spray washers. The evisceration process implies opening of the abdominal cavity and removing the entire viscera pack including the crop. After evisceration the carcasses are often rinsed following chilling and further processing. (70, 71)

The slaughtering process of pigs is shown in Fig 1.3.2. Most fattening pigs are slaughtered at the age of 150 to 180 days. The pigs are delivered to the lairage pens where they usually spend between 2 and maximal 24 hours without being fed to reduce the amount of faeces excretions but with access to clean drinking water. Then the animals are moved in groups of 5-6 from holding pens to the slaughtering area, where they are stunned before slaughter. Typically, a

carbon dioxide chamber or application of electrical current to the head are used. Further, sticking is done by cutting the main blood vessels in the upper chest (anterior vena cava and bicarotid trunk) and the pigs are allowed to bleed out. Afterwards, the pigs are scalded by horizontal or vertical water scalding processes. By horizontal scalding the pigs are held in a tank at 60°C to 70°C for 5-10 minutes. The vertical scalding is carried out in spray water systems, where the pig carcasses are permanently sprayed with warm water. Subsequently, the pigs are mechanically dehaired and singed to burn off any remaining hairs on the carcass. Subsequently by variety of shaving and polishing devices, the singed and remaining hairs are removed following preevisceration wash and evisceration. Finally, the carcasses are splitted in two, washed to remove blood clots and bone dust and moved into the chillers until they are processed further. (72, 73).



*- wastewater

Figure 1.3.2 Flow scheme of pig slaughtering and accruing of wastewater. Modified according to [70, 71]

In order to ensure proper food safety and high product quality with a long shelf life, large amounts of water are necessary during processing. This leads to high amounts of wastewater that accrues at different processing steps (Fig. 1.3.1-1.3.2). The primary wastewater sources can be divided into three areas: (1) truck washing and animal sheds (green line); (2) slaughtering process and cutting (red line); (3) stomach, intestine, and entrails cleaning (yellow line) (74).

The accruing process waters and wastewater in poultry and pig slaughterhouses are contaminated by numerous bacteria which mainly origin from livestock faeces. The gastrointestinal tract of pigs and chickens harbors up to 10^8-10^{11} cfu/g bacteria including those with pathogenic potential such as *Clostridium*, *Streptococcus*, *Enterococcus*, *Campylobacter*, *Salmonella*, *Helicobacter* and *Escherichia* (75). Moreover, it may contain various viruses, helminths, protozoa, fungi and Archaea (75).

Wastewater occurring due to the cleaning of animal transport trucks and holding pens contains bedding material, faeces and urine. In the poultry slaughterhouses, bird's stress and struggling during unloading can cause leakage of faeces which are the main source of contamination by

cleaning of transport crates. Furthermore, muscle contractions and convulsions during stunning can also cause faeces to leak from cloaca. Perimortem defecation at the end of the bleeding process is common as well. Furthermore, dirt, faeces and ingesta are brought to the scalding tanks by each carcass. However, enteric bacteria are mostly eliminated by heat. Along with loose feathers and debris, washing water of plucked carcasses contains bacteria from their external surfaces. Similarly, process water used for dehairing of pigs contains hair and other detritus. Moreover, during poultry evisceration, ruptures of the intestinal tract, caecum and crop may occur. This results in spilling of the gut content and subsequent contamination of the equipment, carcasses and water used to rinse the carcasses to clear away blood residues, loosen tissues and debris. Likewise, during the evisceration process of pigs, an accidental cutting of the stomach and intestines is possible that results in spilling of visceral content and microbial contamination. (72, 73, 76)

During the slaughtering process and later during the cleaning of production facilities, especially during prerinsing, pollutant loads related to the slaughtered livestock are being permanently feeding into the main wastewater stream of the slaughterhouse. Thus, slaughterhouse wastewater usually exhibits a high organic content due to solved fibers, proteins and fats and contains considerable amounts of organic carbon (TOC), phosphorus (TP), nitrogen (TN) and suspended solids (TSS) (77). Furthermore, it possesses a high chemical oxygen demand (COD) and a 5-day biochemical oxygen demand (BOD_5) (77). Common characteristics of slaughterhouse wastewater are summarized in Tab 1.3.1 (78).

Table 1.3.1 General chemical characteristics of slaughterhouse wastewater according to [76]

Parameter	**Range**	**Mean**
TOC (mg/L)	80-1300	550
BOD_5 (mg/L)	140-4600	1,200
COD (mg/L)	500-16000	4,200
TN (mg/L)	50-850	440
TSS (mg/L)	260-6500	1,170
pH	4.90-8.10	6.95
TP (mg/L)	25-210	50
Orto-PO_4 (mg/L)	20-95	45
Orto-P_2O_5 (mg/L)	10-80	20
K (mg/L)	0.01-100	90
Color (mg/L Pt scale)	170-400	280
Turbidity (FAU*)	200-300	270

* FAU, formazine attenuation units.

Furthermore, slaughterhouse wastewater contains alkaline and acidic detergents as well as disinfectants such as chlorine and chlorine-releasing compounds, quaternary ammonium compounds, ampholytic compounds, phenolic compounds and paracetic acid used for cleaning and sanitizing of producing facilities (78). Moreover, despite the prescribed withdrawal periods, it may contain residues of antibiotics commonly used in veterinary medicine, such as tylosin, erythromycin, roxithromycin, lincomycin, trimethoprim, ciprofloxacin, norfloxacin, ofloxacin, sulfamethoxazole and chlorotetracycline as described by Chang and colleagues (2010) (79).

Because of the above mentioned contaminants and high organic strength, the direct discharge of untreated livestock wastewater to surface waters is impractical and should be avoided because of the environmental pollution and possible negative effects on human health. Moreover, the fee on wastewater discharge for slaughterhouses is based on the pollution load. Only after a pre-treatment in on-site wastewater treatment systems, the slaughterhouses in Germany discharge their wastewater either directly into a stream or other receiving body (direct dischargers) or to the municipal WWTP (indirect dischargers) (80, 81).

A typical flow scheme of slaughterhouse wastewater treatment is shown in Fig 1.3.3 (76, 78). The pre-treatment of livestock wastewater is mostly done by mechanical procedures. During this treatment step, the TSS are reduced by separating all large solid particles ranging from 0.5 mm to 30 mm by screeners and sieves. Mesh screening reduces TSS by >60%. Due to the dissociation of solid particles, the BOD_5 can be decreased by up to 30% (78, 82). Furthermore, fat, oil and greases are removed using grease traps (82). After removal of suspended solids, a low reduction of the bacterial burden and their antibiotic resistance genes (ARGs) ranging from 0.09 to 0.55 log units can be observed (83). Prior to the further effluent treatment on-site or in municipal WWTPs by different physicochemical and/or biological methods, the pH of wastewater is adjusted in the equalisation tank (82).

Physicochemical treatment usually involves separation of solids from the liquid. It is mostly based on dissolved air flotation (DAF), coagulation and flocculation, electrocoagulation or different membrane technologies (reverse osmosis, nanofiltration, ultrafiltration, microfiltration). During DAF, fat, grease and other light solids are carried to the tank surface forming a sludge blanket and the scum is constantly removed. In addition to fat and grease flotation, blood coagulants such as ferric chloride and aluminum sulfate can be used to promote protein precipitation. During DAF, moderate to high removal of nutrients as well as reducing of COD (30-90%) and BOD_5 (70-80%) can be achieved. (78, 82)

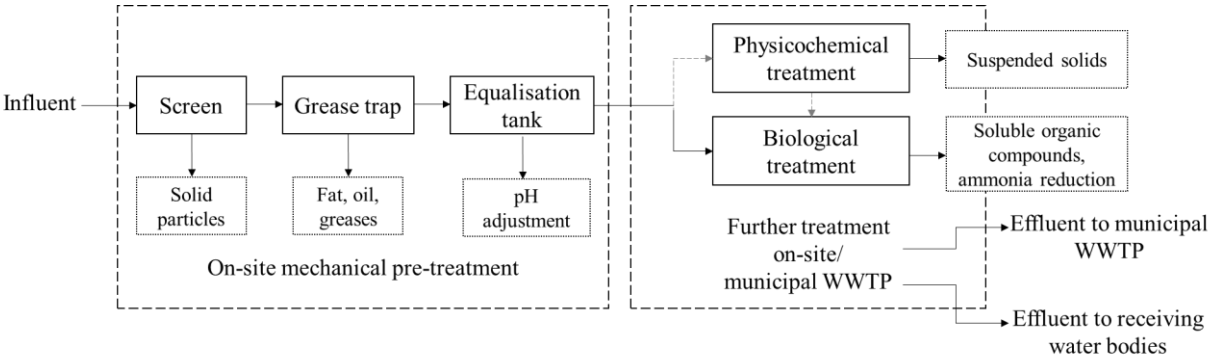


Figure 1.3.3 Flow scheme of slaughterhouse wastewater treatment. Modified according to [76, 78]

Biological treatment is done in order to remove the remained soluble organic compounds and for reduction of ammonia into nitrate and nitrite. It may include various combinations of aerobic and anaerobic digestions using microorganisms. It can decrease BOD_5 by up to 90% and reduce the bacterial load by 1.1 to 3.4 log units (84).

Furthermore, up to now, advanced oxidation processes (AOPs) are becoming an interesting complimentary treatment option to current biological methods which are not designed to remove bacteria. AOPs (ozone treatment, gamma radiation, UV/H_2O_2, UV/ozone treatment) are currently discussed as effective technologies to inactivate microorganisms, especially antibiotic-resistant bacteria and pathogens (84). Study of Jäger and colleagues (2018) reported a high reduction efficacy of facultative pathogenic bacteria and their ARGs for ozone treatment of 98.4% to below the detection limit (10^1 cell equivalents per 100 mL) (84). The combination of UV and ozone treatment showed also a high reduction of the bacterial load and ARGs of 98.4%-99.0% (83). Thus, AOPs can be considered to enhance the quality of wastewater effluents for water reuse purposes when the receiving water body is used for crop irrigation or as a raw water reservoir. In Switzerland since 2015, all WWTPs with high loads (>80,000 inhabitants) and those in the catchment areas of lakes and on rivers with impact on drinking water have been equipped with advanced wastewater treatment systems based on oxidation (i.e. ozonation) (85).

Direct dischargers in the EU with carcass production capacity greater than 50 tons per day need to comply with chemical limits described by EU directive 2010/75/EU (annex I 6.3. a 2010/75/EU) shown in the Tab 1.3.2. (80)

Table 1.3.2 Standard limits for slaughterhouse wastewater discharge in the EU according to EU directive 2010/75/EU.

Parameter, mg/L	EU standards
BOD_5	25
COD	110
Ammonium nitrogen (NH_4-N)	10
TN	18
TP	2

However, at present, no legal limits or reduction levels have been fitted for microbiological pollutants in wastewater in the EU, even though the occurrence of important pathogenic microorganisms with zoonotic potential including species of *Campylobacter*, *Bartonella*, *Salmonella* and *Shigella* causing campylobacteriosis, bartonellosis, salmonellosis and shigellosis, respectively, is well documented in livestock wastewater (86). Moreover, data on the occurrence, phenotypic and genotypic properties of ESKAPE bacteria and ESBL-producing *E. coli* in process waters and wastewater from German poultry and pig slaughterhouses is still lacking. In addition, there is a gap in knowledge regarding their further dissemination into surface waters.

1.4. Research questions and outline of the thesis

The main objective of this thesis is the investigation of clinically-relevant antibiotic-resistant bacteria in process waters and wastewater from poultry and pig slaughterhouses and the assessment of the bacterial dissemination into surface waters.
These objectives lead to the following research questions:

1. What ESKAPE-bacteria and ESBL-producing *E. coli* are prevalent in process waters and wastewater from German poultry and pig slaughterhouses? (chapters 2, 3)

2. What are the characteristics of colistin-resistant *Enterobacteriaceae* and ESBL-producing *Klebsiella* spp. from process waters and wastewater from German poultry and pig slaughterhouses? (chapters 4, 5)

3. Do the recovered target bacteria pose a risk for human health? (chapters 2, 3, 4, 5)

4. How is the dissemination of target bacteria from poultry and pig slaughterhouses into surface waters and the associated risk for human health to be assessed? (chapters 2, 3, 4, 5)

In the first part of the thesis (chapter 2), the occurrence and diversity of ESKAPE-bacteria and ESBL-producing *E. coli* in two German poultry slaughterhouses are investigated. For this purpose, process waters and wastewater that accumulate during operation and cleaning of producing facilities in the delivery and unclean areas are screened for the presence of target bacteria. Furthermore, in order to investigate their dissemination into receiving surface waters, effluents from the in-house WWTPs are examined as well. The recovered isolates are characterized for their antimicrobial resistance phenotypes and are further subjected to different molecular typing approaches, such as *spa*-typing for MRSA, phylogenetic typing and MLST for *E. coli* and VRE. Genes encoding extended-spectrum-β-lactamases and carbapenemases as well as mobilizable colistin resistance genes in *Enterobacteriaceae* and non-fermenters are determined. The data are used to assess their dissemination into surface waters and to analyze the possible risks for human health arising from the presence of livestock-asssociated ESKAPE bacteria and ESBL-producing *E. coli* in investigated samples.

In chapter 3 of the thesis, further samples of process waters and wastewater from German pig slaughterhouses accruing during operation and cleaning of producing facilities in the delivery and unclean areas as well as in their in-house and municipal WWTPs are investigated for the presence of ESKAPE-bacteria and ESBL-producing *E. coli*. Recovered target bacteria are then characterized regarding their phenotypic resistance to clinically important antibiotics and are further epidemiologically typed. Genetic basis of ESBL, CRE and VRE phenotypes as well as colistin-resistance is examined. The generated data are used to identify the potential risks for human health coming up from target bacteria. Moreover, by collecting samples in the on-site preflooders, their subsequent spread into surface waters is also assessed.

In chapter 4, the emergence and characteristics of colistin-resistant *Enterobacteriaceae* (*E. coli, Klebsiella* spp., *Enterobacter cloacae* complex) in process waters and wastewater from German poultry and pig slaughterhouses as well as in their in-house WWTPs are investigated. Antimicrobial resistance of the recovered bacteria is assessed applying epidemiological and clinical breakpoints. Furthermore, polymorphisms of genes encoding PmrAB as well as *mcr*-mediated colistin-resistance (*mcr-1 to -9*) and their ability to transfer this resistance are determined as well. Moreover, the further spread of colistin-resistant *Enterobacteriaceae* into surface waters via municipal WWTPs is analyzed.

In the last chapter (chapter 5), selected ESBL-producing, and colistin-resistant isolates of *Klebsiella* spp. recovered from poultry and pig slaughterhouses as well as municipal WWTPs are characterized in terms of their population structure and antimicrobial resistance genes using whole-genome sequencing. The data are used for the assessment of their relevance for human medicine and possible risks for human health.

The last chapter (chapter 6) displays the conclusions of the entire thesis. It answers the above mentioned research questions in regard to the results from the chapters 2-5.

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2. ESKAPE-bacteria and Extended-Spectrum-β-Lactamase-Producing-producing *Escherichia coli* from wastewater and process water from German poultry slaughterhouses

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2.1. Abstract

Wastewater of livestock slaughterhouses is being considered as a source of antimicrobial-resistant bacteria with clinical relevance and may thus be important for their dissemination into the environment. To get an overview on their occurrence and characteristics, we have investigated process water (n=50) from delivery and unclean areas as well as wastewater (n=32) from in-house wastewater treatment plants of two German poultry slaughterhouses (S1, S2).

The samples were screened for ESKAPE-bacteria (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp.) and *Escherichia coli*. Their antimicrobial resistance phenotypes, ESBL (extended spectrum β-lactamase), carbapenemase and mobilizable colistin resistance genes were determined. Selected ESKAPE-bacteria were epidemiologically classified using different molecular typing techniques.

At least one of the target species was detected in 87.5% (n=28/32) of the waste- and 86.0% (n=43/50) of the process water samples. The vast majority of the recovered isolates (94.9%, n=448/472) was represented by *E. coli* (39.4%), *A. calcoaceticus-baumannii* (ACB)-complex (32.4%), *S. aureus* (12.3%) and *K. pneumoniae* (10.8%), which were widely distributed in the delivery and unclean areas of the individual slaughterhouses including their wastewater effluents. *Enterobacter* spp., *Enterococcus* spp. and *P. aeruginosa* were less abundant and made up 5.1% of the isolates. Phenotypic and genotypic analyses revealed that the recovered isolates exhibited diverse resistance phenotypes and β-lactamase genes. In conclusion, wastewater effluents from the investigated poultry slaughterhouses exhibited clinically relevant bacteria (*E. coli*, MRSA, *K. pneumoniae*, species of the ACB-and *E. cloacae*-complexes) that contribute to the dissemination of clinically relevant resistances (i.e. $bla_{\text{CTX-M/SHV}}$, *mcr*-1) in the environment.

2.2. Importance

Bacteria from livestock may be opportunistic pathogens and carriers of clinically relevant resistance genes, as many antimicrobials are used both in veterinary and human medicine. They may be released into the environment from wastewater treatment plants (WWTPs) that are influenced by wastewater from slaughterhouses, thereby endangering public health. Moreover, process water that accumulates during slaughtering of poultry is an important reservoir for livestock-associated multidrug-resistant bacteria and may serve as a transmission vector to occupationally exposed slaughterhouse employees. Mitigation solutions aiming at the reduction of the bacterial discharge into the production water circuit as well as intervention of their further

transmission and dissemination need to be elaborated. Furthermore, the efficacy of in-house WWTPs needs to be questioned. Reliable data on the occurrence and diversity of clinically relevant bacteria within slaughtering production chain and in the WWTPs effluents in Germany will help to assess their impact on public and environmental health.

2.3. Introduction

Nowadays, antimicrobial-resistant bacteria involved in community and healthcare associated infections give cause for serious concern for global public health. Thus, the treatment of these infections is currently one of the main challenges for humanity (1). Together with *Escherichia coli* (*E.*), multidrug-resistant ESKAPE-bacteria (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) cause the majority of life-threatening bacterial infections in health care facilities among critically ill and immunocompromised patients, worldwide (2, 3). However, neither commensal *E. coli* nor ESKAPE-bacteria are generally pathogenic (4) as most of them (i.e. *E. coli*, *S. aureus*, *K. pneumoniae*, *Enterobacter* spp., *Enterococcus* spp.) are natural colonizers of humans (5) and animals (i.e. livestock) (6–8). In contrast, *Acinetobacter* spp. and *P. aeruginosa* are prevalent in soil and aquatic environments, and information on their natural occurrence in animals or whether they are associated with transmission from animals to humans is scarce (9–11).

After discharge into the environment through feces and wastewater, ESBL producing Enterobacteriaceae (i.e. *E. coli*, *K. pneumoniae*, *Enterobacter* spp.) and *Enterococcus* spp. may be highly prevalent in soil, plants and surface water, and may thus pose a risk for the colonization of humans (12–15), pets (16) and livestock (17, 18). Interaction with environmental pollutions (19, 20), as well as contaminated rural environments (21, 22) and food products (23, 24) can under unfortunate circumstances influence the composition of the microbial community of humans and animals by a colonization with resistant ESKAPE-bacteria.

One of the main properties of ESKAPE-bacteria is their ability to efficiently adapt to altered environmental conditions by exchanging genetic material with other microorganisms via horizontal gene transfer (25–27). The acquisition of resistance determinants by clinically relevant bacteria can lead to an increased frequency of treatment failures and severity of human infections, especially if resistances concern antimicrobials classified as critical or highly important for human medicine (27, 28). Notable examples are the emergence of (i) extended-spectrum β-lactam (ESBL)-/fluoroquinolone-resistant Enterobacteriaceae (3MDRO) (29, 30)

in health-care settings (31) and in the poultry-production chain (19, 32), (ii) carbapenem-resistant Enterobacteriaceae in broilers, pigs and meat products (33, 34) as well as (iii) ESBL-producing *E. coli* carrying mobilizable colistin resistance (*mcr)* genes (35). Emergence of antimicrobial resistances in ESKAPE-bacteria is often attributed with an inappropriate use of antibiotics in human and veterinary medicine (36) followed by successive dissemination into the environment, and transfer to animals and humans. Results of various studies on related isolates of MRSA (methicillin-resistant *S. aureus*), VRE (vancomycin-resistant enterococci), ESBL-producing *E. coli, K. pneumoniae, A. baumannii* from different compartments (i.e. humans, livestock, food) (37–41) support the hypothesis of livestock as a source for clinically relevant bacteria.

Due to high numbers of processed animals, waters from different production steps of slaughterhouses represent sources of ESKAPE-bacteria. Furthermore, insufficient treatment in their in-house wastewater treatment plants (WWTPs) could provide a conduit for the discharge of clinically relevant and/or resistant bacteria into the environment and community (42–44) as recently reported for colistin-, carbapenem- and extremely drug-resistant bacteria (XDR) in communal, clinical and urban German wastewater (20, 45).

The objective of the present study was to evaluate the occurrence and diversity of ESKAPE-bacteria and *E. coli* along different slaughtering steps of two German poultry slaughterhouses. For this purpose, process water from washing of poultry transport trucks and vehicles, stunning facilities, scalding and eviscerating as well as wastewater effluents of the in-house wastewater treatment plants were subjected to bacteria specific isolation procedures. Beside species identification and antimicrobial resistance testing, the recovered bacteria were subjected to molecular epidemiological classification (phylogenetic, and multilocus sequence typing (MLST) of *E. coli* and VRE, *spa*-typing of MRSA) and determination of the genetic basis of the β-lactam, carbapenem- and mobilizable colistin resistance. Data were used for comparison of the content of target bacteria in waters obtained from different slaughtering steps. Based on the results the impact of clinically relevant bacteria released into the environment by insufficient wastewater management was assessed.

2.4. Materials and Methods

2.4.1. Sampling and sample preparation

Two German poultry slaughterhouses (S1 and S2) exhibiting different slaughtering capacities above 100,000 chickens per day were investigated. S1 and S2 produce daily 600 m^3 and

3,600 m^3 wastewater, respectively. S1 operates a wastewater treatment plant (WWTP) based on the membrane bioreactor (MBR) technology with immersed ultrafiltration membranes. S2 possesses a conventional biological WWTP. After treatment, effluents are discharged into the pre-flooder and further into surface water bodies.

The collected samples represent various waters that accumulate during the delivery, in the unclean area of poultry slaughtering process as well as in their in-house WWTPs. Samples were taken at seven sampling sites: transport trucks (only S2), transport crates, stunning facilities, scalders, eviscerators, production facilities (only S1), influent and effluent of the in-house WWTPs. Sampling of both slaughterhouses was conducted by five independent visits between December 2016 and September 2018. Three further visits were conducted to obtain additional samples from the in-house WWTPs of S1 and S2 in the same time period. A minimum time interval of one month was kept between two independent sampling visits to minimize a possible carry-over of targeted bacteria from poultry folks originating from the same fattening farm and so to ensure that the individual samplings would be representative for different poultry populations.

From each individual sample, one liter was collected using sterile Nalgene® Wide Mouth Environmental Sample Bottles (Thermo Fisher Scientific, Waltham, MA, USA). Composite samples from pre-cleaning of five poultry trucks after unloading of birds (only S2) and water samples applying by pre-cleaning of the stunning facilities were collected by catching runoffs. In general, all pre-cleaning steps were conducted without using cleaning and disinfection agents. Water samples from cleaning of poultry transport crates and scalding water were taken by immersion of sterile sampling bottles into the sump of the crate washing facility and the scalder tank, respectively. Process water during evisceration was collected as runoffs from eviscerators in operation. Aggregate wastewater from production facilities (only S1) was taken by immersion of sterile sampling bottles into mixing and homogenization containers after the wastewater had run through a mechanical deposition. The samples of influent and effluent of in-house WWTPs were taken as qualified samples according to the German standard methods for the examination of water, wastewater and sludge (DIN 38402-11:2009-02) (46). The samples were labeled and transported to the laboratory cooled in a Styrofoam box at 5±2°C. To get rid of coarse particles (e.g. bedding, feathers), they were manually filtered using stomacher strainer bags with tissue filter (pore size 0.5 mm, VWR, Radnor, PA, USA) and afterwards subjected to cultivation within 24 h after sampling.

2.4.2. Cultivation and identification of antimicrobial-resistant target bacteria

Water samples were screened for Gram-negative ESBL-producing bacteria of the Enterobacteriaceae, non-fermenting *A. baumannii* and *P. aeruginosa* as well as for MRSA (methicillin-resistant *S. aureus*), VRE (vancomycin-resistant enterococci) and carbapenemase-producing Enterobacteriaceae (CPE). To detect ESBL-producing target bacteria and VRE, 100 µL aliquots of serial 10-fold dilutions or 1 mL of undiluted samples were applied to CHROMagar™ ESBL and CHROMagar™ VRE plates (MAST Diagnostica, Reinfeld, Germany) for cultivation. Furthermore, 10 and 100 mL aliquots of the in-house WWTPs effluent were filtered through sterile 0.45 µm, 47 mm mixed cellulose nitrate filters (GE Healthcare, Chicago, IL, USA) and placed on selective agar plates. To inhibit the growth of accompanying bacteria, all agar plates were incubated at 42°C for 18-24 h (ESBL-producing target bacteria) and for 42-48 h (VRE).

MRSA were isolated following the recommendations of the National Reference Laboratory for Staphylococci with some modifications. For this purpose, 100 mL of water samples were (i) filtered through sterile 0.45 µm, 47 mm mixed cellulose nitrate filters (only effluents of the in-house WWTPs) or (ii) centrifuged for 15 min at 5,000×g and 4°C. The filters or resulting pellets were transferred to 100 mL of Mueller Hinton broth (MHB, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 6.5% NaCl for pre-enrichment. After incubation at 37°C for 18-24 h under shaking (150 rpm), 1 mL of the pre-enrichment broth was transferred to 9 mL of Tryptic Soy Broth (TSB, Sigma-Aldrich, St. Louis, MO, USA) supplemented with aztreonam (50 mg/L) and cefoxitin (3.5 mg/L). Inoculated selective pre-enrichment broth was cultivated for 18-24 h at 37°C. Afterwards a 10 µl loop of culture was streaked out on CHROMagar™ MRSA (MAST Diagnostica, Reinfeld, Germany) screening agar and incubated at 42°C for 18-24 h.

For the isolation of CPE, a selective pre-enrichment was carried out. Therefore, 10 ml water samples were subjected to filtration through 0.45 µm membrane filters. The filters were incubated at 42°C for 18-24 h in Mossel Broth (Sigma-Aldrich, St. Louis, MO, USA) under aerobic conditions to inhibit the growth of accompanying flora (i.e. Gram-positive microorganisms). Thereafter, 100 µl of the selective pre-enrichment broth was plated on CHROMagar™ mSuperCarba (MAST Diagnostica, Reinfeld, Germany) plates and incubated at 42°C for 18-24 h.

Whenever possible, up to four presumptive colonies per sampling site of *E. coli*, *Klebsiella* spp., *Enterobacter* spp. and *Citrobacter* spp., *Acinetobacter* spp., *Pseudomonas* spp., MRSA as well as VRE were picked from the selective plates and sub-cultured on Columbia Agar

comprising 5% sheep blood (MAST Diagnostica, Reinfeld, Germany) at 37°C for 18-24 h. Presumptive coliform bacteria were confirmed by streaking on Chromocult Coliform Agar (Merck, Darmstadt, Germany) and oxidase testing. The non-fermenting *Acinetobacter* spp. and *Pseudomonas* spp. were sub-cultured on CHROMagar™ *Acinetobacter* agar (MAST Diagnostica, Reinfeld, Germany) and Cetrimide agar (Merck, Darmstadt, Germany), respectively, and confirmed by oxidase testing. Potential VRE colonies were streaked onto Slanetz Bartley agar (Merck, Darmstadt, Germany).

Species identification was conducted using MALDI-TOF MS employing a VITEK MS mass spectrometer (bioMérieux, Marcy l'Etoile, France) equipped with the Myla™ software. All isolates were purified on Columbia Agar comprising 5% sheep blood and preserved in cryotubes (Mast Diagnostics, Reinfeld, Germany) at -70°C.

2.4.3. Antimicrobial susceptibility testing

Gram-negative bacteria were tested against 17 antimicrobials or antimicrobial combinations by microdilution method according to protocols of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) using Micronaut-S MDR MRGN-Screening system (MERLIN, Gesellschaft für mikrobiologische Diagnostika GmbH, Bornheim-Hersel, Germany). Resistance testing of Gram-positive bacteria was conducted using the MICRONAUT-S MRSA/GP testing panel. Results were interpreted according to clinical cut-off values (EUCAST Version 9.0) in order to determine the resistance profiles of ESKAPE-bacteria of livestock origin against medically important antimicrobials for humans and to assess their clinical relevance in human medicine. 3MDRO classification of the isolates was done according to the recommendations of the Commission for Hospital Hygiene and Infection Control of 2012 (KRINKO) at the Robert Koch-Institute Berlin, i. e. intermediate was interpreted as resistant (47).

Isolates resistant to combinations of β-lactam/β-lactamase inhibitor combinations were further screened for AmpC enzyme production using the AmpC test D69C (Mast Diagnostica, Reinfeld, Germany).

2.4.4. Molecular detection and typing

For molecular analyses, the template DNA for PCR experiments was prepared from bacterial suspensions in10 mM Tris-EDTA pH 8.0 (Sigma-Aldrich, St. Louis, MO, USA) according to the TE boiling lysate method (48). ESBL-producing coliforms were screened for the presence of β-lactamase genes belonging to the bla_{TEM}, bla_{SHV} and $bla_{\text{CTX-M}}$ families. To this end,

detection assays were used as previously described (49–51). For subtyping, *bla*$_{CTX-M}$-positive samples were further investigated as previously described (52). Sanger sequencing was performed at Microsynth Seqlab (Göttingen, Germany) using PCR amplicons purified with the innuPREP DOUBLEpure Kit (Analytik Jena AG, Jena, Germany). Sequence analysis was conducted with Chromas lite v.2.6.5 (Technelysium Pty Ltd), BioEdit v.7.2.5 (53) and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/).

According to the recommendations of EUCAST (54), isolates of Enterobacteriaceae picked from the CHROMagar ESBL plates (meropenem cut-off of >0.125 mg/L) as well as from the CHROMagar mSuperCarba plates, were screened for the presence of the carbapenemases genes *bla*$_{NDM}$, *bla*$_{IMI}$, *bla*$_{VIM}$, *bla*$_{OXA-48}$, *bla*$_{KPC}$ and *bla*$_{GES}$ by multiplex realtime TaqMan PCR assays (55, 56). *Acinetobacter* spp. isolates were investigated for the presence of *bla*$_{PER}$, *bla*$_{GES}$, and *bla*$_{VEB}$ by PCR and sequencing (57).

Detection of *mcr*-genes among colistin-resistant isolates (MIC>2 mg/L) was conducted by conventional PCR (58). As PCR controls, the isolates *E. coli* R2749 (*mcr*-1), *E. coli* KP37 (*mcr*-2), *S.* Typhimurium SSI_AA940 (*mcr*-3), *S.* Typhimurium R3445 (*mcr*-4), *E. coli* 10E01066 (*mcr*-5) were used. Sequence-based typing of *mcr*-1 amplicons was performed as previously described (59).

Determination of phylogenetic groups (A, B1, B2, C, D, E, F, clade I) of *E. coli* and MLST of selected isolates (i.e. extraintestinal pathogenic *E. coli* (ExPEC), 3MDRO carrying *bla*$_{CTX-M}$) was conducted using the methods of Clermont (60) and Wirt et al. (61), respectively. Sequence based MLST-typing was performed using EnteroBase (http://enterobase.warwick.ac.uk).

For *spa*-typing of MRSA isolates, the *Staphylococcus* protein A repeat region was amplified and sequenced as previously described (62). *spa*-types were predicted using the SpaServer Ridom software (http://www.spaserver.ridom.de). Vancomycin-resistant enterococci were screened for *vanA*, *vanB*, *vanC1* and *vanC2* genes by multiplex PCR assay as previously described (63) and MLST typed using the method of Homan (64). Sequence data was analyzed using PubMLST (https://pubmlst.org/efaecium).

2.5. Results

2.5.1. Clinically relevant resistant bacteria were detected in the majority of the investigated samples

Within this study, 41 samples were collected from the individual slaughterhouses S1 and S2 at seven sampling points: transport trucks (only S2, n=5), transport crates (each n=5), stunning

facilities (each n=5), scalding water (each n=5), eviscerators (each n=5), production facilities (only S1, n=5), influent (each n=8) and effluent (each n=8) of the in-house WWTPs. Further information on the sampling campaign dates is summarized in Table A1.

Overall, 92.7% (n=38) and 80.5% (n=33) of the samples from S1 and S2, respectively, were positive for at least one of the ESKAPE-target bacteria or ESBL-producing *E. coli* (Fig. 2.5.1). Detailed information about the proportion of positive samples per detected species at each sampling point in S1 and S2 is given in Table A1.

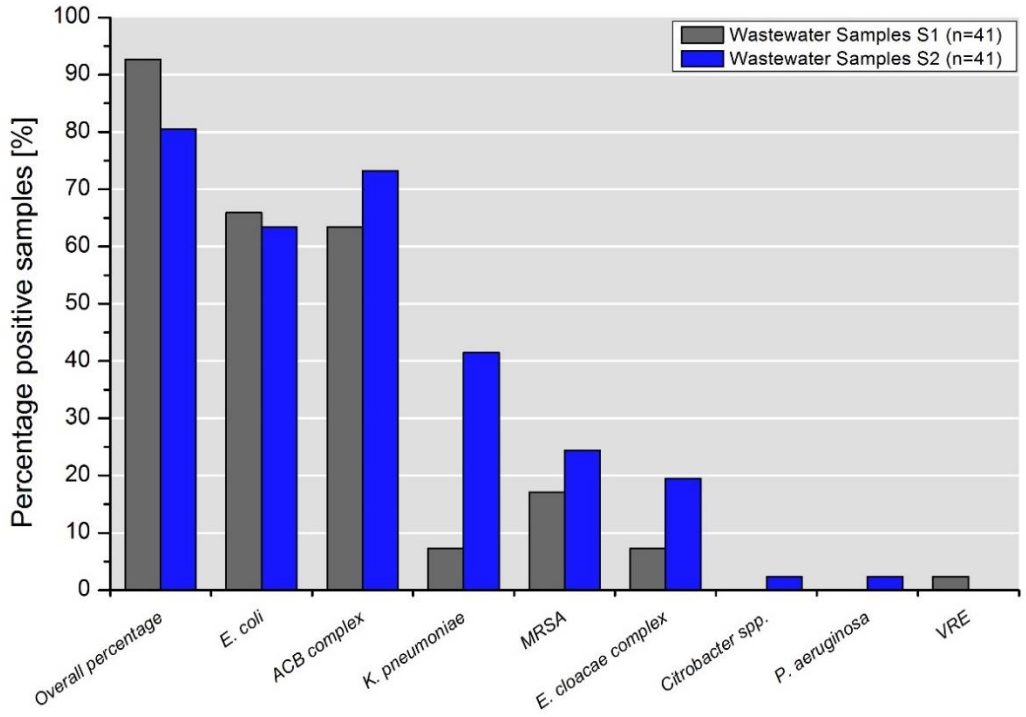

Figure 2.5.1 Percentage of positive samples per target bacteria in S1 and S2.

Escherichia coli (65.9%, n=27) and isolates of the ACB-complex (63.4%, n=26) growing on ESBL selection plates were detected in S1 at all seven sampling points (Fig. 2.5.2). VRE were detected only in one sample (2.4%) from cleaning of poultry transport crates, Fig. 2.5.2A. Interestingly, 75% of effluent samples (n=6) of the S1 WWTP were positive for ACB-complex growing on ESBL agar plates, whereas ESBL-producing *E. coli* (25.0%, n=2) and MRSA (12.5%, n=1) were detected less frequently (Table A1). Similar to slaughterhouse S1, isolates of the ACB-complex and *E. coli* were selected on ESBL plates in 73.2% (n=30) and 63.4% (n=26) of S2, respectively. In contrast to S1, ESBL-producing *K. pneumoniae* were detected in 41.5% (n=17) of S2 at six out of seven sampling points (Fig. 2.5.2B).

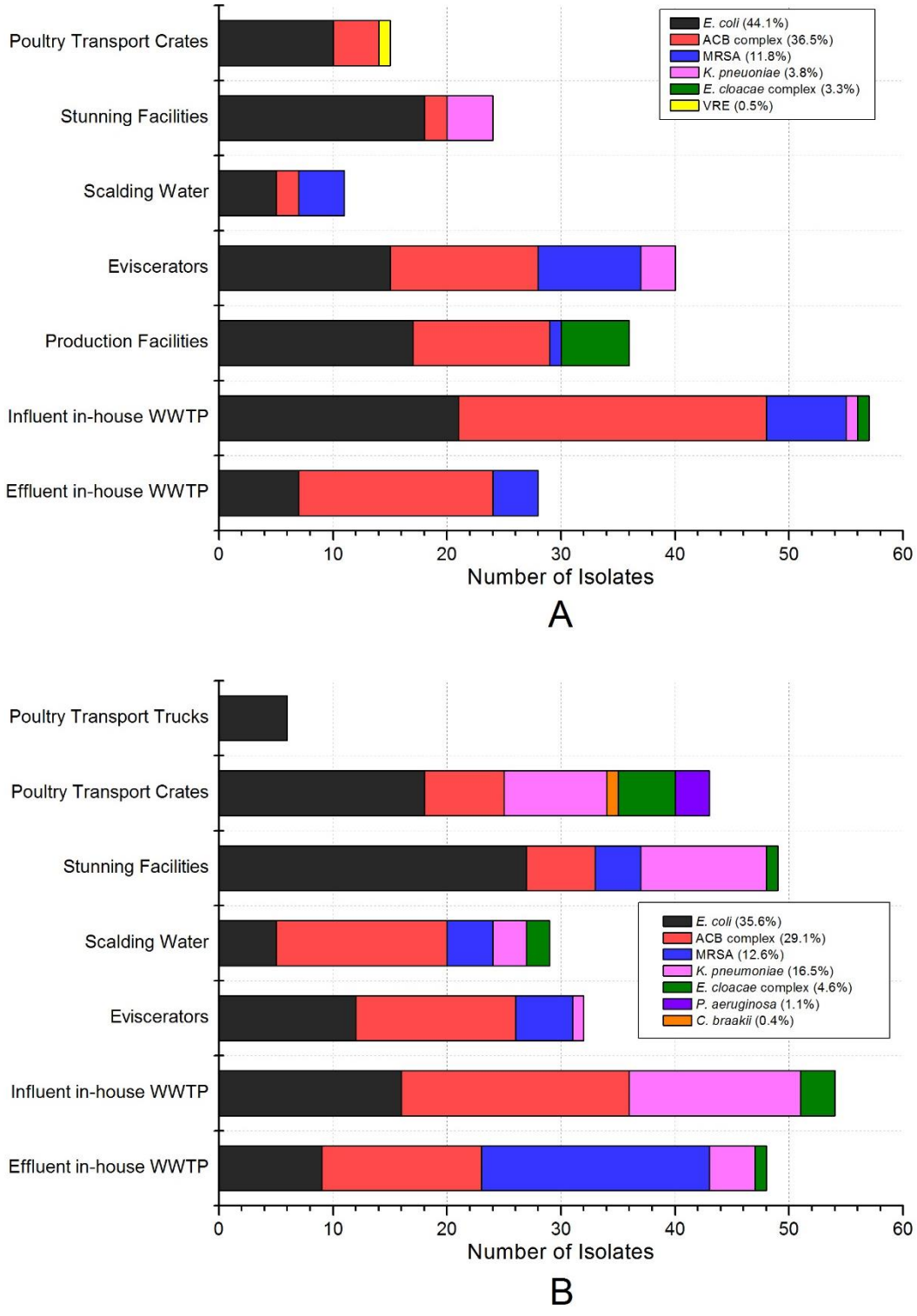

Figure 2.5.2 Occurrence of target bacteria across the sampling points in the slaughterhouses (A) S1 (n=211) and (B) S2 (n=261).

Furthermore, growth of *Citrobacter* spp. and *P. aeruginosa* occurred sporadically in one sample (each 2.4%). Effluent samples of the S2 WWTP were positive for ACB-complex isolates (75.0%, n=6), MRSA (62.5%, n=5), *E. coli* (37.5%, n=3), *K. pneumoniae* (25.0%, n=2) and isolates (12.5%, n=1) of the *E. cloacae*-complex (Table A1).

2.5.2. Target bacteria exhibited a high diversity of antimicrobial resistance phenotypes

An overview on the antimicrobial resistance of the investigated target bacteria is presented in Figure 2.5.3. It has to be kept in mind, however, that the strains were isolated from selective agar plates containing cephalosporins, oxacillin or vancomycin, therefore susceptibility to the selective agents cannot be expected.

Almost all *E. coli* isolates (n=186) showed resistance to penicillins (piperacillin) and 3rd generation cephalosporins (cefotaxime, ceftazidime). The levels of 3MDRO resistant against three antibiotics (i.e. piperacillin, ciprofloxacin and 3rd generation cephalosporins) were at 50.5% (S1, n=47/93) and 40.9% (S2, n=38/93). However, only one *E. coli* isolate from S2 (1.1%, n=1) expressed resistance against ciprofloxacin, 3rd generation cephalosporins and piperacillin in combination with tazobactam. While 10.8% (S1, n=10) and 8.6% (S2, n=8) of the isolates were resistant to colistin, all isolates were susceptible to meropenem, imipenem and amikacin.

Similar to *E. coli*, almost all KEC isolates (*Klebsiella* spp., *E. cloacae*-complex, *Citrobacter* spp., n=71) from slaughterhouse S1 (n=15) and S2 (n=56) showed resistance against piperacillin and 3rd generation cephalosporins. However, in comparison to the *E. coli* isolates, the percentages of 3MDRO bacteria were higher in S1 (60.0%, n=9) and S2 (67.9%, n=38). Furthermore, none of the KEC isolates from S1 and 19.6% of the S2 KEC (n=11) were resistant against ciprofloxacin, 3rd generation cephalosporins and piperacillin in combination with tazobactam. In contrast to S1, 17.9% (n=10) and 26.8% (n=15) of KEC isolates from S2 exhibited resistance against tigecycline and ceftolozan/tazobactam, respectively. Furthermore, 17.9% (n=10) of the KEC isolates from S2 were resistant to colistin.

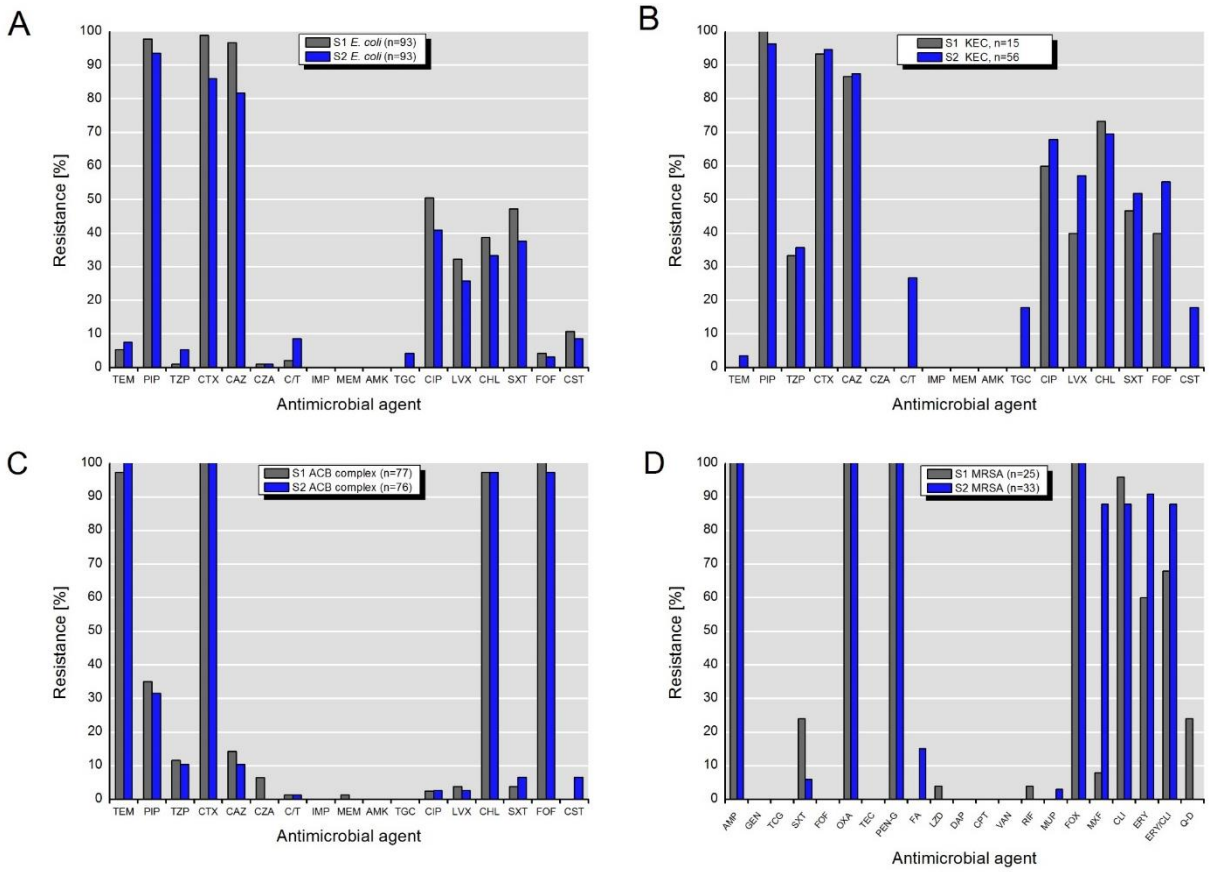

Figure 2.5.3 Resistance to antimicrobial agents detected among isolates of (A) *E. coli*, (B) *K. pneumoniae*, *E. cloacae*-complex, *Citrobacter* spp., (C*) ACB-complex and (D) MRSA.

Abbreviations for antimicrobial agents: TEM, temocillin; PIP, piperacillin; TZP, piperacillin-tazobactam; CTX, cefotaxime; CAZ, ceftazidime; CZA, ceftazidime-avibactam; C/T, ceftolozane-tazobactam; IMP, imipinem; MEM, meropenem; AMK, amikacin; TGC, tigecycline; CIP, ciprofloxacin; LVX, levofloxacin; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin; AMP, ampicillin; GEN, gentamicin; OXA, oxacillin; TEC, teicoplanin; PEN-G, penicillin G; FA, fusidic acid; LZD, linezolid; DAP, daptomycin; CPT, ceftaroline; VAN, vancomycin; RIF, rifampicin; MUP, mupirocin; FOX, cefoxitin; MXF, moxifloxacin; CLI, clindamycin; ERY, erythromycin; Q-D, synercid (quinupristin-dalfopristin).
For temocillin by Enterobacteriaceae a breakpoint of S (≤32) and R (>32) from British Society for Antimicrobial Chemotherapy (BSAC) was used (BSAC 2016), as there are currently no EUCAST or CLSI breakpoints.
* Species of ACB-complex are considered intrinsically resistant against temocillin, cefotaxime, chloramphenicol and fosfomycin

While all isolates of the *A. calcoaceticus-baumannii*-complex (ACB, n=153) from S1 and S2 were resistant to cefotaxime (this species is considered intrinsically resistant to cefotaxime, fosfomycin and trimethoprim according to EUCAST Expert Rules v. 3.1, 2016), their resistance levels against piperacillin (S1, 35.1%; S2, 31.6%) and ceftazidime (S1, 4.3%; S2, 10.5%) were lower. The rates of 3MDRO were equally low at 2.6% (n=2). A resistance against colistin was detected only in the ACB-complex isolates from S2 (6.6%, n=5). While all isolates were susceptible against imipenem, amikacin and tigecycline, one isolate from S1 was meropenem-resistant.

AmpC β-lactamase production was detected only in some isolates exhibiting resistance against combinations of β-lactam/β-lactamase inhibitor (S1, n=24; S2, n=42). Of these, 8.3% (n=2) were detected in the slaughterhouse S1 and 7.1% (n=3) in S2, representing isolates of the ACB-complex (n=2) from S1 as well as isolates of the *E. cloacae*-complex (n=2) and *K. pneumoniae* (n=1) from S2.

Antimicrobial resistance testing of MRSA (n=58) from S1 (n=25) and S2 (n=33) showed that all isolates were resistant to oxacillin, ampicillin, penicillin G and cefoxitin. MRSA from S2 showed high rates of resistance to erythromycin (90.9%) and erythromycin/clindamycin (87.9%). Furthermore, almost all MRSA from S2 (87.9%, n=29) were resistant to moxifloxacin, whereas the percentage of such resistance among the isolates from S1 was 8.0% (n=2).

The single VRE isolate from S1 was identified as *E. faecium* and was determined to be resistant against oxacillin, penicillin G, gentamycin, clindamycin, daptomycin, erythromycin, vancomycin, combination of erythromycin and clindamycin as well as cefoxitin.

2.5.3. Characterization of β-lactamase genes (blaESBL and carbapenemase genes)

ESBL *E. coli* (n=186), *K. pneumoniae* (n=51), the *E. cloacae*-complex (n=19) and *Citrobacter* spp. (n=1) were screened for the presence of *bla*-genes of the enzymes SHV, TEM and CTX-M.

Among 93 *E. coli* from slaughterhouse S1, the most common gene was bla_{TEM} (51.6%, n=48), followed by $bla_{\text{CTX-M}}$ (33.3%, n=31) and bla_{SHV} (15.1%, n=45). Further analysis revealed following subtypes TEM-116, TEM-52c and TEM-1. Among CTX-M positive isolates, the subtypes CTX-M-15 and CTX-M-1 were identified. bla_{SHV}-carrying isolates exhibit the variants SHV-12 and SHV-2a (Fig. 4).

Similar to the *E. coli* isolates from S1, bla_{TEM} (46.2%, n=43/93) was the most frequent gene among the *E. coli* isolates from S2, followed by $bla_{\text{CTX-M}}$ (29.0%, n=27) and bla_{SHV} (24.7%,

n=23). Further sequencing revealed that TEM-52, CTX-M-1, SHV-12 and TEM-116 were present in the vast majority of the isolates (81.5%). The minority of *bla* subtypes (18.5%) were represented by TEM-1, CTX-M-15, SHV-2, SHV-2a as well as SHV-38 and TEM-20 (Fig. 2.5.4).

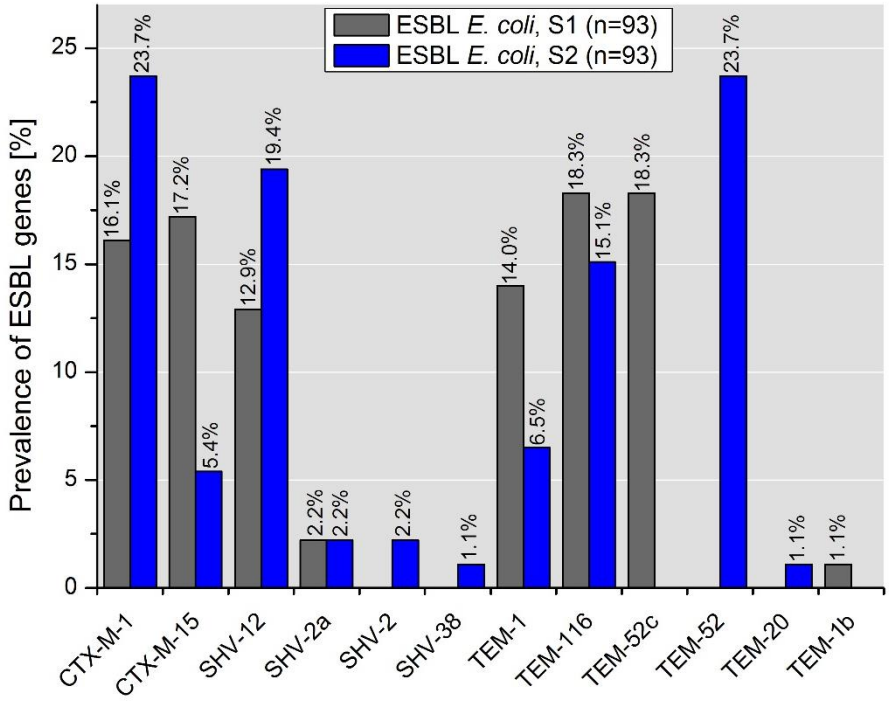

Figure 2.5.4 Distribution of single ESBL types in *E. coli* isolates from the slaughterhouses S1 and S2.

The majority of the isolates of the *E. cloacae*-complex (84.2%, n=16/19) were negative for the tested ESBL genes. Only two isolates from S1 (33.3%) and one from S2 (7.7%) carried $bla_{\text{SHV-12}}$. Among bla_{ESBL} genes of *K. pneumoniae* from slaughterhouse S1 (n=8), SHV-2 was produced by 50.0% of the isolates, followed by SHV-28 (25.0%), SHV-27 and SHV-1 (each 12.5%). Similar to the S1 isolates, the ESBL genotype of *K. pneumoniae* (n=43) from S2 was mainly represented by bla_{SHV} subtypes (97.7%). While the majority of the isolates (79.1%) expressed SHV-2, SHV-27 and SHV-1, SHV-28, SHV-25, CTX-M-1 as well as combinations of SHV-2/TEM-1b, SHV-27/TEM-52b was only produced by some *K. pneumoniae* (Fig. 2.5.5). Enterobacteriaceae isolates from CHROMagar ESBL plates (meropenem cut-off >0.125 mg/L, n=10) as well as from the CHROMagar mSuperCarba plates (n=22) were negative for the tested carbapenemase genes in the molecular screening. However, we found two isolates of the ACB-complex (2.6%) from S1 that were positive-tested for bla_{PER} and bla_{GES}.

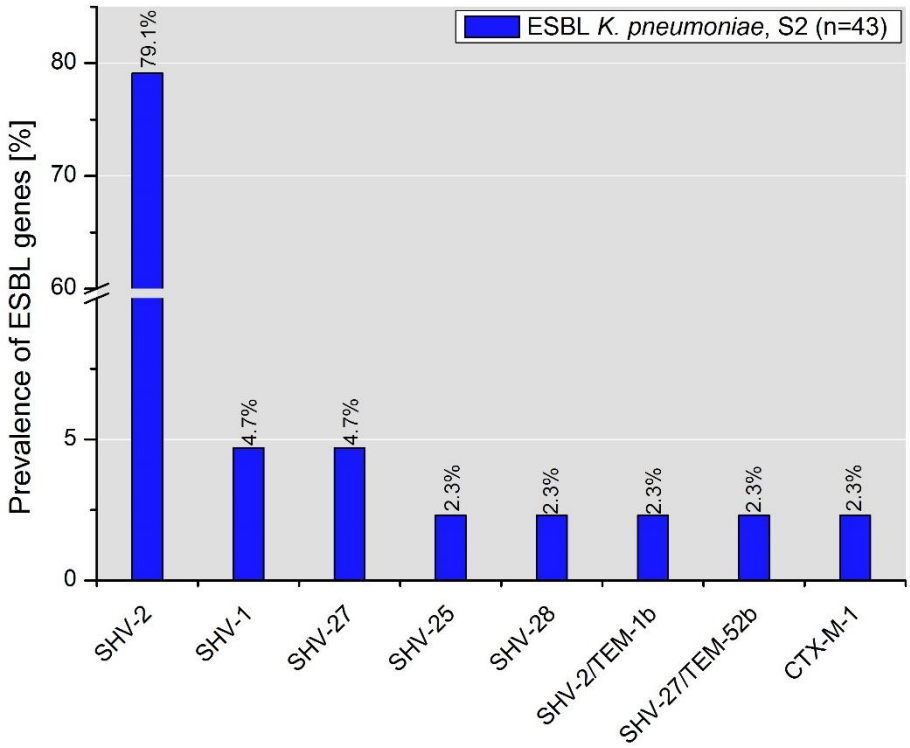

Figure 2.5.5 Distribution of single ESBL types in *K. pneumoniae* isolates from the slaughterhouse S2.

Among isolates from S1, resistance to colistin was observed only in *E. coli* (10.8%, n=10), while in S2, isolates of *E. coli* (8.6%, n=8), *K. pneumoniae* (14.0%, n=6), the *E. cloacae*-complex (33.3%, n=4), the ACB-complex (6.5%, n=5) and *P. aeruginosa* (n=2) were detected. Among colistin-resistant *E. coli* isolates from S1 and S2, 80% (n=8) and 62.5% (n=5), respectively, carried the molilizable colistin resistance gene mcr-1. Beside *E. coli*, *mcr-1* was also detected in 50% of the *K. pneumoniae* (n=3). Sanger sequencing of *mcr*-1 amplicons revealed that all analyzed isolates exhibited the *mcr-1.1*.

2.5.4. Phylogenetic and MLST-typing of E. coli confirmed that the isolates mainly belong to the commensal bacteria

The majority of *E. coli* from slaughterhouse S1 (67.7%, n=63) belonged to the phylogroups C (34.4%, n=32) and B1 (33.3%, n=31), which are mainly represented by commensal isolates. The less abundant phylogenetic groups of the *E. coli* isolates from S1 are A (8.6%, n=8), E (4.3%, n=4) and F (3.2%, n=3). The virulence-associated groups B2 and D were represented by 14.0% (n=13) and 2.2% (n=2) of the isolates, respectively. These isolates mainly originated from cleaning samples of eviscerators and aggregate wastewaters from the production facilities (each 33.3%, n=5), but also from the influent of the in-house WWTP (20.0%, n=3) as well as

from cleaning samples of poultry transport crates and effluent of the in-house WWTP (13.4%, n=2). The rate of 3MDRO resistance among ExPEC isolates (26.7%, n=4) was lower in comparison to overall 3MDRO resistance level among ESBL-producing *E. coli* from S1 (50.5%, n=47). The large majority of the B2 isolates (84.6%, n=11) carried $bla_{\text{SHV-12}}$, whereas the remaining B2 isolates and D isolates harbored $bla_{\text{TEM-52c}}$ (each 15.4%, n=2).

Similar to *E. coli* isolates from S1, one of the most predominant phylogenetic groups among the S2 *E. coli* was B1 (36.6%, n=34). The phylogroups A and C exhibited equal proportions of 17.2% (n=16), followed by group E and F (each 14.0%, n=13). One isolate recovered from the effluent of the in-house WWTP belonged to group D (1.1%) and no isolates of group B2 were detected.

MLST was performed on isolates (i) belonging to phylogenetic groups B2 and D (n=16), (ii) recovered from the effluent of the in-house WWTPs (n=12) as well as from (iii) *E. coli* carrying either $bla_{\text{CTX-M-1}}$ or $bla_{\text{CTX-M-15}}$ and expressing a 3MDRO phenotype (n=34). Overall, 71.0% (n=44) were assigned to twelve known STs and 29.0% (n=18) exhibited 13 not yet assigned STs.

MLST-typing of *E. coli* from phylogroups B2 and D (S1, n=15) revealed that ST4994 was the most predominant sequence type among group B2 isolates (84.6%, n=11). Other individual isolates were classified as ST135 (n=1) or belong to a yet unknown sequence type (n=1). Among isolates of the phylogroup D (n=2, $bla_{\text{TEM-52c}}$) the sequence types ST69 and ST648 were detected. Isolates recovered from the effluent of the WWTPs (n=6) were assigned to ST361 (n=2/6, group C, $bla_{\text{CTX-M-15}}$), whereas the remaining isolates (n=4) exhibited yet unassigned types. Among the *E. coli* isolates carrying $bla_{\text{CTX-M}}$ genes with 3MDRO phenotype (n=19), ST361 (78.9%, n=15) was the most predominant sequence type. Four isolates could not be allocated to previously reported STs.

The isolate of the phylogroup D from S2 was assigned to ST117 and expressed SHV-12. Four further isolates from the in-house WWTP effluent (n=4/8) were assigned to ST10 (n=2/8, group A, $bla_{\text{TEM-52c}}$ and $bla_{\text{SHV-12}}$, respectively), ST101 (n=1/8, group B1, $bla_{\text{CTX-M-1}}$) and ST212 (n=1/8, group B1, $bla_{\text{TEM-52}}$), whereas three isolates had unassigned STs. The *E. coli* isolates harboring $bla_{\text{CTX-M}}$ genes and expressing 3MDRO phenotype (n=15) revealed ten different STs. Of the isolates, 60.0% (n=9) belonged to ST6617 (n=3), ST1485 (n=2), ST4994 (n=2), ST5686 (n=2) and 40.0% (n=6) of them had not yet assigned STs. *Escherichia coli* isolates with not assigned allelic profiles are shown in Table A2.

2.5.5. MRSA isolates from wastewater belong to the clonal complexes CC398 and CC9

MRSA isolates from S1 (n=25) and S2 (n=33) were allocated to six different *spa*-types. Five of them were livestock-associated and belonged to the clonal complexes CC9 (t1430, t13177) and CC398 (t8588, t011, t034), whereas one isolate from S1 (4.0%) was assigned to the health-care associated *spa*-type t045 of the CC5. It was isolated from the aggregated wastewater of the production facilities. The most predominant *spa*-type among MRSA isolates from S1 was t034 (76.0%, n=19), followed by t011 (12.0%, n=3) and t8588 (8.0%, n=2). Of the MRSA strains from the slaughterhouse S2, 75.8% (n=25) belonged to *spa*-type t1430, whereas 24.2% were assigned to *spa*-types t034 and t13177 (each 12.1%, n=4).

2.5.6. Vancomycin-resistant enterococci

The vancomycin-resistant *E. faecium* isolate was allocated to ST1249 and carried the *vanA* gene.

2.6. Discussion

In this study, we have investigated (i) process waters from different stages of the poultry slaughtering process as well as (ii) influents and effluents of in-house wastewater treatment plants of two German slaughterhouses. To our knowledge, it is the first time in Germany that such samples were taken directly in the slaughterhouses and their on-site WWTPs. Our results showed that bacteria with antimicrobial resistances against highly and critically important antimicrobials pollute the receiving water bodies as they survived the passage through the in-house WWTPs. These results clearly demonstrate that additional or alternative treatment steps are necessary before slaughterhouse wastewater can be released into the environment. The inefficacy of conventional biological WWTPs in Germany was also already reported for the treatment of municipal (45) and hospital (20) sewage.

The presence of potential clinically relevant ESKAPE-bacteria (i.e. *A. baumannii*, *K. pneumoniae*) and ESBL-producing *E. coli* in most of the investigated stages of the poultry slaughtering process is not surprising as most of them are able to colonize the gastrointestinal tract of livestock (esp. poultry) which was also described by other authors (40, 65). Bustillo-Lecompte and Mehrvar (2017) justified that their release to the process waters and subsequently into the wastewater is a consequence of the excretion of organic matter during delivery and slaughter from colonized poultry (66). However, to reduce the spread of resistant bacteria into the environment, several mitigation measures during poultry primary production (i.e. breeding

farms, hatcheries, fattening farms), slaughtering process as well as the wastewater treatment process need to be taken into consideration. This includes the improvement of poultry welfare conditions, implementation of strict health and infection control programs as well as reduction of the use of antimicrobials (67, 68).

Furthermore, during poultry processing various intervention options depending on the production step are conceivable but are often not realized on the basis of their estimated cost-benefit ratios. To reduce the release of potentially dangerous bacteria in process water used for cleaning of poultry crates, the use of pre-disinfection equipment prior to the washing step is conceivable (69). Process waters applied during CO_2 stunning and evisceration represent further important reservoirs for dissemination of the targeted bacteria of this study, which were released by defecation, fecal leakages and gastrointestinal disruptions. Another strategy to reduce the dissemination is a general reduction of clinically relevant bacteria on the surface and in the gastrointestinal tract of the animals. This can be reached by inclusion of probiotics and/or prebiotics in the feed (70), administration of oral bacteriophage cocktails (71) and competitive exclusion cultures (72). Futhermore, non-immersion scalders combined with decontamination of carcasses with hot water, could decrease the cross-contamination with resistant bacteria (73). Besides, using moisturized hot air would decrease the amount of produced wastewater. Moreover, use of advanced wastewater treatment technologies and its disinfection need to be considered. Hembach et al. (2019) proposed to combine oxidative, adsorptive, and membrane-based technologies in order to prevent environmental contamination with antibiotic resistant determinants and facultative pathogenic bacteria (i.e. ESKAPE bacteria) (74).

Unlike hospital and urban effluents, where the occurrence of XDR and carbapenemase-producing bacteria is frequently described (20, 75, 76), waste- and process water from the investigated poultry slaughterhouses did not exhibit such high risk bacteria. This emphasizes the importance of restricted use of carbapenems in human medicine. Moreover, the use of aminopenicillins and their β-lactamase inhibitor combinations, (fluoro)quinolones as well as $3^{rd}/4^{th}$ generation cephalosporins in livestock, should be limited to the absolutely necessary extent (77). They have wider spectrum of action and thus more likely to select multidrug resistant organisms, thereby compromising the activity of these antimicrobials for treatment of severe infections in human medicine (77).

Our results on the prevailing *E. coli* showed a strong congruence with data published in previous papers (78, 79). Furthermore, in this study, *E. coli* of the phylogroups B2, D and F, implicated as extraintestinal pathogens (ExPEC) (80), were recovered from all sampling points of both slaughterhouses. This emphasizes an increased risk of transmission of such ExPEC

clones to the personnel involved into particular operations in delivery and unclean areas of the slaughtering process (81).

In general, ESBL-producing *E. coli* of this study showed higher resistance rates to fluoroquinolones than the isolates from retail chicken meat (20.8%) reported by Casella (82). The increased fluoroquinolone resistance rate in isolates of this study may be caused by the use of enrofloxacin in slaughtered broiler herds. However, reliable data on the use of fluoroquinolones in the flocks are lacking.

While the majority of the isolates could be epidemiologically linked to poultry, some of the determined sequence types/clonal lineages are also attributed as high risk clones (i.e. ST69, ST10, ST648 and ST117) emerging in human infections in different countries (39, 83, 84). Isolates belonging to ST69, ST10 and ST212 were detected in cleaning of transport crates and effluents of the WWTPs. In general, *E. coli* of these sequence types are high risk clones that have been isolated from broilers and poultry meat (85) as well as from various patients with infections in different countries (84). Previously, ST212 *E. coli* were identified as enterotoxigenic *Escherichia coli* (ETEC) that have been recovered from surface water, pigs, broilers and humans (86, 87).

In this study, the majority of the ESBL-producing *E. coli* exhibited genes that are coding for CTX-M-1, TEM-116, TEM-52 and SHV-12 β-lactamases. These enzymes have already been reported in isolates of poultry and humans (19, 88). Among our isolates, $bla_{\text{CTX-M-1}}$ belongs to the most abundant determinants in ESBL-producing *E. coli*, which correlates well with prevalence data for $bla_{\text{CTX-M-1}}$ (18.0-69.0%) in isolates from chickens and chicken meat in Germany (89, 90). Similar to other studies, *E. coli* isolates carrying $bla_{\text{SHV-2}}$, $bla_{\text{SHV-2a}}$ and $bla_{\text{TEM-20}}$ were only sporadically detected in chicken and retail chicken meat (91, 92). In contrast to other studies from Germany and the Netherlands (89, 93, 94), where low percentages (0.0-5.2%) of $bla_{\text{CTX-M-15}}$-producing *E. coli* in chickens and poultry products have been identified, 12.2% of the ESBL-producing *E. coli* from wastewater of slaughterhouse S1 carried this gene. This may be due to a possible acquisition of $bla_{\text{CTX-M-15}}$ plasmids from human strains, as it has been already shown for animal *E. coli* strains in the UK (95). CTX-M-15 is one of the most frequently encountered ESBL-enzymes in human clinical isolates of various countries (96). Partially it is due to the clonal spread and predominance of a subset of ExPEC lineages in the human field that are commonly associated with $bla_{\text{CTX-M}}$ genes (particularly with $bla_{\text{CTX-M-15}}$), e.g. ST131, ST69 and ST10 (39, 97). However, the abundance of such ExPEC clones in poultry production in Germany is moderate and often they are associated with pAmpC rather than $bla_{\text{CTX-M}}$ (78). In this context, the transfer of $bla_{\text{CTX-M-15}}$ in *E. coli* by mobile genetic elements

between humans, livestock and the environment through the food chain (98) and occupational exposure (99) may play a primary role.

In previous studies ESBL-producing *K. pneumoniae* were only sporadically identified in German broilers during slaughter (100, 101), whereas in this study, 63.4% of the samples throughout almost all sampling points in S2 tested positive- for *K. pneumoniae*. In contrast to samples from S2, only 7.3% of the samples of S1 exhibited *K. pneumoniae*. These different proportions might be caused by the content of colonized flocks that were supplied by different fattening farms. Taking into consideration that there are only a few breeding companies and hatcheries in Germany, a vertical transmission through the production chain cannot be excluded. Moreover, the majority of the *K. pneumoniae* isolates carried $bla_{\text{SHV-2}}$, which has also been frequently found in isolates from egg shells, broilers and humans (102, 65). The results of our study are consistent with the observations that resistance genes of the SHV β-lactamase family are ubiquitous in ESBL producing *K. pneumoniae* (103, 104). However, in contrast to the isolates occurring in environmental sources in rural areas (105) as well as those cultured from patients specimens (106), almost all *K. pneumoniae* isolates from this study lack genes of the CTX-M/TEM families. This emphasizes the need for further studies applying high-resolution technologies, such as whole genome sequencing, to better elucidate their epidemiology and clinical relevance.

To date, only limited data on *Enterobacter* spp. exhibiting resistance against cephalosporins of $3^{rd}/4^{th}$ generation in poultry is available. Overall, the occurrence of bacteria of the *E. cloacae*-complex (1.23%) in retail poultry meat seems to be low as previously reported for Germany (91). ESBL-producing bacteria of the *E. cloacae*-complex (15.8%) exhibited only the ESBL enzyme SHV-12. These data are in good agreement with the observations of Towne et al. (2010) (107) who detected $bla_{\text{SHV-12}}$ in 8.7% of clinical *Enterobacter* spp. isolates. Steadily increasing abundance of ESBL-encoding isolates of *E. cloacae* complex along with the inducible production of AmpC as well as its constitutive de-repression could lead to near pan-resistance to β-lactam antibiotics diminishing the already limited number of therapeutic options (108–110).

Bacteria of the ACB-complex belong to the most important nosocomial pathogens, which are able to survive in competitive and demanding environments (111). However, reliable data on the impact of livestock-associated isolates on human and animal health are lacking. Wilharm et al. (2017) indicated linkages between livestock isolates and human clinical isolates, suggesting that *A. baumannii* might have zoonotic potential (40). Despite of the importance of these bacteria, data on emergence and antimicrobial resistance of bacteria of the ACB-complex in

broilers are scarce (40, 112). However, poultry meat retailed in different countries showed the highest contamination rates among raw meat with species of the ACB-complex (26.7%-48.0%) (113).

As bacteria of the ACB-complex were sporadically isolated from chicken, goslings, ducks and wild birds (40, 114), these animals might play an important role as natural reservoirs for these organisms. Given that *Acinetobacter* spp. are considered intrinsically resistant to cefotaxime, temocillin, fosfomycin and chloramphenicol (115), the ACB-complex isolates in our study contrast with human and animal clinical strains, which exhibited high levels of resistance against fluoroquinolones and carbapenems (116, 117). Almost all tested isolates lacked acquired bla_{ESBL} genes like $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}, bla_{VEB}, bla_{PER} and bla_{GES} which would be a genetic evidence of an acquired resistance against β-lactams (111). However, their resistance against 3rd generation cephalosporins might be a consequence of an increased expression of the chromosomal bla_{ADC} gene or other mechanisms (118).

The results of this study shown that the majority of the detected MRSA lineages belong to the CC398 and ST9, which are the most common LA-MRSA in Europe (119). The *spa*-types t034, t011 and t1430 have been already identified among isolates from chicken and meat products in countries with pronounced conventional farming like Denmark (120), Germany and the Netherlands (121). Furthermore, they were also detected in environmental samples from broiler barns (14), poultry slaughterhouses and its personnel (122) as well as in human patients in Norway and different countries of the European Union/European Economic Area countries (38, 123). Interestingly, isolates of the *spa*-types t034 and t011 represent the most frequent LA-MRSA recovered from hospital inpatients and ambulatory patients in Germany in regions with high livestock production (38). Another notable finding was the detection of the *spa*-type t13177 in MRSA from the effluent of the WWTPs. Isolates of this type were sporadically detected in fresh broiler meat and retail chicken meat in Germany (124) and Switzerland (125), respectively. Unlike the other LA-MRSA, these isolates carried genes coding for major staphylococcal enterotoxins that may cause toxic shock-like syndromes and implicate food poisoning (126).

The antibiotic resistance patterns of MRSA isolates in this study are similar to that of Rosenberg Goldstein et al. 2012 (12). However, the observed differences between isolates from S1 and S2 may be due to distinct prevalent clonal lineages detected in the two slaughterhouses. In another study (122), 95.0% of t1430 and only 11.5% of t034 MRSA were resistant to ciprofloxacin, while these were the most predominant *spa*-types in MRSA isolates from S1 and S2. It has been

postulated that t1430 is a poultry-associated MRSA type (122) and that its high resistance against moxifloxacin might be due to the usage of (fluoro)quinolones in the poultry industry. Within this study, only one vancomycin-resistant *E. faecium* ST1249 was recovered from cleaning samples of poultry transport crates. VRE ST1249 has been previously isolated in 3.7% of the chicken products from the United Kingdom (127). The occurrence of VRE in livestock is related to glycopeptide avoparcin (128, 129) that was used for growth promotion in Germany between 1975 and 1996 (130). However, Johnsen et al. 2011 (131) and Andersson et al. 2010 (132) suggest that the reversibility of acquired glycopeptide resistance will be slow and could last >25 years. Our findings and other reports (133, 134) reinforce this theory. Furthermore, it is presumed that without the selective pressure of avoparcin, co-selection by macrolides that are often used to treat poultry can occur (135). Moreover, copper added to the feed can also exert a selective effect on VRE (136).

2.7. Conclusion

Process- and wastewater from poultry slaughterhouses are important reservoirs for antimicrobial resistant bacteria with clinical relevance. The ubiquitous distribution of enterobacteria and MRSA with resistances to highly and critically important antimicrobials in process- and wastewater of poultry slaughterhouses is worrisome as they (i) may colonize slaughterhouse workers and (ii) could be reintroduced into the food chain by cross-contamination during carcass-processing. Furthermore, (iii) they were released into the environment via surface waters due to insufficient treatment within in-house WWTPs. New measurements to reduce the input of resistant bacteria into the slaughterhouses and their consequent excretion into process- and wastewater as well as strategies for improvement of discharge water status and treatment processes need to be taken into consideration.

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3. Antibiotic-resistant bacteria and antimicrobial residues in wastewater and process water from German pig slaughterhouses and their receiving municipal wastewater treatment plants

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3.1. Abstract

Slaughterhouse process- and wastewater are considered as a hotspot for antibiotic-resistant bacteria and antimicrobial residues and may thus play an important role for their dissemination into the environment. In this study, we investigated occurrence and characteristics of ESKAPE bacteria (*E. faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, *Enterobacter* spp.) and ESBL (extended spectrum β-lactamase) -producing *E. coli* in water samples of different processing stages of two German pig slaughterhouses (S1/S2) as well as their municipal wastewater treatment plants (mWWTPs). Furthermore, residues of various antimicrobials were determined.

A total of 103 water samples were taken in delivery and dirty areas of the slaughterhouses S1/S2 (n=37), their in-house WWTPs (n=30) and mWWTPs including their receiving water bodies (n=36). The recovered isolates (n=886) were characterized for their antimicrobial resistance pattern and its genetic basis.

Targeted species were ubiquitous along the slaughtering and wastewater chains. Phenotypic and genotypic analyses revealed a broad variety of resistance phenotypes and β-lactamase genes. Carbapenemase-producing Enterobacteriaceae (CPE), vancomycin-resistant enterococci (VRE) and healthcare-associated (HA) MRSA were recovered only from mWWTPs and their preflooders. In contrast, the *mcr-1* gene was exclusively detected in *E. coli* from S1/S2. Residues of five antimicrobials were detected in 14.9% (10/67) of S1/S2 samples in low range concentrations (≤1.30 µg/L), whereas 91.7% (33/36) of mWWTPs samples exhibited residues of 22 different antibiotics in concentrations of up to 4.20 µg/L.

Target bacteria from S1/S2 and mWWTPs exhibited differences in their abundances, resistance phenotypes and genotypes as well as clonal lineages. S1/S2 samples exhibited bacteria with zoonotic potential (e.g. MRSA of CC398, *E. coli* of significant clones), whereas ESKAPE bacteria exhibiting resistances of clinical importance were mainly detected in mWWTPs. Municipal WWTPs seem to fail to eliminate these bacteria leading to a discharge into the preflooder and a subsequent dissemination into the surface water.

3.2. Introduction

Development of antimicrobial resistance (AMR) and their spread in the environment is nowadays a major concern in public health (1). Therefore, the World Health Organization (WHO) developed a global priority list of important antimicrobial-resistant bacteria to guide research, discovery, and development of novel antibiotics (2). Within this prioritization list, the group of ESKAPE bacteria (*Enterococcus* spp., *Staphylococcus aureus*, *Klebsiella*

pneumoniae, Acinetobacter baumannii, *Pseudomonas aeruginosa*, *Enterobacter* spp.) as well as *Escherichia coli* gained a high impact, as they are a leading cause of life-threatening infections in healthcare settings, worldwide (3).

Inappropriate and frequent use of antimicrobials as therapeutics and growth promoters in animal husbandry has led to an increased antimicrobial resistance in livestock-associated bacteria by promoting the development of novel resistances (e.g. mobile colistin resistance genes) (4, 5). Furthermore, an exposure to antimicrobial agents used in animal husbandry can cause the development of cross-resistances to antimicrobials that are routinely used in the human medicine (6, 7). This might result in antibiotic treatment failure (3, 8).

In the last years, dissemination of ESKAPE bacteria (e.g. MRSA) and ESBL-producing *E. coli* was shown to be not only restricted to the medical and healthcare systems but includes also habitats with anthropogenic or agricultural influence (9). Livestock are carriers of ESKAPE bacteria and ESBL-producing *E. coli* and can disseminate them to humans by direct contact (3) and cross-contamination of food products (10, 11). However, animals are also a source for a release of antimicrobial-resistant bacteria to the environment (e.g. surface waters) (12). Furthermore, animals treated with antimicrobials excrete their unmetabolized residues and could introduce them into slaughterhouses, if withdrawal periods were not observed (13). Actually, despite strict hygiene rules established in the slaughterhouses in Germany, 12.1% of pork meat and 71.9% of poultry meat samples, were contaminated with ESBL/pAmpC-*E. coli* (14). Here, one possible cross-contamination route could be the process water that accumulates at different steps in the slaughtering process (e.g. scalding and eviscerating water) and comprises various multidrug-resistant (MDR) bacteria as shown for poultry slaughterhouses (15).

German slaughterhouses usually possess in-house wastewater treatment plants (WWTPs) and afterwards discharge their pretreated wastewater either into a municipal WWTP or directly into a water body (e.g. river). However, no legal limits or reduction levels have been fitted for microbiological contamination of wastewater in Germany. Thus, in-house and municipal WWTPs receiving, *inter alia*, the wastewater from slaughterhouses, serve as a reservoir for the spread of antimicrobial-resistant bacteria with clinical relevance into the environment (15). Such environmental (16, 17) and foodborne exposure (14) of the general population might have an impact on colonization probability and/or infection caused by ESKAPE bacteria. Moreover, occupational exposure of farmers and slaughterhouse workers to slaughter animals, products and contaminated working environment also poses an elevated risk (18),

So far, no data on the occurrence, phenotypic and genotypic properties of ESKAPE bacteria, ESBL-producing *E. coli* as well as antimicrobial residues in process- and wastewater from German pig slaughterhouses have been published. Thus, this study aimed to evaluate the occurrence of ESKAPE bacteria, ESBL-producing *E. coli* and antimicrobial residues in process- and wastewater accumulating in the delivery and dirty areas of two German pig slaughterhouses as well as in their in-house WWTPs. Moreover, their further dissemination through the municipal WWTPs into receiving water bodies was also investigated. Recovered bacteria were subjected to the antimicrobial resistance-testing and determination of genetic basis of the β-lactam-, carbapenem-, vancomycin- and mobilizable colistin resistance genes. Furthermore, ESBL-producing *E. coli* and MRSA strains were classified using different epidemiological typing methods.

3.3. Materials and methods

3.3.1. Sampling sites and sample preparation

Sample acquisition was conducted in two independent German pig slaughterhouses (S1 and S2) exhibiting different slaughtering capacities of >3.000 pigs per day. Daily wastewater amounts of 2,100 m^3 (S1) and 550 m^3 (S2) were pre-treated in conventional aerobic biological wastewater treatment plants (WWTPs) on-site. S1 additionally operates a physical-chemical WWTP using flotation and precipitation (flocculation). After pre-treatment, the wastewater of both slaughterhouses is released into the municipal WWTPs (mWWTPs), where it further passes a conventional aerobic activated sludge process and which possess different population equivalents of 190,000 and 41,000 for mWWTP-S1 (8,000 m^3/day) and mWWTP-S2 (5,800 m^3/day), respectively. Thereafter, the effluents are discharged into rivers.

Sampling of process- and wastewater was performed between March 2017 and July 2018. A minimum time interval of four weeks was kept between two independent sampling campaigns to decrease the risk of possible carryover of the targeted bacteria from pig herds originating from the same fattening farm. Beside delivery (animal transporters, holding pens) and unclean areas (scalding and dehairing water, aggregate wastewater from production facilities) as well as the in-house WWTPs of the slaughterhouses (in- and effluent), sampling was also conducted at the municipal WWTPs (in- and effluent) receiving the pre-treated wastewater from S1 and S2. Additionally, preflooders (i.e. rivers) upstream and downstream the discharge points were also chosen as sampling sites. At each site, one liter was collected in sterile polyethylene Nalgene® Wide Mouth Environmental Sample Bottles (Thermo Fisher Scientific, Waltham,

MA, USA). With the aim for getting an overview on the occurrence of the target species, no technical replicates of the water samples were taken, which might be considered as a limitation of the study.

Overall, 103 water samples were collected and investigated within this study. While 67 samples originated from both pig slaughterhouses (S1/S2), 36 were recovered from the respective mWWTPs and their receiving water bodies. In S1 and S2, wastewater of pig transporters (n=10), wastewater of holding pens (n=7), scalding and dehairing water (n=10), aggregate wastewater of producing facilities (n=10), the influent and effluent of the biological (n=20) and chemical-physical (n=10) WWTPs were sampled. Furthermore, samples from the mWWTPs were taken from the influent (n=9) and effluent (n=9), on-site pre-flooder upstream (n=9) and downstream (n=9) the discharge point.

Water used for the cleaning of pig transporters was collected in the on-site wash facility after passing through the screens and removal of coarse impurities by immersion of the sterile sampling bottles into the mixing and homogenization container. Water used for cleaning of holding pens was collected by catching the surface runoffs in open drains (only S1). Furthermore, wastewater that accumulates during slaughter due to process-related pre-cleaning of producing facilities in stunning, slaughtering, evisceration and cutting areas (aggregate wastewater) was taken by immersion of sterile sampling bottles into the mixing and homogenization container. Additionally, water from scalding was sampled by immersion of sterile sampling bottles into the combined scalding and dehairing tank. The samples of influents and effluents of the on-site and municipal WWTPs were taken as qualified samples in accordance with the German standard methods for the examination of water, wastewater and sludge (DIN 38402-11:2009-02). Furthermore, water bodies receiving treated wastewater (i.e. on-site preflooders) were sampled 50 m upstream and downstream the discharge point by plunging the sample bottles three meters off shore.

All samples were labeled and transported to the laboratory cooled in a Styrofoam box at 5±2°C. To remove residual solids (e.g. bristles, meat particles, bedding), the samples were filtered using Stomacher® 400 Classic strainer bags with tissue filter (pore size 0.5 mm, VWR, Radnor, PA, USA). The samples were processed for cultivation assays within 24 h after their collection.

3.3.2. Cultivation, identification and susceptibility testing of target antimicrobial resistant bacteria

Detailed information on cultivation and detection of Gram-negative, ESBL-producing bacteria (*E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Citrobacter* spp. and non-fermenters *A. baumannii*

and *P. aeruginosa*), carbapenem-resistant Enterobacteriaceae (CRE), MRSA (methicillin-resistant *S. aureus*) and VRE (vancomycin-resistant enterococci) has already been published (15).

Up to four presumptive targeted bacterial colonies per species and sampling point were picked and sub-cultured on Columbia Agar with 5% sheep blood (MAST Diagnostica, Reinfeld, Germany) at 37°C for 18-24 h (15). Confirmation of the species was done by MALDI-TOF MS (bioMérieux, Marcy-l´Étoile, France) equipped with the Myla™ software. The isolates were purified on Columbia Agar with 5% sheep blood and archived in cryotubes (Mast Diagnostics, Reinfeld, Germany) at −70°C.

Isolated bacteria were further subjected to antimicrobial susceptibility testing by microdilution method according to protocols of the European Committee on Antimicrobial Susceptibility Testing (EUCAST v 9.0) using Micronaut-S MDR MRGN-Screening system (MERLIN, Gesellschaft für mikrobiologische Diagnostika GmbH, Bornheim-Hersel, Germany) for Gram-negative bacteria and MICRONAUT-S MRSA/GP testing panel for Gram-positives. The results were evaluated based on clinical cut-off values recommended by EUCAST, whereas the intermediate test results were interpreted as resistant. Targeted Gram-negative bacteria were classified according to the recommendations of the Commission for Hospital Hygiene and Infection Control of 2012 at the Robert Koch-Institute Berlin. Organisms expressing resistance or intermediate susceptibility against three antibiotic groups (ureidopenicillins (piperacillin), 3rd generation cephalosporins (cefotaxime or ceftazidime), fluoroquinolones (ciprofloxacin)) are defined as 3MDRO multidrug-resistant organisms. 4MDRO are additionally resistant against carbapenems such as imipenem or meropenem (19).

AmpC enzyme production was tested in isolates resistant against β-lactam/β-lactamase inhibitor combinations by disc diffusion method using commercial AmpC test D69C (Mast Diagnostica) enabling the detection of both plasmid-mediated and chromosomal AmpC, whether inducible or derepressed (20).

3.3.3. Detection and analysis of selected resistance genes

Template DNA in all PCR reactions was prepared by resuspending of 1 µL loopful of bacterial colonies from fresh agar plates in 10 mM Tris-EDTA pH 8.0 (Sigma-Aldrich, St. Louis, MO, USA) and heating according to the TE boiling lysate method (21).

ESBL-producing Enterobacterales were screened for *bla* genes encoding SHV, TEM, CTX-M (groups 1, 2, 8 and 9) enzymes by PCR as previously described (22–25). Isolates of the *A. baumannii-calcoaceticus* complex were examined for the presence of *bla*$_{PER}$, *bla*$_{GES}$, and

bla$_{VEB}$ genes by PCR and sequencing (26). Resulting PCR amplicons were purified with the innuPREP DOUBLEpure Kit (Analytik Jena AG, Jena, Germany) and Sanger sequenced at Microsynth Seqlab (Göttingen, Germany). Obtained sequences were analyzed using open access programs Chromas lite v.2.6.5 (Technelysium Pty Ltd), BioEdit v.7.2.5 (27) and NCBI BLAST (http://blast.ncbi.nlm.nih.gov/).

Enterobacterales isolates that were picked from the CHROMagar ESBL plates and exhibited a meropenem resistance MIC of >0.125 mg/L as well as those from the CHROMagar mSuperCarba plates were screened for carbapenemases VIM, IMI, NDM, KPC, OXA-48 and GES by multiplexed real-time *Taq*Man PCR assays (28, 29).

Colistin-resistant isolates (MIC >2 mg/L) were analyzed by conventional multiplex PCR for the presence of *mcr-1* to *mcr-5* genes (30). Sequence-based typing of *mcr-1* amplicons was conducted as described by (31).

Vancomycin-resistant enterococci were screened for *vanA, vanB, vanC1* and *vanC2* genes by conventional multiplex PCR assay described by (32).

3.3.4. Molecular typing of resistant bacterial isolates

Assignment of *E. coli* isolates into phylogenetic groups (A, B1, B2, C, D, E, F, clade I-V) and multilocus sequence-typing (MLST) of selected isolates was conducted using previously published procedures (33, 34). Determination of the sequence types was performed using EnteroBase (http://enterobase.warwick.ac.uk/species/index/ecoli).

MRSA isolates were *spa*-typed by amplifying and sequencing of *Staphylococcus* protein A repeat region as recommended by (35). Assignment to *spa* types was done using Ridom *spa* server database (http://www.spaserver.ridom.de).

3.3.5. Determination of antimicrobial residues

The samples were prepared and analyzed for 45 antibiotics and two metabolites (N-acetylsulfamethoxazole and anhydroerythromycin) as described previously (36). Briefly, after dilution and filtration through hydrophilic PTFE filters from Macherey-Nagel (Düren, Germany), samples were analyzed by high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (MSMS). We analyzed the following antibiotic classes: β-lactams (i.e. penicillins, cephalosporins and carbapenems), tetracyclines, fluoroquinolones, sulfonamides (as well as their synergist trimethoprim), macrolides, lincosamides, glycopeptides, oxazolidinones, nitroimidazoles, spiramycin and tylosin. All analyzed antibiotics including their limit of quantification are given in Table A1. According to the

conventional method, the limit of detection of each individual analyte was one third of the limit of quantification (LOQ).

3.4. Results

3.4.1. Composition of clinically relevant bacteria in slaughterhouses and mWWTP samples.

Overall, 95.5% of S1/S2 and 97.2% of mWWTPs samples were positive for at least one of the antimicrobial-resistant target bacteria (Fig. 3.4.1). *E. coli* strains able to grow on ESBL selection plates were isolated most frequently, with 85.1% of S1/S2 and 97.2% of mWWTPs samples being positive at all sampling points (Fig. 3.4.2, 3.4.3). MRSA were more often isolated in S1/S2 (80.6%) than in mWWTPs (16.7%). In contrast to their ubiquitous occurrence in the slaughterhouses, at the municipal WWTPs these bacteria were only detected in the effluent (Fig. 3.4.2, 3.4.3). Furthermore, MRSA were recovered in the receiving water bodies downstream of discharge points (Fig. 2, 3). Vancomycin-resistant *E. faecium* were detected across all sampling points in mWWTPs including on-site pre-flooder (61.1%), whereas S1/S2 samples yielded negative-results. Furthermore, 63.9% of mWWTPs samples were positive for *K. oxytoca*, whereas its occurrence in S1/S2 was observed only sporadically (3.0%). Detailed information on the proportion of positive samples per detected species and their frequency at each sampling point in S1/S2 and the respective mWWTPs is given in Table A2.

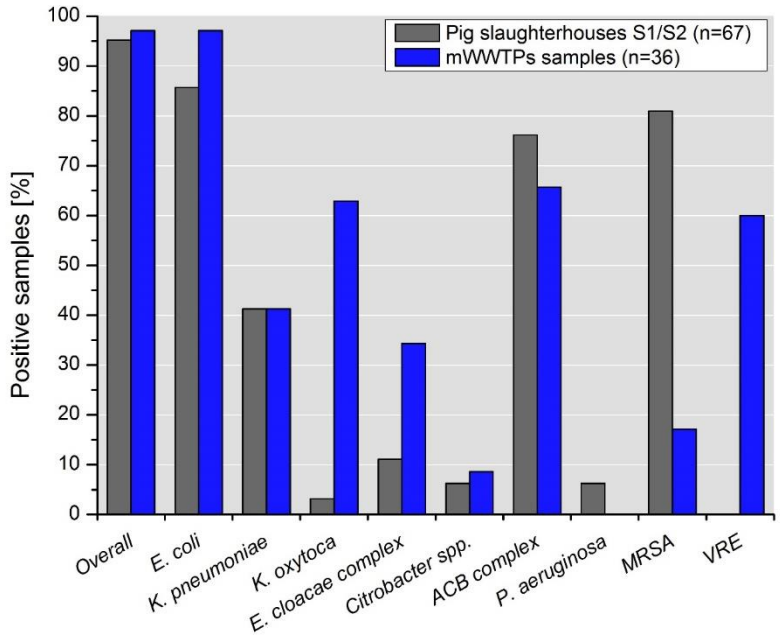

Figure 3.4.1 Percentage of positive samples per target bacteria in the pig slaughterhouses S1/S2 and the municipal WWTPs receiving their wastewater (mWWTPs). ACB (*Acinetobacter calcoaceticus-Acinetobacter baumannii*) complex, MRSA (methicillin-resistant *Staphylococcus aureus*), VRE (vancomycin-resistant enterococci).

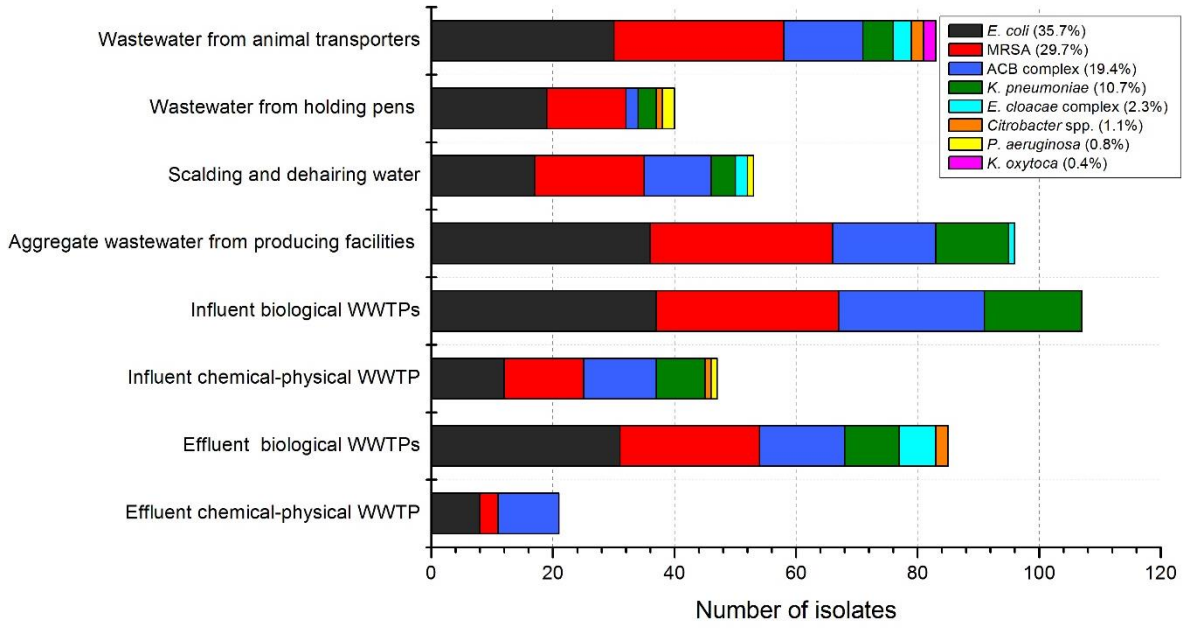

Figure 3.4.2 Occurrence of target bacteria across the sampling points in the pig slaughterhouses S1/S2 (n=532).

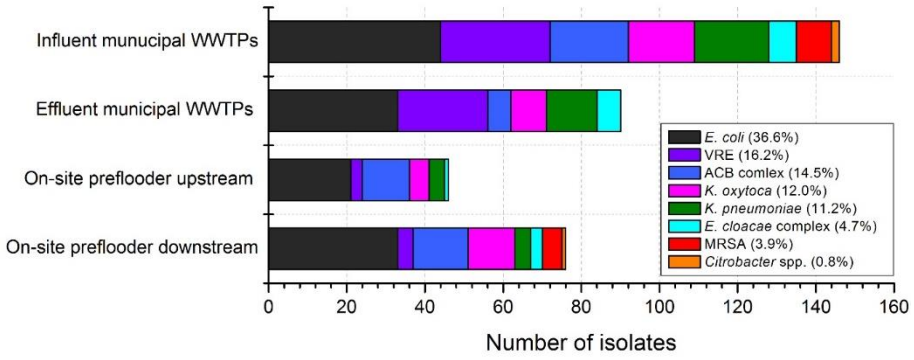

Figure 3.4.3 Occurrence of target bacteria across the sampling points in the municipal WWTPs receiving wastewater from the investigated pig slaughterhouses including their on-site preflooders (n=358).

3.4.2. Antimicrobial resistance pattern of recovered isolates.

Escherichia coli isolates from S1/S2 (n=190) and mWWTPs (n=131) were resistant to or showed only intermediate susceptibility to antimicrobials of different classes (Fig. 3.4.4A), but were still susceptible against carbapenems (IMI, MEM). Overall, *E. coli* isolates from mWWTPs had a higher 3MDRO rate (52.7%, 69/131) that those from S1/S2 (27.9%, 53/190) and exhibited higher resistance or intermediate susceptibility against piperacillin-tazobactam and the newly approved antibiotic combination ceftolozane-tazobactam.

In comparison to isolates of *Klebsiella* spp., the *E. cloacae* complex and *Citrobacter* spp. (KEC) from S1/S2 (n=77), the KEC isolates from mWWTPs (n=103) exhibited higher resistance levels

to almost all tested antimicrobials (Fig. 3.4.4B) and a higher percentage of 3MDRO resistance (S1/S2: 29.9%, mWWTPs: 53.4%). In contrast to isolates from S1/S2, they showed resistance or intermediate susceptibility against amikacin (8.7%, 9/103), imipenem (11.7%, 12/103) and meropenem (13.6%, 14/103) as well as exhibited higher levels of resistance against combinations of β-lactam/β-lactamase inhibitor (TZP, CZA, C/T).

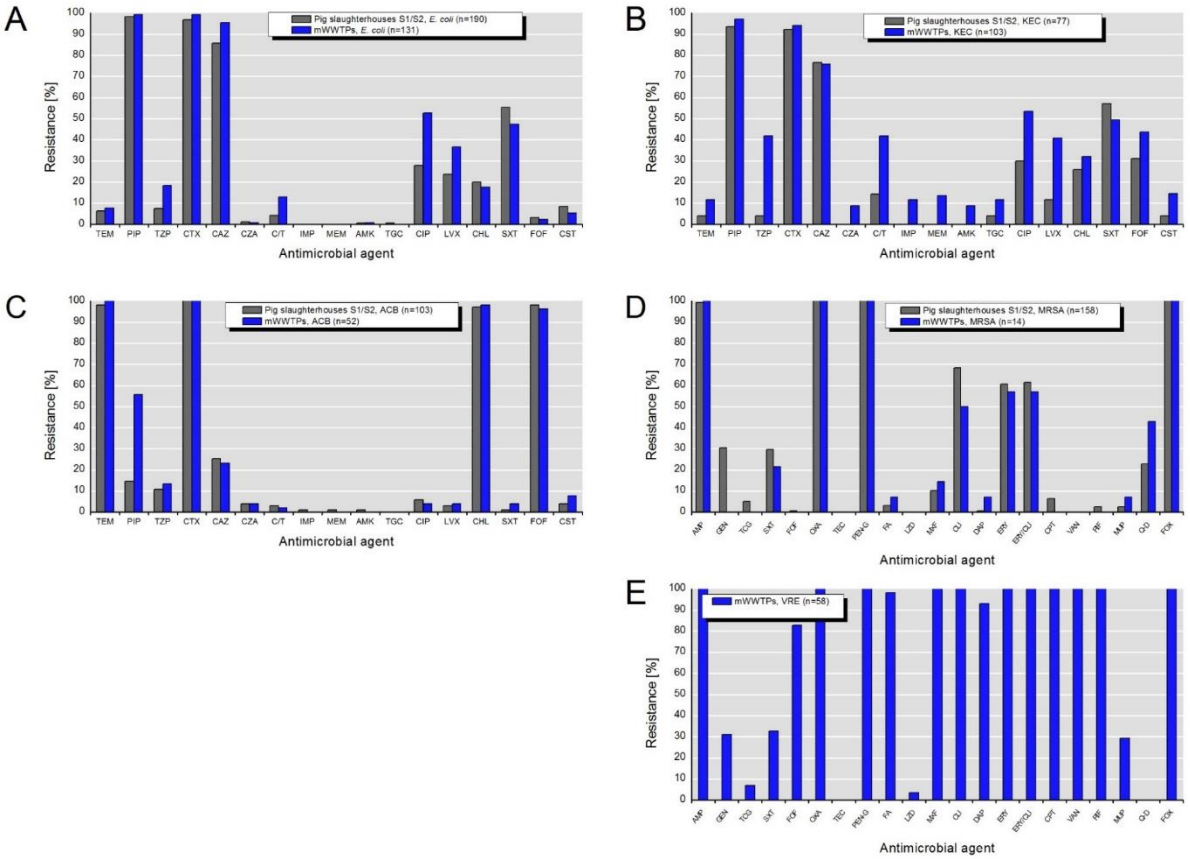

Figure 3.4.4 Resistance to antimicrobial agents detected among isolates of (A) *E. coli*, (B) *K. pneumoniae*, *E. cloacae* complex, *Citrobacter* spp., (C*) ACB complex, (D) MRSA and (E) VRE.

Abbreviations for antimicrobial agents: TEM, temocillin; PIP, piperacillin; TZP, piperacillin-tazobactam; CTX, cefotaxime; CAZ, ceftazidime; CZA, ceftazidime-avibactam; C/T, ceftolozane-tazobactam; IMP, imipinem; MEM, meropenem; AMK, amikacin; TGC, tigecycline; CIP, ciprofloxacin; LVX, levofloxacin; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colistin; AMP, ampicillin; GEN, gentamicin; OXA, oxacillin; TEC, teicoplanin; PEN-G, penicillin G; FA, fusidic acid; LZD, linezolid; DAP, daptomycin; CPT, ceftaroline; VAN, vancomycin; RIF, rifampicin; MUP, mupirocin; FOX, cefoxitin; MXF, moxifloxacin; CLI, clindamycin; ERY, erythromycin; Q-D, synercid (quinupristin-dalfopristin).
For temocillin by Enterobacteriaceae a breakpoint of S (≤32) and R (>32) from British Society for Antimicrobial Chemotherapy (BSAC) was used (BSAC 2016), as there are currently no EUCAST or CLSI breakpoints.
* Species of ACB-complex are considered intrinsically resistant against temocillin, cefotaxime, chloramphenicol and fosfomycin.

Isolates of *A. calcoaceticus-baumannii* (ACB) complex from S1/S2 (n=103) and mWWTPs (n=52) exhibited equally low 3MDRO resistance levels of 5.8% (6/103) and 3.8% (2/52), respectively (Fig. 3.4.4C). All isolates but one isolate from S1/S2 were carbapenem (IMI, MER) and amikacin susceptible. Furthermore, resistance against colistin was detected in 3.9% (4/103) and in 7.7% (4/52) of the isolates from S1/S2 and mWWTPs, respectively.

Among the isolates from S1/S2 (n=41) and mWWTPs (n=99) exhibiting resistance or intermediate susceptibility against combinations of β-lactam/β-lactamase inhibitor (i.e. piperacillin-tazobactam, TZP; ceftazidime-avibactam, CZA; ceftolozane-tazobactam, C/T), 14.6% (6/41) and 10.1% (10/99) were determined to produce AmpC β-lactamases, respectively. In S1/S2 they were represented by isolates of the ACB complex (4/6) and *Citrobacter* spp. (2/6). In mWWTPs, additionally to ACB complex (4/10) and *Citrobacter* spp. (2/10), also some isolates of the *E. cloacae* complex (4/10) exhibited this phenotype.

MRSA isolates showed resistance to most of the tested antimicrobials, with exception of teicoplanin, linezolid and vancomycin (Fig. 3.4.4D). Interestingly, in comparison to the MRSA isolates from mWWTPs (n=14), S1/S2 isolates exhibited a broader resistance pattern, in particular against gentamicin (30.4%, 48/158) and to lower extent (<6.3%) against ceftarolin, tigecyclin and rifampicin.

Of the antimicrobials used for the characterization of VRE isolates from mWWTPs, only teicoplanin and quinupristin/dalfopristin were determined to be susceptible (Fig. 4E). Low resistance rates (including intermediate susceptibility) were further observed against tigecycline (6.9%, 4/58) and linezolid (3.4%, 2/58).

3.4.3. Characterization of resistance genes

Isolates of *E. coli* (n=321), *Klebsiella* spp. (n=142), the *E. cloacae* complex (n=29) and *Citrobacter* spp. (n=9) with an ESBL phenotype were further molecularly screened for genes encoding β-lactamases of the TEM, SHV and CTX-M families.

Among the *E. coli* isolates from S1/S2 (n=190), the most prevalent family of genes was $bla_{\text{CTX-M}}$ (76.3%), bla_{TEM} (11.6%) and bla_{SHV} (2.2%). Interestingly, bla_{TEM} was not responsible for ESBL phenotype, but coded for broad-spectrum beta-lactamase (BSBL) TEM-1. However, eight isolates (4.2%) carried $bla_{\text{SHV-12}}$, $bla_{\text{CTX-M-137}}$ and $bla_{\text{CTX-M-1}}$ in addition to $bla_{\text{TEM-1}}$. Furthermore, 5.5% were negative for all tested ESBL genes. Overall, the majority of S1/S2 isolates (65.2%) harbored $bla_{\text{CTX-M-1}}$ (Fig. 3.4.5).

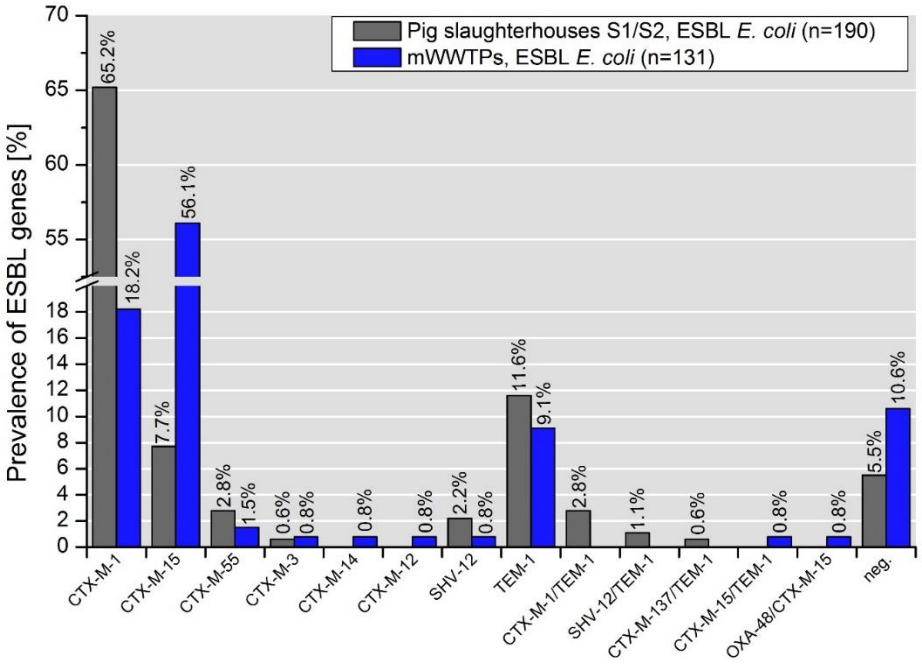

Figure 3.4.5 Distribution of single ESBL types in E. coli isolates from the pig slaughterhouses S1/S2 and municipal WWTPs receiving their wastewater (mWWTPs).

Of the *E. coli* isolates from mWWTPs (n=131), 78.6% exhibited a *bla*$_{\text{CTX-M}}$ genotype, 9.2% *bla*$_{\text{TEM-1}}$ and 9.9% yielded no positive result (Fig. 5). Only one isolate (0.8%) was shown to carry *bla*$_{\text{SHV-12}}$. Furthermore, unlike S1/S2 isolates, *bla*$_{\text{CTX-M-15}}$ was harbored by the majority of mWWTPs isolates (56.5%).

The majority of the S1/S2 *K. pneumoniae* isolates (61.4%, 35/57) carried *bla*$_{\text{CTX-M-1}}$ in combinations with *bla*$_{\text{SHV-1/-11/-27/-60}}$ and/or *bla*$_{\text{TEM-1B}}$, whereas *bla*$_{\text{CTX-M-15}}$ alone or in combination with *bla*$_{\text{SHV-1/-11/-28/-133}}$ and/or *bla*$_{\text{TEM-1A/1B}}$ was detected in 32.4% of the isolates (Fig. 3.4.6). Genes of the SHV family (*bla*$_{\text{SHV-28}}$, *bla*$_{\text{SHV-33}}$) were carried only by 2.9% of the isolates from both slaughterhouses. In contrast to S1/S2 isolates, the vast majority of *K. pneumoniae* isolates recovered in mWWTPs (82.5%, 33/40) harbored *bla*$_{\text{CTX-M-15}}$ in combination with *bla*$_{\text{TEM-1A}}$, *bla*$_{\text{TEM-1B}}$ and/or *bla*$_{\text{SHV-1/-11/-28}}$. Further genes of the SHV family (*bla*$_{\text{SHV-31}}$ and *bla*$_{\text{SHV-69}}$) were carried by 7.5% and 5.0% of mWWTPs isolates, respectively. One isolate (2.5%) carried a combination of *bla*$_{\text{SHV-69}}$/*bla*$_{\text{TEM-1B}}$.

Both isolates of *K. oxytoca* recovered from S1/S2 samples were negative for the tested ESBL genes. Furthermore, only 9.1% of the *K. oxytoca* isolates (4/43) from mWWTPs carried *bla*$_{\text{CTX-M-9}}$ and 4.5% a combination of *bla*$_{\text{CTX-M-1}}$/*bla*$_{\text{SHV-1}}$/*bla*$_{\text{TEM-1B}}$.

Among the isolates of the *E. cloacae* complex from S1/S2 (n=12), only five (41.7%) carried *bla*$_{\text{CTX-M-1}}$. Whereas among the *Citrobacter* spp. isolates (n=6) from slaughterhouses, all harbored *bla*$_{\text{CTX-M-1}}$ and two of them in combination with *bla*$_{\text{TEM-1}}$. Among the isolates of the

E. cloacae complex from mWWTPs (n=17), only five were positive for $bla_{CTX\text{-}M\text{-}15}$ (29.4%). Two of three *Citrobacter* spp. isolates from mWWTPs carried $bla_{CTX\text{-}M\text{-}15}$, and $bla_{SHV\text{-}12}$.

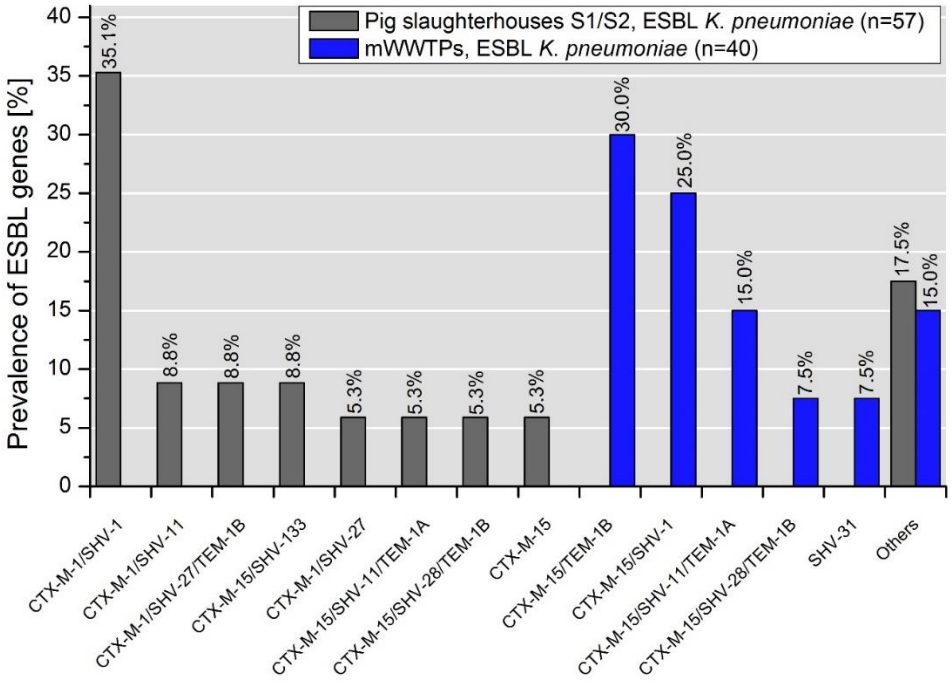

Figure 3.4.6 Distribution of single ESBL types in *K. pneumoniae* isolates from the pig slaughterhouses S1/S2 and municipal WWTPs receiving their wastewater (mWWTPs).

Of the isolates from CHROMagar ESBL plates (meropenem MIC >0.125 mg/L) as well as from the CHROMagar mSuperCarba plates, which were recovered from S1/S2 (n=21) and mWWTPs (n=32), only one *E. coli* carried a carbapenemase-encoding gene $bla_{OXA\text{-}48}$ in combination with $bla_{CTX\text{-}M\text{-}15}$. Furthermore, one *C. freundii* harbored bla_{KPC} together with $bla_{SHV\text{-}12}$. Both of them originated from the influent of mWWTPs.

The mobilizable colistin resistance gene *mcr-1* was not detected among the colistin-resistant isolates from mWWTPs (n=14). However, *mcr-1.1* was detected in 34.8% of slaughterhouse isolates (8/23) that expressed resistance against colistin and was carried only by *E. coli*.

All VRE isolates (n=58) carried *vanB* genes.

3.4.4. Phylogenetic typing of ESBL-producing *E. coli* isolates

Escherichia coli isolates from S1/S2 (n=190) mainly belonged to groups B1 (43.0%), A (26.3%) and C (20.0%), which typically contain commensal isolates (Fig. 3.4.7). Only six isolates recovered from wastewater used for cleaning of pig transporters and influent of the in-house WWTPs were assigned to groups B2 (1.6%, 3/190) and D (1.6%, 3/190) which comprise extraintestinal pathogenic *E. coli* (ExPEC). In contrast, virulence associated groups B2 and D

were represented by 29.8% (39/131) of mWWTP isolates. Furthermore, they were detected at all sampling points including effluents and on-site preflooders.

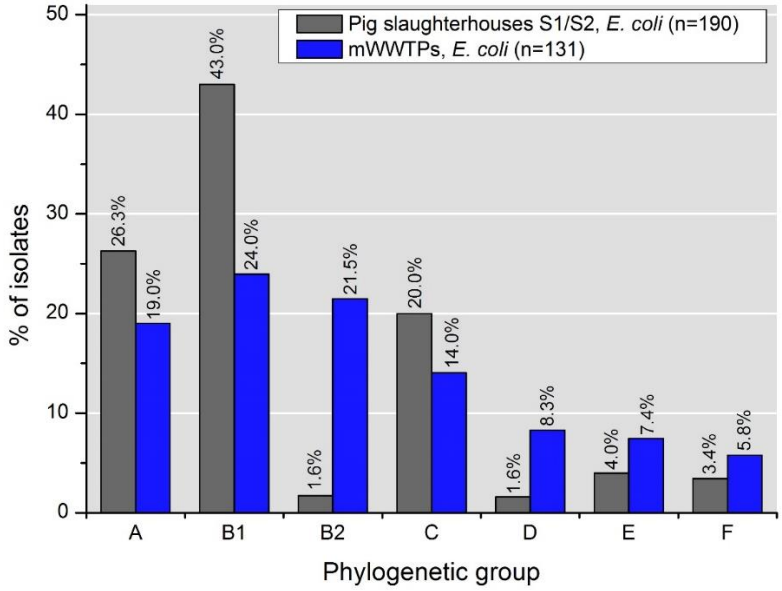

Figure 3.4.7 Assignment of *E. coli* isolates from S1/S2 and mWWTPs into phylogenetic groups.

MLST was performed on 70 isolates including 34 from S1/S2 and 36 from mWWTPs covering all sampling points. The chosen isolates: (i) exhibited a 3MDRO resistance phenotype and harbored $bla_{\text{CTX-M}}$, (ii) were resistant against new combinations of β-lactam/β-lactamase inhibitor and/or (iii) belonged to phylogenetic groups B2/D. In total, 47 isolates (67.1%) were assigned to 30 previously described sequence types (STs) (Table 3.4.1), while 33 isolates (32.9%) exhibited allele variants resulting in 16 STs that up to now have not been described within the prevailing MLST scheme (Table A3).

Table 3.4.1 MLST distribution of ESBL-producing *E. coli* isolates recovered from pig slaughterhouses S1/S2 and the municipal WWTPs including their receiving water bodies.

MLST of S1/S2 *E. coli* isolates		**MLST of mWWTPs *E. coli* isolates**	
Sequence type	n	Sequence type	n
ST641	3	ST131	5
ST10	2	ST69	3
ST224	2	ST91	2
ST117	2	ST648	2
ST359	2	ST95	1
ST542	2	ST405	1
ST5703	2	ST10	1
ST1170	1	ST46	1
ST354	1	ST5686	1
ST1284	1	ST59	1
ST3595	1	ST167	1
ST398	1	ST6617	1

Table 3.4.1 (continued)

ST453	1	ST8900	1
ST1431	1	ST200	1
ST5784	1		
ST101	1		
ST744	1		
New STs	9	New STs	14
Total	34	Total	36

3.4.5. *spa*-typing of MRSA isolates

MRSA isolates from S1/S2 (n=158) and mWWTPs (n=14) were assigned to twelve different *spa* types, which mainly comprise livestock-associated MRSA of CC398. The majority of S1/S2 isolates (89.5%, 141/158) belonged to t011 (54.5%) and t034 (35.0%), whereas the remaining 10.5% were represented by t8100 (3.3%), t2011, t2576 (each 1.6%) and t4224, t1793, t2971, t9266 as well as t899 (each 0.8%). Similar to S1/S2 isolates, the mWWTPs isolates were mostly allocated to *spa* types t034 and t011 (57.1%, 8/14), followed by t2011 (28.6%, 4/14). Two isolates recovered from the influent of mWWTPs exhibited *spa* types t003 and t005 of healthcare-associated CC5, respectively.

3.4.6. Antibiotic pollution

Antibiotic residues were detected in 14.9% (10/67) of the samples from S1/S2 in wastewater used for cleaning of pig transporters, waiting pens, scalding water, aggregate wastewater from production facilities as well as in the influents of the in-house WWTPs. The most frequently detected antimicrobial was doxycycline (7.5%, 5/67), followed by chlortetracycline (4.5%, 3/67) and sulfadiazine (3.0%, 2/67). Tetracycline and trimethoprim were detected each only in one sample (1.5%, 1/67). Compared to the samples from mWWTPs and their receiving water bodies, the S1/S2 samples exhibited low concentrations within the range of 0.06 µg/L (e.g. trimethoprim) up to 1.30 µg/L (e.g. chlortetracycline).

In 91.7% (33/36) of the samples from mWWTPs and their receiving water bodies, residues of 22 different antibiotics in ranges from 0.02 µg/L to 4.20 µg/L were detected. Most of the antimicrobials (21) were detected in the influents, whereas 12, 11 and 6 were present in the effluents, preflooders downstream and upstream the discharge point, respectively. All results on mWWTPs and their receiving water bodies are presented in Table 3.4.2.

Table 3.4.2 Antibiotic residues in samples from the municipal WWTPs receiving wastewater from the investigated pig slaughterhouses S1/S2.

Antibiotics[a]	**Influent mWWTPs-S1/S2 (n=9)**			**Effluent mWWTPs-S1/S2 (n=9)**			**Preflooder downstream (n=9)**			**Preflooder upstream (n=9)**		
	min, [µg/L]	max, [µg/L]	frequency[b], [%]	min, [µg/L]	max, [µg/L]	frequency, [%]	min, [µg/L]	max, [µg/L]	frequency, [%]	min, [µg/L]	max, [µg/L]	frequency, [%]
AMX	0.14	0.14	11.1		–			–		0.11	0.11	11.1
AMP	0.69	0.69	11.1		–			–			–	
AZM	0.08	0.90	88.9	0.09	0.57	100	0.09	0.32	55.6		–	
CTC	3.00	3.00	11.1		–			–			–	
CIP	0.16	4.20	66.7		–			–			–	
CLR	0.07	0.47	77.8	0.06	0.20	100	0.07	0.16	66.7		< LOQ (0.04)	
CLI	0.05	0.07	22.2	0.03	0.07	100		–			< LOQ (0.01)	
DO	0.24	0.24	11.1		–			–			–	
ERY	0.03	0.19	77.8	0.04	0.17	100	0.03	0.11	55.6	0.04	0.04	11.1
ERY,-Dehydrato	0.02	0.10	66.7	0.02	0.07	88.9	0.02	0.04	44.4	0.02	0.02	11.1
FLX	0.06	0.06	11.1		–			–			–	
LZD	0.17	0.17	11.1		–			–			–	
MNZ	0.16	0.16	11.1		< LOQ (0.04)			–			–	
OFX	0.28	2.50	77.8	0.21	1.10	66.7	0.31	0.51	33.3		–	
PIP	0.33	0.38	22.2	0.09	0.57	44.4	0.11	0.11	11.1		–	
SPM		< LOQ (0.08)			< LOQ (0.06)			–			–	
RXM	0.05	0.11	55.6	0.06	0.13	55.6	0.09	0.09	11.1		–	
SDM		–			–			–		0.06	0.06	11.1
SXM	0.10	0.98	88.9	0.13	0.45	100	0.03	0.35	88.9	0.02	0.03	55.6
SXM-N4-Acetyl	0.25	1.40	88.9	0.24	0.43	44.4	0.14	0.14	11.1		–	
TMP	0.10	0.29	88.9	0.04	0.16	88.9	0.02	0.12	55.6		–	
TYL	0.30	0.30	11.1	0.07	0.33	22.2	0.12	0.12	11.1	0.06	0.06	11.1
VAN	0.48	0.48	11.1		–			–			–	

Table 3.4.2 (continued)
[a]Abbreviations for antimicrobial agents: AMX, amoxicillin; AMP, ampicillin; AZM, azithromycin; CTC, chlortetracycline; CIP, ciprofloxacin; CLR, clarithromycin; CLI, clindamycin; DO, doxycycline; ERY, erythromycin; ERY,-Dehydrato, metabolite of erythromycin; FLX, flucloxacillin; LZD, linezolid; MNZ, metronidazole; OFX, ofloxacin; PIP, piperacillin; SPM, spiramycin; RXM, roxithromycin; SDM, sulfadimidine; SXM, sulfamethoxazole; SXM-N4-Acetyl, metabolite of sulfamethoxazole; TMP, trimethoprim; TYL, tylosin; VAN, vancomycin.

[b]Only samples with concentrations >LOQ were used for the evaluation.

3.5. Discussion

Little data on ESKAPE bacteria and antibiotic residues in process waters and wastewater from slaughterhouses have been published so far (37–39). Mostly, for the determination of antibiotic resistance patterns of bacteria from livestock and food, antimicrobials less critically important for human medicine and epidemiological cut-off (ECOFF) values are applied. Moreover, the sales of antimicrobial veterinary medicinal products in different European countries vary and range between 3.1 mg/PCU (population correction unit) in Norway to 423.1 mg/PCU in Cyprus, with Germany (89.0 mg/PCU) being above the median of 61.9 mg/PCU (40). Furthermore, the prescribing patterns of the various antibiotic classes differ as well (40). Such contrasting antimicrobial consumption behaviors result in different antimicrobial resistance patterns as it shown for zoonotic and indicator bacteria from animals and food in the EU (41). Thus, data obtained in this work cannot be considered of general validity. Nevertheless, our study provides new important insights into characterization of ESKAPE bacteria from German pig slaughterhouses and their relevance for human medicine.

3.5.1. ESBL-producing *E. coli*

The frequent occurrence of ESBL-producing *E. coli* emphasizes its high prevalence in the pig primary production sector and general population as well as its ubiquitous dissemination in the (aqueous) environment.

The higher 3MDRO rate as well as resistance rate against piperacillin in combination with tazobactam (TZP) of *E. coli* isolates recovered in mWWTPs, highlights importance of carbapenems, amikacin and tigecyclin as last resort antibiotics. The TZP-resistant isolates were confirmed as negative for AmpC production. Other possible mechanisms causing such a phenotype could be a hyperproduction of TEM-1 (42, 43) production of inhibitor resistant TEM-variants (44) and co-production of other β-lactamases, e.g. CTX-M-190, OXA-1 (45, 46). However, most of the TZP-resistant isolates carried *bla*$_{\text{CTX-M-15}}$.

ESBL-producing *E. coli* isolates from mWWTPs carried mostly *bla*$_{\text{CTX-M-15/1}}$. The detected ESBL genes are similar to those reported in the study of (47) for municipal WWTPs in UK, while *bla*$_{\text{CTX-M-1}}$ was most frequently detected in isolates from livestock and meat. Such a high abundance (74.0%, 97/131) of these *bla*$_{\text{CTX-M}}$ subtypes also reflects the current situation in hospital and ambulatory patients in Germany (48, 49) as well as in the community, together with the low incidence of acquired carbapenem resistance genes (50). *Escherichia coli* isolates carrying *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{KPC}}$ positive *C. freundii* were both detected in the influent of mWWTPs and might originate from clinics since the effluent wastewater from hospitals in Germany is discharged into the municipal WWTPs in most cases without prior treatment (51, 52). The negative influence of untreated hospital sewage on the occurrence of CPE in German and Swiss municipal WWTPs was already shown by (16) and (53), respectively. However, the incidence of CPE in our study was low and comparable with that of (16) who recovered CPE with a low abundance in wastewater from a rural system in Germany that was not influenced by hospitals.

Interestingly, all *mcr-1*-encoding *E. coli* isolates were recovered from slaughterhouses. This result agrees with the findings of (54, 55) indicating that *mcr-1* is more prevalent in livestock than in humans. However, based on the remarkable genetic plasticity of some multi-resistance plasmids in human Gram-negative pathogens (56, 57), an acquisition of certain antimicrobial resistance determinants from livestock bacteria (e.g. *mcr*-genes) via interaction with polluted (rural) environments and food products cannot be excluded.

ESBL-producing *E. coli* isolates from mWWTPs exhibited a high percentage of ExPEC clones of 29.8%, which comprise of global extraintestinal pathogenic lineages (ST131, ST69, ST648, ST95, ST405, ST10, ST167) (58). These clones are responsible for the majority of human extraintestinal infections worldwide and are associated with UTIs, bacteremia, infections of skin and soft tissues, respiratory tract as well as pneumonia, prostatitis and peritonitis (59, 58). Interesting, only 3.2% of the ESBL-producing *E. coli* isolated in the investigated pig slaughterhouses belonged to the virulence-associated groups B2 and D. However, the detected clones of ST354, ST1284, ST453, ST1431, ST101, ST1170 have already been described in German patients in hospitals, in outpatients with UTIs as well as in healthy persons (50, 48). Furthermore, ST117 that also belongs to major human ExPEC STs and ST10, was also detected in the pig slaughterhouses and were already described in pigs with diarrhea (58, 60). This emphasizes the zoonotic potential of livestock- and slaughterhouse-associated *E. coli* to colonize and infect humans, in particular occupationally exposed workers of in-house and municipal WWTPs as well as slaughterhouse employees.

3.5.2. *K. pneumoniae* and *K. oxytoca*

The vast majority of *K. pneumoniae* isolates recovered in S1/S2 as well as in mWWTPs carried $bla_{\text{CTX-M}}$ genes always in combination with $bla_{\text{SHV-1/-11/-28}}$ and/or $bla_{\text{TEM-1B/-1A}}$. Falgenhauer and colleagues reported also on *K. pneumoniae* isolates recovered from recreational and surface water in Germany carrying $bla_{\text{CTX-M-15}}$ in combination with $bla_{\text{OXA-1}}$, $bla_{\text{SHV-28}}$ and/or $bla_{\text{TEM-1}}$ and expressing a MDR phenotype (61). These findings are in contrast to the previously published results on occurrence of *bla* genes in *K. pneumoniae* isolates from waste- and process water originating from German poultry slaughterhouses, where bla_{SHV} with 98% (50/51) was the most predominant ESBL genotype (15). The high abundance of $bla_{\text{CTX-M-15}}$ in *K. pneumoniae* isolates from mWWTPs indicates its wide dissemination in the community, whereas the majority of *K. pneumoniae* isolated in the pig slaughterhouses carried $bla_{\text{CTX-M-1}}$.

Interestingly, 41.7% of the KEC isolates from mWWTPs were TZP-resistant, whereas the rate of such isolates from the pig slaughterhouses was at 3.9%. TZP resistance can be conferred by hyperproduction of SHV β-lactamases (62). Furthermore, carbapenem-nonsusceptible *K. pneumoniae* isolates were detected only in the influents of mWWTPs. However, as they were negative for the tested carbapenemases genes, diverse range of other β-lactamases combined with alterations or loss of outer membrane proteins (OMP) is also conceivable (63).

K. oxytoca was almost exclusively isolated in mWWTPs. It may be related to the fact that the investigated mWWTPs treat also wastewater from hospitals and *K. oxytoca* is associated with nosocomial infections in hospitalized patients, including children and neonates (64). However, it is also typically disseminated in the general population (64).

In S1/S2 *K. oxytoca* only occurred in the wastewater used for washing of pig transporters where the entry from other sources cannot be excluded, since *K. oxytoca* is ubiquitous in the environment (65). This species is known for its cross-species infecting capability (66). Nevertheless, there is no data on colonization or infections caused by *K. oxytoca* in pigs.

The recovered isolates were all negative for tested ESBL genes. However, other mechanisms that were not investigated in this study could also account for the resistant phenotype, e.g. carriage of chromosomal β-lactamases of the OXY group (67).

3.5.3. Methicillin-resistant *S. aureus*

The wide distribution of livestock-associated (LA) MRSA in process- and wastewater of the investigated pig slaughterhouses should raise caution regarding possible colonization of the personnel involved into processing of pigs, where large amounts of contaminated wastewater accrue (e.g. work in the holding pens, slaughtering, dehairing and evisceration). Increased

prevalence of MRSA carriage among slaughterhouse workers with occupational exposure to slaughter animals and products in comparison to the general population has already been described by (68) and (69). Furthermore, the study of (70) indicated that LA-MRSA caused up to 10% of all human MRSA infections in Germany in regions with high density livestock-farming. LA-MRSA of CC398 identified in this study (t011, t034, t2011, t2576) made up 16.8% of all isolates recovered from hospital and ambulatory patients in Germany (70).

Workers involved in operation of the on-site wastewater treatment facilities of slaughterhouses and municipal WWTPs, as well as residents in their proximity could also become colonized through occupational transmission and aerosolized bacteria (71). Yet, to our best knowledge, no studies have evaluated MRSA carriage rates among these populations in Germany.

In contrast to the wastewater from the investigated pig slaughterhouses, HA-MRSA were isolated only in the mWWTPs. The detected strains were recovered in the effluents and belonged to the widely disseminated in Germany and other European countries pandemic clones of *spa* type t003 (CC 5), "Rhine-Hessen pandemic strain", as well as to *spa* type t005 (CC 22), "Barnim pandemic strain" (72). Thompson and colleagues reported on HA-MRSA in untreated hospital wastewaters, their subsequent discharge into the mWWTPs and their survival through the treatment process (73). Furthermore, they postulated that hospitals were adding to the load of MRSA entering mWWTPs emphasizing their role as a source for HA-MRSA.

In comparison to the wastewater samples from the pig slaughterhouses (80.6%, 54/67), MRSA had a low prevalence in municipal WWTPs (16.7%, 6/36). Especially considering the fact that an optimized cultivation protocol with a pre-enrichment step, including resuscitation of injured bacteria, was used (15). This could be due to the low MRSA colonization rate of 0.5-1.5% of the general population in Germany (70) and the fact that *S. aureus* mostly resides on the skin as well as mucous membranes and an intestinal colonization is rather atypical (74). The result of the present study is in consent with studies of (75, 76) which have demonstrated that MRSA has a low abundance in municipal WWTPs.

3.5.4. Vancomycin-resistant enterococci

In this study, VRE were only detected in the municipal WWTPs including the up- and downstream preflooders indicating inadequate wastewater treatment by conventional WWTPs resulting in the pollution of the receiving water bodies. Despite the fact that VRE carrying *vanA*/*vanB* are an important cause of nosocomial infections in healthcare facilities (77), they have also been frequently isolated outside clinical settings, e.g. in rivers and lakes in the Rhine-Main metropolitan area of Germany (78), USA (79) and the Netherlands (80). These findings

point out a critical role of surface waters as an important dissemination pathway for VRE and demonstrate a possible transmission route to humans.

Wastewater from healthcare facilities could be considered as a source of VRE, as a direct selection of VRE in clinical sewage is conceivable (36). However, (80) isolated VRE at four out of six WWTPs effluent locations in the Netherlands that didn't receive hospital effluents indicating their presence in the general population as well. To better understand the role of hospital wastewater in the dissemination of VRE through the mWWTPs into surface waters, more investigations are needed, e.g. comparison of VRE isolates from mWWTPs with those recovered from invasive enterococcal infections through the high-resolution technologies such as SNP-based WGS typing.

Absence or low prevalence of VRE in livestock in Germany is in consent with the GERMAP report of Federal Office of Consumer Protection and Food Safety (81). Furthermore, it is unlikely that nowadays agriculture in Germany plays a significant role in development of glycopeptide-resistant enterococci and their spreading into surface water, as glycopeptides are not approved for use in animals and the use of avoparcin in feed as growth promoter was banned in Germany in 1996 (82). However, as *vanA* positive VRE of ST1249 was recently reported in wastewater from German poultry slaughterhouses (15), their co-selection by macrolides (83) and copper (84) added to the feed could not be completely excluded.

3.5.5. Antibiotic residues

Antimicrobials detected in S1/S2 mostly belonged to tetracyclines and sulfonamides. According to (85), tetracyclines are most frequently prescribed to treat infections in fattening pigs in Germany. In 2018, 178t of antimicrobials of this class were sold to the veterinarians in Germany making up 24.6% of the total amount (86). However, no antibiotic residues were detected in the effluents of the in-house WWTPs of the investigated slaughterhouses. The removal of the antibiotics can be attributed to adsorption and oxidation by activated sludge as well as photodegradation (87). Furthermore, in case of tetracyclines this could be due to their conversion into analytically-camouflaged metabolites through formation of calcium-, magnesium chelate complexes that are difficult to detect in the aqueous phases using liquid chromatography/mass spectrometry (88). However, currently there is a knowledge gap in Germany regarding the pollutant fractions attributable to pig slaughterhouses.

The antimicrobial residues detected in process- and wastewater of S1/S2 could originate from muscle and fat tissue as well as from the intestines of the slaughtered animals, as the permissible maximum values for antimicrobial residues in live animals and animal products in the European

Union are set at 100 µg/kg for chlortetracycline and tetracycline in the muscle tissue of pigs (89). In case that the withdrawal periods were not observed before the pigs went to slaughter, feces and urine could also be a source of antibiotic pollution in investigated samples. Some of commonly used veterinary antibiotics exhibit excretion rates of up to 90% (90). However, is unlikely that the withdrawal periods of these antibiotics were not strictly adhered to, as in 2014 only 0.02% (2/9533) of the analyzed samples of pork in Germany contained residues of tetracyclines exceeding the set limits (91).

The most frequently detected antimicrobials in mWWTPs (≥77.8%, ≥7/9) belonged to the antibiotic classes that are moderately persistent in surface water systems such as macrolides (azithromycin, clarithromycin, erythromycin, roxithromycin) and "potentiated" sulfonamides (sulfamethoxazole and trimethoprim), as well as fluoroquinolones (ofloxacin, ciprofloxacin). This partially reflects the antibiotic consumption in Germany in human medicine (92). Furthermore, some of the detected substances (i.e. ampicillin, azithromycin, ciprofloxacin, metronidazole, ofloxacin) exceeded their PNECs (Predicted No Effect Concentration) (93). Thus, it cannot be ruled out that these substances would exert a selective pressure in favor of antibiotic-resistant bacteria. Interestingly, even after the dilution of the effluent from the mWWTPs with the recipient water, the concentrations of azithromycin and ofloxacin still exceeded their PNECs of 0.25 µg/L and 0.5 µg/L, respectively. Especially fluoroquinolones play an important role in shaping and dissemination of virulent resistant clones, as already described for *P. aeruginosa* ST235 (94) and *E. coli* ST131 (95).

Furthermore, out of the 16 tested β-lactams, only amoxicillin, ampicillin and piperacillin were detected, whereas β-lactam antibiotics (aminopenicillins and cephalosporins) were the most commonly prescribed drug classes in Germany in 2018-2019 (92). With the exception of piperacillin, they were only detected in the influent of mWWTPs with low frequencies (11.1%, 1/9), confirming their instability in water and degradation during conventional activated sludge treatment through hydrolysis of the β-lactam ring (96). Nevertheless, twelve substances were still detected with frequencies up to 100% in the effluents of mWWTPs, indicating their ineffectiveness in removing of macrolides, sulfonamides as well as (fluoro)quinolones and enabling their further accumulation in the environment by formation of stable residues, e.g. with soil components (97).

3.6. Conclusions

Process- and wastewater from pig slaughterhouses constitute an important reservoir of antibiotic resistant bacteria with clinical relevance. ESBL-producing *E. coli* of clinically

relevant STs and *K. pneumoniae* as well as LA-MRSA mostly of CC398 were widely distributed in process- and wastewater accruing in delivery and unclean production areas. It poses a risk to human health, since they may colonize and infect slaughterhouse employees with occupational exposure to contaminated waters. Furthermore, despite strict hygiene management strategies established in German slaughterhouses, antibiotic-resistant bacteria could be re-introduced into the food chain by cross-contamination during processing of pig carcasses. Moreover, in-house WWTPs of pig slaughterhouses are a significant input source of livestock-associated bacteria with zoonotic potential into the municipal WWTPs. However, the most important resistant bacteria of ESKAPE group that pose a potential threat to humans (CPE, VRE carrying *vanB*, HA-MRSA of CC 5 and CC 22) were mainly detected in human wastewater that was also a hotspot for antimicrobial residues with some of them exceeding their PNECs. Nevertheless, the incidence of CPE in untreated wastewater from mWWTPs was low, as only two isolates of *E. coli* producing OXA-48, and KPC-producing *C. freundii* were detected (2/501). Due to the limited number of screened samples of already mixed hospital and municipal wastewater, it was not possible to determine the effect of hospital wastewater on the level of CPE, VRE and HA-MRSA in the investigated municipal WWTPs. Furthermore, due to inadequate wastewater treatment by municipal WWTPs, ESKAPE bacteria were discharged into receiving water bodies (i.e. rivers) enabling their further dissemination into the general population, e.g. by contact with humans and animals. However, to estimate the risk of the exposure via surface water and possible health consequences for humans, more studies are needed to clarify the persistence of resistant bacteria and their extracellular DNA in surface waters.

In order to protect human and environmental health regarding pollution with AMR and antibiotic residues, prescription and consumption patterns of antibiotics in livestock production need to be further reconsidered. Furthermore, evaluation of relevant pollution hotspots of surface waters and implementation of advanced wastewater treatment technologies need to be encouraged.

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4. Colistin-resistant *Enterobacteriaceae* isolated from process waters and wastewater from German poultry and pig slaughterhouses

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4.1. Abstract

Due to the high prevalence of colistin-resistant *Enterobacteriaceae* (i.e. *Escherichia coli*, *Klebsiella* spp., *Enterobacter cloacae* complex) in poultry and pigs, process water and wastewater from slaughterhouses were considered as a hotspot for isolates carrying plasmid-encoded, mobilizable colistin resistances (*mcr*-genes). As an incomplete treatment of the waters within in-house and municipal wastewater treatment plants (WWTPs) will further contribute to their spread via surface water, questions on the diversity of the prevailing isolates, plasmid types and their transmissibility arise.

Samples taken in the poultry slaughterhouses yielded the highest occurrence of colistin-resistant *Enterobacteriaceae* (40.2%, 33/82), followed by municipal WWTPs (25.0%, 9/36) and pig slaughterhouses (14.9%, 10/67). Noteworthy, MCR-1-producing *K. pneumoniae* and *E. coli* were detected in scalding waters and preflooders of municipal WWTPs. A total of 70.8% (46/65) of *E. coli* and 20.6% (7/34) of *K. pneumoniae* isolates carried *mcr-1* on a variety of transferable plasmids with incompatibility groups IncI1, IncHI2, IncX4, IncF and IncI2, while in the majority of colistin-resistant *mcr*-negative *E. coli* and *K. pneumoniae* isolates non-synonymous polymorphisms in *pmrAB* were detected.

Our findings demonstrated high occurrence of colistin-resistant *E. coli* and *K. pneumoniae* carrying *mcr-1* on transferrable plasmids in poultry and pig slaughterhouses and indicate their dissemination into surface water.

4.2. Introduction

Since the 1950s, colistin (polymyxin E) has been extensively used in the European animal production (1) to prevent/treat gastrointestinal infections caused by Gram-negative bacteria (e.g. diarrhoea in pigs caused by *Escherichia coli* and *Salmonella* spp. as well as colibacillosis in poultry) (2). Moreover, it was also used in a lower dosage as a feed additive until the ban of antimicrobial growth promoters in the European Union (EU) in 2006 (3). In 2016, colistin was classified as a highly important antimicrobial (VHIA) in the veterinary sector by the World Organisation for Animal Health (OIE).

Despite its nephrotoxicity and neurotoxicity, colistin was re-introduced into human therapy to treat infections caused by multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa* or carbapenemase-producing Enterobacteriaceae (CPE) (4). Due to its high impact, the World Health Organisation (WHO) included colistin into the group of the "highest priority critically important antimicrobials" for human medicine (5). Alongside with other antibiotics of the last resort (e.g. tigecycline, amikacin and the new combinations of ceftazidime-avibactam and ceftozolane-tazobactam), its use is restricted to clinical cases for which no alternative options are available (6).

In Gram-negative bacteria, colistin interacts with lipopolysaccharide (LPS) and phospholipids in the outer cell membrane. Due to the competitive displacement of divalent cations Ca^{2+} and Mg^{2+} from the phosphate groups of membrane lipids (7), both cell membranes are disrupted leading to the leakage of intracellular contents and subsequent bacterial death.

Before 2015, colistin resistance in Enterobacteriaceae was assumed to be caused on chromosomal mutations in genes (esp. *pmrA/B* and *phoP/Q* and *mgrB*) encoding regulatory proteins that influence transcription of enzymes that modify the lipopolysaccharide (8, 9). But the description of the first plasmid-encoded, mobilizable colistin resistance gene (*mcr-1*) in Chinese *E. coli* from livestock and retail meat as well as in clinical *K. pneumoniae* isolates (10, 11) raised serious public health concern on the emergence of colistin-resistant bacteria.

Further studies on the genetic basis of colistin-resistant bacteria resulting in the discovery of nine additional *mcr* genes. While *mcr-2* to *mcr-8* being detected mostly in *E. coli* and *K. pneumoniae* isolates from pigs and poultry (12–18), *mcr-9* and *mcr-10* were discovered in clinical strains of *S. enterica* serotype Typhimurium (19) and *Enterobacter roggenkampii*, respectively (20). However, *mcr-1* is the most frequently identified type one among currently described genes (21). Its occurrence is often associated with a variety of plasmids, including IncX4, IncF, IncHI1, IncHI2, IncI2, IncY and broad-host-range plasmids IncP (22–25).

Furthermore, *mcr-1* is often bracketed by IS*Apl1* insertion sequence enabling their broad dissemination by transposition (26, 27).

Due to a high number of colonized animals, slaughterhouses might represent a significant source of introduction of *mcr* genes into the food chain, e.g. despite strict hygiene standards, through possible contamination of carcasses and products (28, 29). Furthermore, slaughterhouse workers with occupational exposure to colonized animals and contaminated process water as well as employees of the WWTPs are exposed to an increased risk of colonization (30). Moreover, due to insufficient wastewater treatment by in-house and municipal WWTPs, livestock wastewater might be an important route for dissemination of *mcr-1*-carrying bacteria into the environment (31).

On the basis of the high prevalence of colistin-resistant *Enterobacteriaceae* in livestock feces, these bacteria might accumulate in process waters and wastewater from slaughterhouses. These waters might represent potential reservoirs that can contribute to a broad spread of the resistance to other environmental ecosystems including surface waters. So far, no data on the occurrence and characteristics of colistin-resistant *Enterobacteriaceae* in process waters and wastewater from German poultry and pig slaughterhouses have been reported. Furthermore, information on the impact of slaughterhouse wastewaters for the dissemination of this resistance is scarce and needs to be determined. Thus, this study aimed to evaluate their occurrence in the delivery and unclean areas of German poultry and pig slaughterhouses as well as in their in-house WWTPs. Moreover, their further spread into surface waters via municipal WWTPs was also investigated. To test this hypothesis, colistin-resistant *Enterobacteriaceae* (*E. coli*, *Klebsiella* spp. and *E. cloacae* complex) were isolated by plating on selective agar plates. Recovered bacteria were characterized for their antimicrobial resistance patterns, polymorphisms of genes encoding PmrAB as well as *mcr*-mediated colistin-resistance (*mcr-1* to *mcr-9*) and their ability to transfer this resistance. Furthermore, *E. coli* strains were assessed for their phylogenetic composition in order to determine the presence of extra-intestinal pathogenic *E. coli* (ExPEC) clones known to be implicated in a variety of diseases in humans and animals.

4.3. Materials and methods

4.3.1. Sampling and sample preparation

Sampling and sample preparation of process waters and wastewater taken in poultry and pig slaughterhouses, their in-house wastewater treatment plants (WWTPs) as well as municipal WWTPs and on-site preflooders has been already described previously (32, 33)

A total of 185 water samples were included in the study. Briefly, 82 samples of process water and wastewater accruing in the delivery and unclean areas were collected from two poultry slaughterhouses. Samples were taken at seven sampling sites: transport trucks, transport crates, stunning facilities, scalders, eviscerators, production facilities, influent and effluent of the in-house WWTPs. From each individual sample, one liter was collected using sterile Nalgene® Wide Mouth Environmental Sample Bottles (Thermo Fisher Scientific, Waltham, MA, USA). For more details please see (32).

Further 67 samples of process water and wastewater were collected from the delivery (animal transporters, holding pens) and unclean areas (scalding and dehairing water, aggregate wastewater from production facilities) as well as the in-house WWTPs (in- and effluent) of two pig slaughterhouses. Additionally, 18 samples were collected from the influents (n=9) and effluents (n=9) of the municipal mWWTPs receiving the wastewater from the investigated pig slaughterhouses for the final treatment Their on-site preflooders upstream (n=9) and downstream the discharge points (n=9) were sampled as well. At each site, one liter was collected in sterile polyethylene Nalgene® Wide Mouth Environmental Sample Bottles (Thermo Fisher Scientific, Waltham, MA, USA). For more details please see (33).

4.3.2. Cultivation, identification and susceptibility testing of target polymyxin-resistant lactose-fermenting Enterobacteriaceae

Water samples were screened for polymyxin-resistant lactose-fermenting Enterobacteriaceae (*Escherichia coli*, *Klebsiella* spp., *Enterobacter cloacae* complex) using SuperPolymyxin medium (34). For cultivation, aliquots of 100 µl and 1 ml of the original samples were applied onto SuperPolymyxin plates and incubated under aerobic conditions at 37°C for 18-24 h. When possible, up to three colonies of lactose fermenters were picked based on their morphology and sub-cultured on Columbia Agar with 5% sheep blood (MAST Diagnostica, Reinfeld, Germany) at 37°C for 18-24 h.

Identification of the isolates species was conducted by MALDI-TOF MS as previously described (32).

The antimicrobial susceptibility testing of the isolates and transconjugants was performed by applying two different antibiotic susceptibility testing panels as well as epidemiological and clinical breakpoints. The first scheme (A) was based on broth microdilution according to CLSI guidelines (M07-A9) following application of epidemiological cut-off values of European Committee on Antimicrobial Susceptibility Testing (EUCAST) as recommended for isolates from livestock and food. The second one (B) was applied in order to assess the clinical relevance

of recovered colistin-resistant isolates in human medicine. For this purpose, they were tested against clinically important antimicrobials for humans by microdilution method as previously described (32). Moreover, MICRONAUT MIC-Strips Colistin (MERLIN Diagnostika GmbH, Bornheim-Hersel, Germany) were used to test the colistin concentrations of up to 64 mg/L.
Also, isolates of *E. coli*, *K. pneumoniae* and *E. cloacae* complex that were cultivated from the same samples on CHROMagar™ ESBL plates (MAST Diagnostica, Reinfeld, Germany) as described previously by (32) and showed resistance to colistin, were included in this study.

4.3.3. Moleculartyping of resistant bacterial isolates

Cell lysates prepared by boiling of bacterial suspensions (35) were used as template for PCR. Determination of phylogenetic groups (A, B1, B2, C, D, E, F, clade I-V) of *E. coli* was conducted according to a previously published method of Clermont (36).

4.3.4. PCR screening for *mcr-1* to *-9* and Sanger-sequencing of the amplicons

Isolates were screened for *mcr-1* to *mcr-5* as well as *mcr-6* to *mcr-9* genes using the multiplex PCR protocols as described by (37) and (38), respectively. As positive controls the isolates *E. coli* R2749 (*mcr-1*), *E. coli* KP37 (*mcr-2*), *S.* Typhimurium SSI_AA940 (*mcr-3*), *S.* Typhimurium R3445 (*mcr-4*), *E. coli* 10E01066 (*mcr-5*) and *S.* Infantis 15-SA01028 (*mcr-9*) were used. The artificially synthesized positive controls for *mcr-6*, *mcr-7* and *mcr-8* were kindly provided by the Department for Biological Safety of German Federal Institute for Risk Assessment (BfR) (Berlin, Germany). PCR products were separated by electrophoresis on a 1.0% agarose-TBE gel and stained with midori green (Labomedic Medizin- und Labortechnik GmbH, Bonn, Germany). Sequence-based typing of *mcr-1* (39) amplicons was performed at Microsynth Seqlab (Göttingen, Germany).

4.3.5. XbaI PFGE-profiling of *mcr-1*-positive *E. coli* and *K. pneumoniae* isolates and *mcr-1* localization

The phylogenetic relationship of the *mcr-1*-carrying *E. coli* and *K. pneumoniae* was assessed XbaI macrorestriction via pulsed-field gel electrophoresis (PFGE) according to the PulseNet protocol (40). Plasmidal localization of the *mcr* genes was determined by S1-PFGE followed by southern blotting and DNA-DNA hybridization against a digoxigenin-labeled PCR amplicon as previously described (Hammerl et al., 2018). The size of *mcr*-carrying plasmids was predicted on the basis of the S1-PFGE pictures with Bionumerics (Applied Math, Sint Marten-Latem, The Netherlands; version 7.5) using *Salmonella* Braenderup (H9812) as size marker.

4.3.6. Conjugation assays

In vitro conjugation experiments were conducted in liquid medium using the plasmid-free rifampicin-resistant *E. coli* recipient strain CV601 GFP at a donor:recipient ratio of 1:1 as previously described (41). Transconjugants were selected after incubation at 37°C for 24-48 h under selective conditions on lysogeny broth (LB) agar (Sigma-Aldrich, St. Louis, MO, USA) containing colistin sulfate (1 µg/ml) and rifampicin (200 µg/ml) (w/v). Isolates that did not yield transconjugants were further subjected to filter mating assays with the rifampicin-resistant, lactose-negative *E. coli* recipient strain W3110 at a donor:recipient ratio of 1:1 (42). The selection of transconjugants was done on MacConkey agar (Sigma-Aldrich, St. Louis, MO, USA) containing colistin sulfate (1 µg/ml) and rifampicin (200 µg/ml) after incubation at 37°C for 24 to 48 h under selective conditions. Potential transconjugants were subjected to PCR to confirm the presence of the *mcr* genes. Those transconjugants obtained with *E. coli* CV601 as recipient were additionally examined for GFP fluorescence using fluorescence microscope Axio Scope.A1 (Carl Zeiss Microscopy GmbH, Jena, Germany).

4.3.7. Transformation assays

mcr-1-positive isolates that did not generate any transconjugants were further submitted to transformation experiments using NEB® 10-beta electrocompetent *E. coli* cells (New England Biolabs, Ipswich, MA, USA) and MicroPulser Electroporator (BioRad, Hercules, CA, USA) according to manufacturer's protocols. Plasmid DNA was extracted from overnight cultures of *mcr-1*-positive isolates using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol. The transformants were selected on LB agar (Sigma-Aldrich, St. Louis, MO, USA) containing colistin sulfate (1 µg/ml). The transconjugants and transformants were cryopreserved at −20°C using cryotubes (Mast Diagnostics, Reinfeld, Germany) until further analysis.

4.3.8. Plasmid replicon typing

Plasmid DNA was extracted from overnight cultures of *E. coli* CV601 and W3110 transconjugants using GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer's protocol. The presence of IncF and IncI plasmids were conducted by RT-PCR 5`-nuclease assays (TaqMan RT-PCR) as previously described (41). Plasmids from transconjugants that could not be detected by RT-PCR were further investigated by PCR-Based Replicon Typing (PBRT). Therefore, PCR amplification on plasmid DNA was performed using primers for the 30 different replicons (HI1, HI2, I1, I2, X1, X2, X3, X4, L, M,

N, FIA, FIB, FIC, FII, FIIS, FIIK, FIB KN, FIB KQ, W, Y, P1, A/C, T, K, U, R, B/O, HIB-M, and FIB-M), which are representative for the major plasmid incompatibility groups among Enterobacteriaceae (43, 44).

4.3.9. Amplification and sequencing of *pmrA* and *prmB* genes in *mcr*-negative *E. coli* and *K. pneumoniae* isolates

The coding sequences of the *pmrA* and *pmrB* genes in *E. coli* and *K. pneumoniae* were amplified as previously described by (9, 45, 46). PCR amplicons were purified using the innuPREP DOUBLEpure Kit (Analytik Jena AG, Jena, Germany) and sequenced at Microsynth Seqlab (Göttingen, Germany). Genomic DNA from five randomly selected *mcr-1*-negative colistin-susceptible *E. coli* and *K. pneumoniae* isolates (colistin MIC <2 mg/L) originating from the same samples were used as control. Sequence analysis was conducted with Chromas lite v.2.6.5 (Technelysium Pty Ltd) and BioEdit v.7.2.5 (47).

4.4. Results

4.4.1. Detection of *Enterobacteriaceae* in samples from poultry and pig slaughterhouses as well as from mWWTPs

Due to the growth of accompanying bacterial flora that belongs to intrinsically colistin-resistant genera (e.g. *Proteus, Providencia, Morganella*) and colistin-susceptible isolates on the selective agar plates as well as absence of sample replicates, it was not possible to perform accurate quantification of target bacteria. This could be considered as a limitation of this study.

Water samples collected in poultry slaughterhouses yielded the highest percentage of colistin-resistant *Enterobacteriaceae* (40.2%; 33/82) followed by mWWTPs (25.0%, 9/36) and pig slaughterhouses (14.9%, 10/67). Detailed information on species distribution is shown in Fig. 4.4.1.

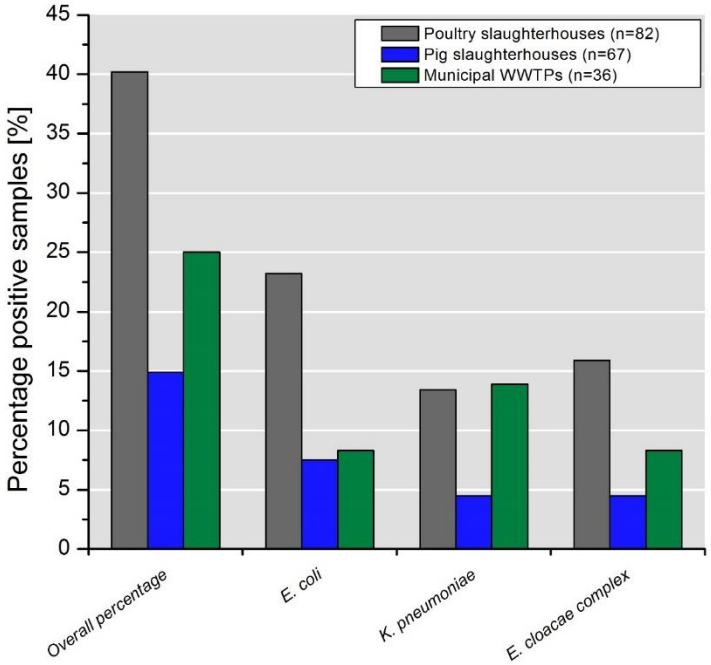

Figure 4.4.1 Percentage of samples containing colistin-resistant target bacteria taken in poultry and pig slaughterhouses as well as in the municipal WWTPs.

In the poultry and pig slaughterhouses the target bacteria were recovered at almost all sampling points as shown in Figure 4.4.2 and 4.4.3, respectively. Interestingly, only one out of nine samples taken in the effluent of the mWWTPs was positive for target colistin-resistant bacteria. Moreover, no colistin-resistant target bacteria were detected in the on-site preflooders upstream the discharge point (Fig. 4.4.3).

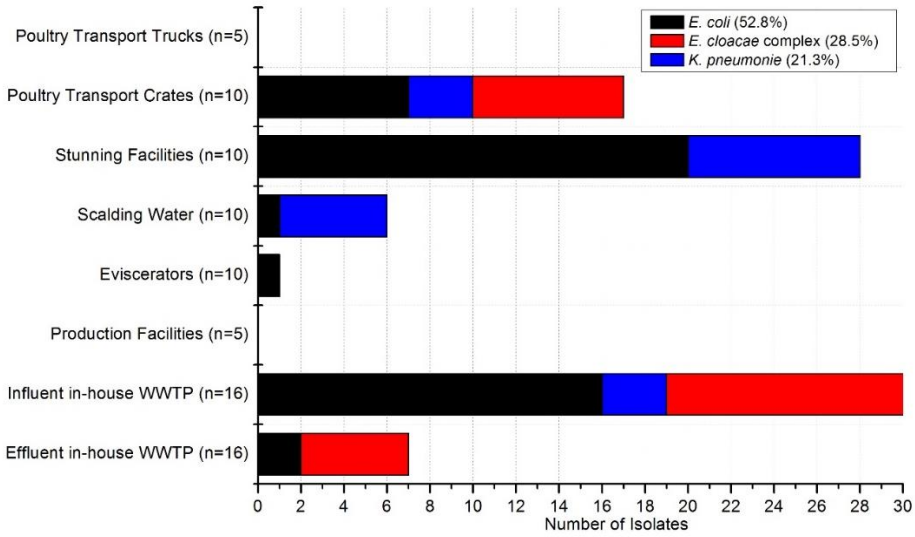

Figure 4.4.2 Occurrence of target bacteria tested as colistin-resistant across the sampling points in poultry slaughterhouses (n=82). Number of samples taken at each sampling point is stated.

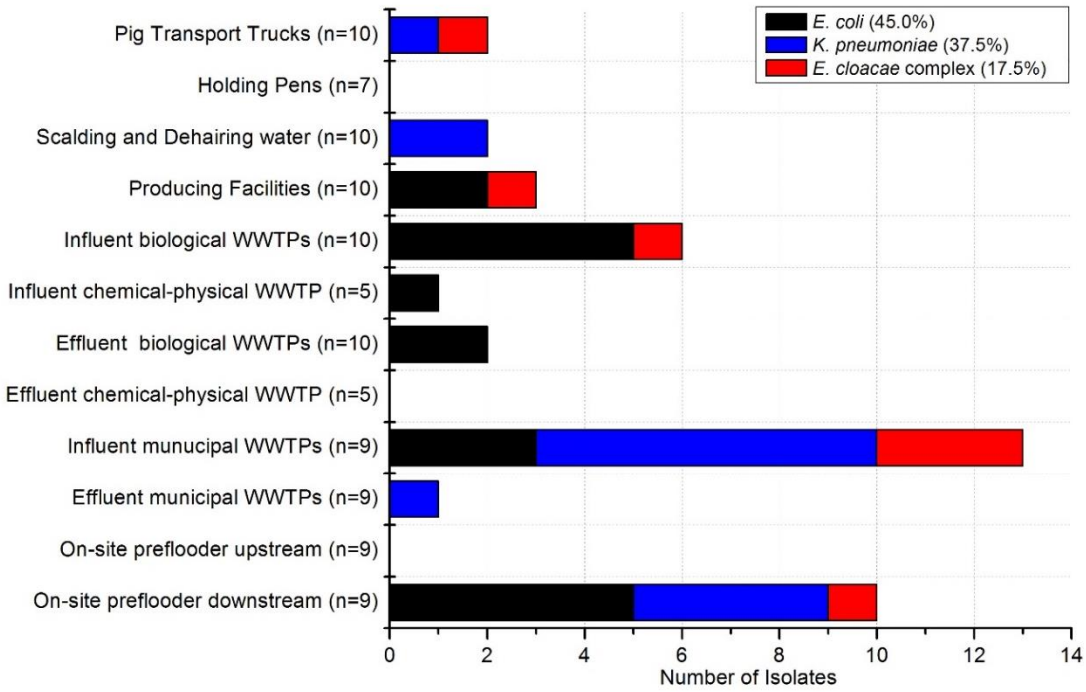

Figure 4.4.3 Occurrence of target bacteria tested as colistin-resistant across the sampling points in pig slaughterhouses (n=67) and in the municipal WWTPs receiving wastewater from the investigated pig slaughterhouses (n=36). Number of samples taken at each sampling point is stated.

Overall, 129 isolates were recovered from 185 samples. Of the isolates, 50.4% were determined as *E. coli*, 26.3% as *K. pneumoniae* and 23.3% as isolates of the *E. cloacae* complex. The most frequently isolated species in poultry and pig slaughterhouses was *E. coli*, whereas in mWWTPs *K. pneumoniae* was more abundant.

4.4.2. Resistance patterns (scheme A (EUCAST) and scheme B (KRINKO))

Isolates of *E. coli*, *K. pneumoniae* and *E. cloacae* complex exhibited various resistance patterns. The resistance rates using epidemiological cut-off values (Fig. 4.4.4) were higher in comparison to those obtained with clinical breakpoints (Fig. 4.4.5).

According to the scheme A, the recovered isolates were either susceptible or expressed low resistance rates to gentamicin, tigecycline and with exception of *E. cloacae* complex to carbapenems (IMP and MEM). The resistance rates to 3rd generation cephalosporins (CTX and CAZ) varied between isolated species and were in the range of 23.5% for *K. pneumoniae* and 46.7% for *E. cloacae* complex. The highest level of multiple drug resistance (MDR, combined resistance to CST, CIP and TET) shown isolates of *E. coli* (49.2%), followed by *K. pneumoniae* (35.3%) and *E. cloacae* complex (33.3%). MICs of antimicrobials with undefined

epidemiological cut-offs for *E. cloacae* complex (AMP, CHL, NAL, SMX, TMP, ETP and FOX) are shown in Tab. 4.4.1.

According to the scheme B, the isolates with exception of *K. pneumoniae* had lower resistance rates to 3rd generation cephalosporins (CTX and CAZ). The differences varied between 12.3% for CAZ by *E. coli* and 26.6% for CTX by *E. cloacae* complex (Fig. 4, 5). Furthermore, they were susceptible to temocillin, ceftazidime-avibactam, imipenem, meropenem, amikacin and, with exception of some *E. cloacae* complex isolates, to tigecycline.

The highest 3MDRO rates (multidrug-resistant organisms with combined resistance to PIP, CTX, CIP) were exhibited by *K. pneumoniae* (26.5%), followed by *E. cloacae* complex (20.6%) and *E. coli* (13.8%). However, if using piperacillin-tazobactam instead of piperacillin, as recommended by (48) for determination of the MDR status, the 3MDRO rates were lower at 5.9% for *K. pneumoniae*, 3.3% for *E. cloacae* complex and 3.1% for *E. coli*.

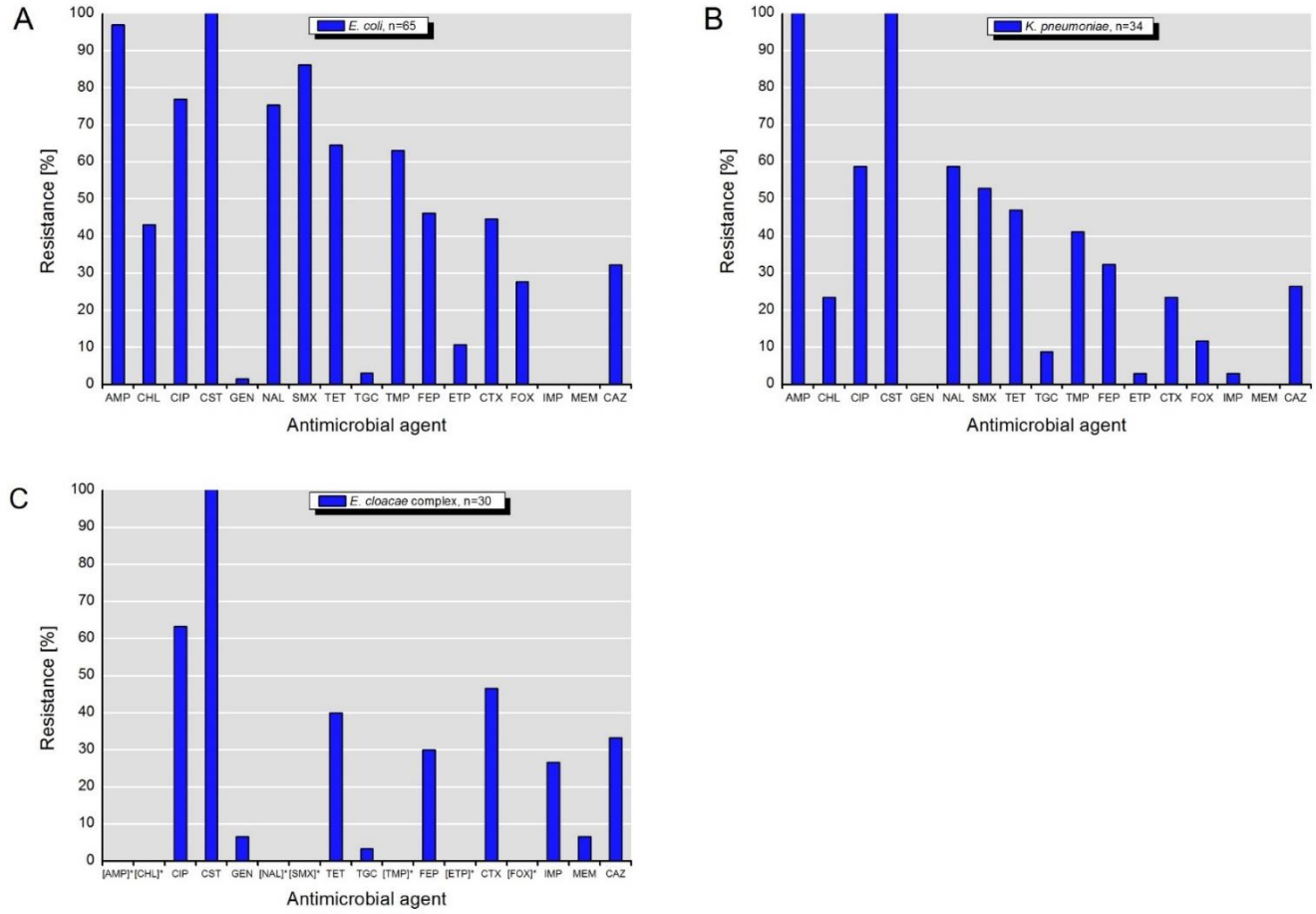

Figure 4.4.4 Resistance to antimicrobial agents detected among target colistin-resistant isolates of (A) *E. coli*, (B) *K. pneumoniae* and (C) *E. cloacae* complex with MICs interpreted according to the epidemiological cut-off values (ECOFFs) of EUCAST (scheme A).

Abbreviations for antimicrobial agents: AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; NAL, nalidixic acid; SMX, sulfamethoxazole; TET, tetracycline; TGC, tigecycline; TMP, trimethoprim; FEP, cefepime; ETP, ertapenem; CTX, cefotaxime; FOX, cefoxitin; IMI, imipenem; MEM, meropenem; CAZ, ceftazidime.
[ ]* - antimicrobials with undefined ECOFFs (Tab. 4.4.1)

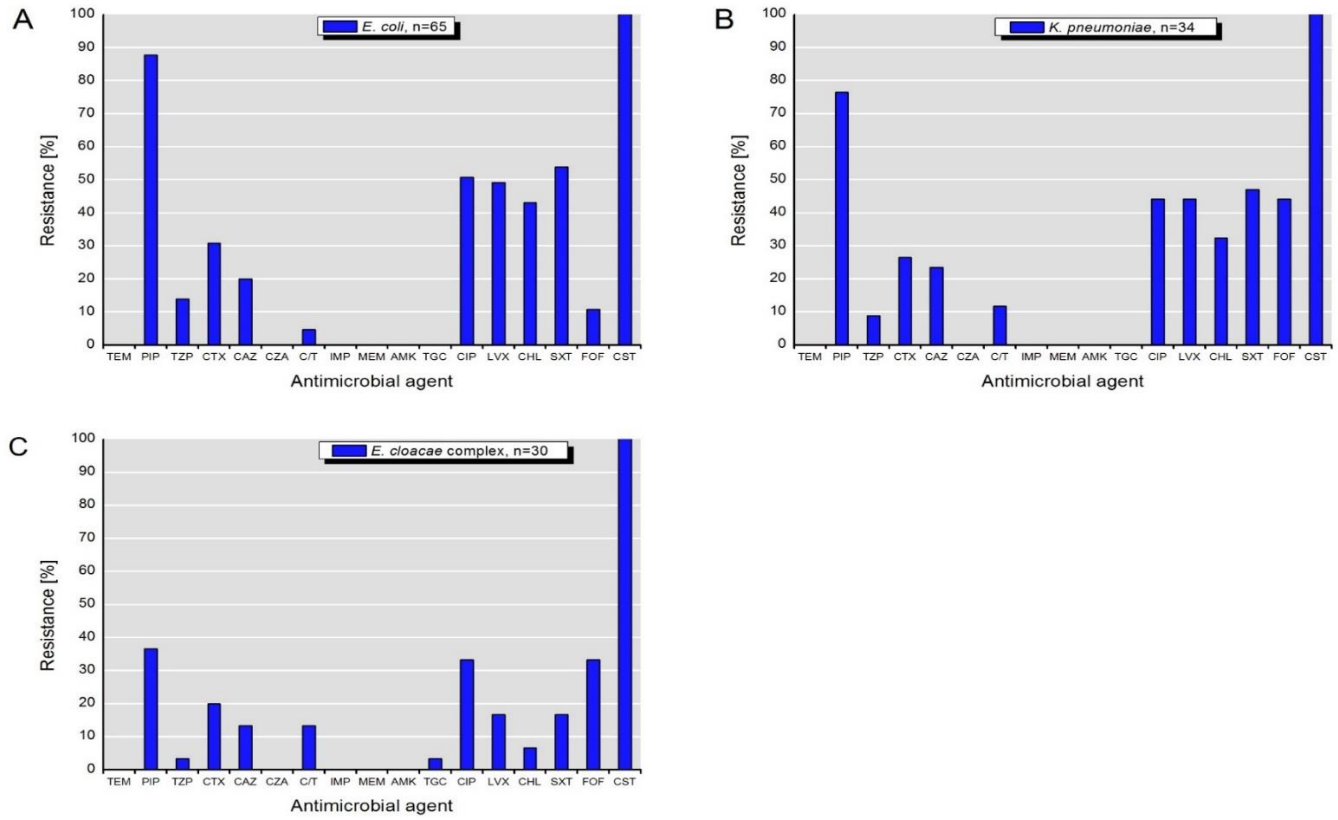

Figure 4.4.5 Resistance to antimicrobial agents detected among target colistin-resistant isolates of (A) *E. coli*, (B) *K. pneumoniae* and (C) *E. cloacae* complex with MICs interpreted according to the clinical breakpoints of EUCAST (scheme B).

Abbreviations for antimicrobial agents: TEM, temocillin; PIP, piperacillin; TZP, piperacillin-tazobactam; CTX, cefotaxime; CAZ, ceftazidime; CZA, ceftazidime-avibactam; C/T, ceftolozane-tazobactam; IMP, imipinem; MEM, meropenem; AMK, amikacin; TGC, tigecycline; CIP, ciprofloxacin; LVX, levofloxacin; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin; CST, colist

Table 4.4.1 MICs (mg/L) of antimicrobials with undefined epidemiological cut-offs for isolates of *E. cloacae* complex tested negative for *mcr-1* to *mcr-9*.

Strain	Species	Origin	AMP	CHL	NAL	SMX	TMP	ETP	FOX
Poultry slaughterhouses									
LWGS-C-4/10-01	*E. asburiae*	Effluent in-house WWTP	> 64	≤ 8	> 128	≤ 8	≤ 0.25		
LWGS-C-4/2-01	*E. asburiae*	Transport crates	16	16	8	> 1024	2		
LWGS-C-4/2-03	*E. asburiae*	Transport crates	32	≤ 8	≤ 4	≤ 8	0.5		
LWGS-C-4/2-16	*E. asburiae*	Transport crates	8	16	≤ 4	256	1		
LWGS-C-4/2-22	*E. asburiae*	Transport crates	8	16	16	≤ 8	0.5	0.03	> 64
LWGS-C-4/5-23	*E. asburiae*	Influent in-house WWTP	16	≤ 8	> 128	≤ 8	≤ 0.25	0.03	> 64
LWGS-C-4/5-24	*E. asburiae*	Influent in-house WWTP	16	≤ 8	64	≤ 8	0.5	0.03	> 64
LWGS-C-4/5-28	*E. asburiae*	Influent in-house WWTP	16	16	16	512	1	0.03	> 64
LWGS-C-4/5-31	*E. asburiae*	Influent in-house WWTP	8	≤ 8	> 128	≤ 8	≤ 0.25	≤ 0.015	> 64
LWGS-C-4/5-33	*E. asburiae*	Influent in-house WWTP	4	≤ 8	> 128	≤ 8	≤ 0.25	≤ 0.015	> 64
LWGS-C-4/5-35	*E. asburiae*	Influent in-house WWTP	8	≤ 8	> 128	≤ 8	0.5	≤ 0.015	> 64
LWGS-C-4/5-41	*E. asburiae*	Influent in-house WWTP	8	≤ 8	> 128	≤ 8	≤ 0.25	≤ 0.015	> 64
LWGS-C-4/5-42	*E. asburiae*	Influent in-house WWTP	16	≤ 8	16	≤ 8	≤ 0.25	≤ 0.015	> 64
LWGS-C-4/5-43	*E. asburiae*	Influent in-house WWTP	8	≤ 8	> 128	≤ 8	≤ 0.25	0.06	> 64
LWGS-C-4/5-44	*E. asburiae*	Influent in-house WWTP	16	≤ 8	16	≤ 8	≤ 0.25	≤ 0.015	> 64
LWGS-C-4/6-04	*E. asburiae*	Effluent in-house WWTP	32	≤ 8	> 128	≤ 8	≤ 0.25		
LWGS-C-4/6-07	*E. asburiae*	Effluent in-house WWTP	32	≤ 8	≤ 4	≤ 8	≤ 0.25	0.12	> 64
LWGS-C-4/5-29	*E. hormaechei*	Influent in-house WWTP	> 64	≤ 8	> 128	≤ 8	≤ 0.25	0.03	> 64
LWGS-C-4/6-05	*E. hormaechei*	Effluent in-house WWTP	> 64	≤ 8	> 128	≤ 8	≤ 0.25		
LWGS-C-4/6-01	*E. kobei*	Effluent in-house WWTP	32	16	≤ 4	≤ 8	≤ 0.25		
LWGS-1/2-22	*E. asburiae*	Transport crates	> 64	≤ 8	≤ 4	≤ 8	≤ 0.25	0.03	> 64
LWGS-4/2-02	*E. asburiae*	Transport crates	> 64	≤ 8	> 128	≤ 8	1	0.5	> 64
LWGS-4/2-04	*E. asburiae*	Transport crates	> 64	16	16	32	1	1	> 64
Pig slaughterhouses and mWWTPs									
LWSS-C-3/2-01	*E. asburiae*	Influent biological WWTP	16	≤ 8	≤ 4	≤ 8	≤ 0.25	≤ 0.015	> 64
LWSS-C-5/10-24	*E. asburiae*	Influent municipal WWTP	8	≤ 8	≤ 4	≤ 8	≤ 0.25	0.03	> 64
LWSS-C-5/10-25	*E. asburiae*	Influent municipal WWTP	64	≤ 8	8	≤ 8	≤ 0.25		
LWSS-C-3/9-03	*E. cloacae*	Producing facilities	16	≤ 8	≤ 4	≤ 8	≤ 0.25		
LWSS-5/3-11	*E. aerogenes*	Pig Transport Trucks	> 64	16	≤ 4	> 1024	> 32	0.06	> 64
LWSS-3/10-33	*E. asburiae*	Influent municipal WWTP	> 64	≤ 8	> 128	> 1024	> 32	2	> 64
LWSS-3/12-25	*E. asburiae*	On-site preflooder downstream	> 64	≤ 8	> 128	> 1024	> 32	0.12	> 64

4.4.3. Phylogenetic groups of *E. coli* (n=65)

The majority of the *E. coli* isolates belonged to the most common phylogroups associated to commensal strains, such as A (32.3%), B1 (24.6%), C (16.9%), F (10.8%), Clade I, II (9.2%) and E (1.5%). Only two isolates (3.0%) recovered from the influent of the in-house WWTP of a poultry slaughterhouse were assigned to extraintestinal pathogenic (ExPEC) group D. Furthermore, one isolate originating from the wastewater used for cleaning of poultry stunning facilities belonged to group B2.

4.4.4. Occurrence of *mcr* genes

Of the *mcr* genes screened, only *mcr-1.1* was detected in 70.8% of *E. coli* and 20.6% of *K. pneumoniae* isolates. Colistin MICs of *mcr-1*-positive *E. coli* isolates ranged from 4 to 8 mg/L, whereas *mcr-1* carrying *K. pneumoniae* isolates expressed higher level of resistance from 4 to >64 mg/L.

In poultry and pig slaughterhouses the *mcr-1.1* carrying isolates of *E. coli* and *K. pneumoniae* were disseminated at almost all sampling points including scalding water and effluents of the in-house WWTPs. Furthermore, *mcr-1.1* positive isolates of *E. coli* were detected in on-site preflooders downstream the discharge point. Detailed information on the isolation source and phenotypic resistance of *mcr-1.1* carrying isolates of *E. coli* and *K. pneumoniae* is given in Tab. 4.4.2.

Table 4.4.2 Characteristics of MCR-1–producing *E. coli* and *K. pneumoniae* isolates and their transconjugants.

Strain	Species	Origin	Colistin MIC, mg/L	Resistance phenotype (epidemiological cut-off values of EUCAST)[a]	Resistance phenotype (clinical breakpoints of EUCAST)[b]	Incompatibility group (kb) of *mcr-1* plasmids	Colistin MIC of transconjugants mg/L	Co-transferred resistance (epidemiological cut-off values of EUCAST)[a]	Co-transferred resistance (clinical breakpoints of EUCAST)[b]
Poultry slaughterhouses									
LWGS-1/5-11	*E. coli*	Influent in-house WWTP	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CHL, SXT, CST	IncF (30)	8		
LWGS-1/5-12	*E. coli*	Influent in-house WWTP	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CHL, SXT, CST	IncF (30)	8		
LWGS-1/7-07	*E. coli*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CHL, SXT, CST	IncX4 (30)	4	CIP, NAL	
LWGS-1/7-09	*E. coli*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CHL, SXT, CST	IncI1 (30)	8	CIP, NAL	
LWGS-1/7-11	*E. coli*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CAZ, CHL, SXT, CST	IncX4 (30)	4	CIP, NAL	
LWGS-1/7-12	*E. coli*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CHL, SXT, CST	IncX4 (30)	4	CIP, NAL	
LWGS-1/8-08	*E. coli*	Eviscerators	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CAZ, CHL, SXT, FOF, CST	IncHI2 (30)	4	CIP, NAL	
LWGS-4/5-12	*E. coli*	Influent in-house WWTP	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TET, FEP	PIP, CTX, CAZ, C/T, CIP, LVX, CHL, CST	IncHI2 (245)	4	AMP, CIP, CTX, CAZ	
LWGS-C-1/5-03	*E. coli*	Influent in-house WWTP	4	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, SXT, CST	IncI1 (30)	4	AMP, SMX, TMP	SXT
LWGS-C-1/5-04	*E. coli*	Influent in-house WWTP	4	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, CST	IncI2 (n.d.*)	4	CIP, NAL	
LWGS-C-1/7-02	*E. coli*	Stunning facilities	4	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, CST	IncX4 (30)	4	CIP, NAL	PIP, CIP, LVX, SXT
LWGS-C-1/7-04	*E. coli*	Stunning facilities	4	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, CST	IncX4 (30)	4	CIP, NAL	
LWGS-C-1/7-06	*E. coli*	Stunning facilities	4	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, SXT, CST	IncX4 (30)	4	CIP, NAL	
LWGS-C-4/2-02	*E. coli*	Transport crates	4	AMP, CIP, CST, NAL, SMX, TET	PIP, CIP, LVX, CST	IncX4 (30)	4	CIP, NAL	
LWGS-C-4/5-10	*E. coli*	Influent in-house WWTP	4	AMP, CST, SMX, TET, TMP	PIP, SXT, CST	IncI1 (360)	4	AMP, SMX, TET, TMP	PIP, SXT

Table 4.4.2 (continued)

LWGS-C-4/5-14	***E. coli***	**Influent in-house WWTP**	**4**	**AMP, CST, SMX, TET, TMP**	**FOF, CST**	**IncI1 (360)**	**4**	**AMP, SMX, TET, TMP**	
LWGS-C-4/6-02	*E. coli*	Effluent in-house WWTP	8	AMP, CST, SMX, TMP	PIP, CIP, LVX, CST	IncHI2 (30)	8	AMP, SMX, TMP	PIP
LWGS-C-4/7-04	*E. coli*	Stunning facilities	4	AMP, CIP, CST, SMX, NAL, TET, TMP	PIP, CIP, SXT, CST	IncF (215)	4	AMP, CIP, NAL, SMX, TMP	SXT
LWGS-C-4/7-07	*E. coli*	Stunning facilities	4	AMP, CIP, CST, SMX, NAL, TET, TMP	PIP, SXT, CST	IncHI2 (215)	4	AMP, CIP, NAL, TET	PIP, SXT
LWGS-4/7-04	*K. pneumoniae*	Stunning facilities	8	AMP, CIP, CST, CTX, NAL, SMX, CAZ, TET, TMP, FEP, FOX	PIP, CTX, CIP, LVX, CHL, SXT, FOF, CST	IncI1 (30)	4	CIP, NAL	
LWGS-4/7-12	*K. pneumoniae*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TET, FEP	PIP, TZP, CTX, CAZ, CIP, LVX, CHL, SXT, FOF, CST	IncX4 (85)	8	CIP, CTX, NAL, CAZ, FOX	
LWGS-4/7-14	*K. pneumoniae*	Stunning facilities	16	AMP, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CAZ, C/T, CIP, LVX, SXT, FOF, CST	IncX4 (30)	4	CIP, NAL	
LWGS-C-4/2-17	*K. pneumoniae*	Transport crates	>64	AMP, CST	PIP, SMX, CST	IncI1 (30)	8		
LWGS-C-4/3-01	*K. pneumoniae*	Scalding water	16	AMP, CIP, CST, NAL	CIP, LVX, CST	IncX4 (30)	4	CIP, NAL	
					Pig slaughterhouses and mWWTPs				
LWSS-5/1-09	*E. coli*	Influent in-house WWTP	8	AMP, CHL, CIP, CST, CTX, FOX, TET, TMP, FEP	PIP, TZP, CTX, CIP, LVX, CHL, SMX, CST	IncI1 (30)	8	TET	
LWSS-5/1-21	*E. coli*	Influent in-house WWTP	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CIP, LVX, CHL, SXT, CST	IncI1 (30)	8	TET	
LWSS-5/1-22	*E. coli*	Influent in-house WWTP	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CIP, LVX, CHL, SXT, CST	IncI1 (30)	4	TET	
LWSS-5/1-23	*E. coli*	Influent in-house WWTP	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CIP, LVX, CHL, SXT, CST	IncI1 (30)	4	TET	
LWSS-5/2-28	*E. coli*	Effluent in-house WWTP	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CIP, LVX, CHL, SXT, CST	IncI1 (30)	4	TET	
LWSS-5/2-29	*E. coli*	Effluent in-house WWTP	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CTX, CIP, LVX, CHL, SXT, CST	IncI1 (30)	4	TET	

Table 4.4.2 (continued)

LWSS-5/6-69	***E. coli***	**Aggregate wastewater from producing facilities**	**8**	**AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP**	**PIP, TZP, CIP, LVX, CHL, SXT, CST**	**IncI1 (30)**	**4**	**TET**
LWSS-5/6-70	*E. coli*	Aggregate wastewater from producing facilities	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, TET, TMP, FEP, ETP	PIP, TZP, CIP, LVX, CHL, SXT, CST	IncI1 (30)	4	TET
LWSS-C-3/1-04	*E. coli*	Influent in-house WWTP	8	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, CHL, CST	IncI1 (30)	4	CIP, NAL
LWSS-C-3/12-05	*E. coli*	On-site preflooder downstream	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TET, FEP	PIP, CST	IncHI2 (n.d.*)	2	CIP, NAL
LWSS-C-3/12-07	*E. coli*	On-site preflooder downstream	4	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TET, TGC, FEP	PIP, CST	IncHI2 (245)	2	
LWSS-C-3/12-08	*E. coli*	On-site preflooder downstream	8	AMP, CHL, CIP, CST, CTX, FOX, NAL, SMX, CAZ, TET, FEP	PIP, TZP, CST	IncHI2 (230)	4	CIP, NAL
LWSS-C-3/5-01	*K. pneumoniae*	Animal transporters	>64	AMP, CHL, CIP, CST, NAL	PIP, CIP, LVX, CHL, CST	IncX4 (30)	4	CIP, NAL
LWSS-C-3/8-01	*K. pneumoniae*	Scalding water	>64	AMP, CHL, CIP, CST, CTX, FOX, NAL, CAZ, FEP	FOF, CST	IncX4 (30)	4	CIP, NAL

[a] Antimicrobial Susceptibility Testing Plates of German Federal Institute for Risk Assessment (BfR) containing sulfamethoxazole (SMX), trimethoprim (TMP), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), tigecycline (TGC), ertapenem (ETP), meropenem (MEM), imipenem (IMI), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), cefepime (FEP), colistin (CST), ampicillin (AMP), gentamicin (GEN). MIC were interpreted according to the epidemiological cut off values of EUCAST.

[b] Micronaut-S MDR MRGN-Screening system containing temocillin (TEM), piperacillin (PIP), piperacillin/tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), imipenem (IMI), meropenem (MEM), amikacin (AMK), tigecycline (TGC), chloramphenicol (CHL), fosfomycin (FOF), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LVX) and colistin (CST). MIC were interpreted according to the clinical breakpoints of EUCAST.

4.4.5. PFGE patterns of colistin-resistant *mcr-1* carrying isolates, location of *mcr-1* genes

Overall, the analysed isolates (n=53, 46 *E. coli* and 7 *K. pneumoniae*) exhibited a broad diversity as they were assigned to 25 different XbaI profiles (20 for *E. coli* and 5 for *K. pneumoniae*). S1 nuclease PFGE, followed by Southern blot hybridization revealed the presence of *mcr-1* carrying plasmids ranging between 30 and 360 kb. Interestingly, the majority of the isolates exhibited a predominant plasmid type of 30 kb. However, we had also determined a substantial number of isolates exhibiting the same XbaI macrorestriction patterns and/or plasmid profiles.

4.4.6. Conjugation experiments, Inc-typing of plasmids

In 67.4% (31/46) of *mcr-1* carrying *E. coli* isolates, the *mcr-1* gene was found to be encoded on plasmids of different Inc-groups that could be conjugated into recipient *E. coli* cells (Tab. 1). Plasmids were affiliated to IncI1 (41.9%), IncHI2 and IncX4 (each 22.6%,), IncF (9.7%) as well as IncI2 (3.2%) types as demonstrated by TaqMan RT-PCR and PBRT method. All seven *mcr-1*-positive *K. pneumoniae* isolates carried the *mcr-1* on self-transmissible IncX4 (71.4%) and IncI1 (28.6%) plasmids. Of note, IncI1-type plasmids carrying *mcr-1* were predominant in all sampling sites. Colistin MICs of transconjugants were either identical or lower than those of the donor strains and ranged from 2 to 8 mg/L.

Conjugation experiments with the applied selection conditions resulted in diverse co-transferred resistance phenotypes. Using epidemiological cut-off values, 81.6% (31/38) of *E. coli* and *K. pneumoniae* transconjugants expressed resistance to further antimicrobials beside colistin. Among the isolates recovered in the poultry slaughterhouses the most frequently co-transferred resistance was to ciprofloxacin and nalidixic acid (70.8%, 17/24), followed by ampicillin (29.2%, 7/24) and trimethoprim/sulfamethoxazole (25.0%, 6/24). Only 8.3% (2/24) of the isolates co-transferred resistance against 3rd generation cephalosporins. In contrast, the majority of the isolates originating from the pig slaughterhouses co-transferred resistance against tetracycline (57.1%, 8/14). The resistance to ciprofloxacin and nalidixic acid was co-transferred by 35.7% (5/14) of the isolates. However, when applying scheme B based on clinical breakpoints, only 15.8% (6/38) of the colistin-resistant transconjugants expressed additional resistances, mostly to sulfamethoxazole-trimethoprim (5/6) and piperacillin (4/6).

Detailed information on Inc-types of *mcr-1* harboring plasmids, colistin MIC of the transconjugants and co-transferred resistance phenotypes of individual isolates is given in Tab. 4.4.2.

4.4.7. *pmrAB* sequences of colistin-resistant *E. coli* and *K. pneumoniae* isolates tested negative for *mcr-1* to *mcr-9*

In 73.7% (14/19) of *E. coli* isolates non-synonymous polymorphisms at the protein level were detected in *pmrA* and *pmrB*. Nucleotide sequence polymorphisms that produce protein variants 15Gly→Arg, 80Ala→Val, 85Thr→Ala, 204Ala→X were found in *pmrA*. Furthermore, eleven variants, 2His→Arg, 10Leu→Arg, 12Gln→x, 14Leu→Pro, 29Ser→x, 44Phe→x, 94Pro→S, 285Ala→Thr, 312Asp→Asn, 333His→Gln, 360Ala→Val, were found in *pmrB*.

In 81.5% (22/27) of *K. pneumoniae* isolates the *pmrA* and *pmrB* genes revealed polymorphic positions that were non-synonymous at the protein level. Additionally, four non-synonymous polymorphisms were found in *pmrA* (37Ala→Thr, 57Glu→Gly, 147Ala→Glu and 217Ala→Val) and six in *pmrB* (2Ala→Ser, 73Pro→x, 74Ser→x, 112Thr→Pro, 157Thr→Pro, 203Ser→Pro). In one *K. pneumoniae* isolate recovered from the on-site preflooder downstream the discharge point, a insertion of nine amino acids (Gln-Leu-Gln-Gln-Leu-Ala-Arg-Val-Gly) was inserted between amino acid residues Glu-201 and Gln-202 of *pmrB*. Detailed information on non-synonymous polymorphisms of individual *E. coli* and *K. pneumoniae* isolates, their origin and resistance phenotypes is given in Tab. 4.4.3 and Tab. 4.4.4, respectively.

Table 4.4.3 PmrAB polymorphisms of colistin-resistant *E. coli* isolates tested negative for *mcr-1* to *mcr-9*.

Strain	Species	Origin	Colistin MIC, mg/L	Resistance phenotype (epidemiological cut-off values of EUCAST)[a]	Resistance phenotype (clinical breakpoints of EUCAST)[b]	PmrAB[c]
Poultry slaughterhouses						
LWGS-C-1/7-07	*E. coli*	Stunning facilities	8	AMP, CIP, CST, NAL, SMX, TET	PIP, CST	2H→R (PmrB) 360A→V (PmrB)
LWGS-C-1/7-08	*E. coli*	Stunning facilities	8	AMP, CIP, CST, NAL, SMX, TET	PIP, CST	2H→R (PmrB) 360A→V (PmrB)
LWGS-C-1/7-11	*E. coli*	Stunning facilities	8	AMP, CIP, CST, NAL, SMX, TET	PIP, CST	2H→R (PmrB) 360A→V (PmrB)
LWGS-C-1/7-12	*E. coli*	Stunning facilities	8	AMP, CIP, CST, NAL, SMX, TET	PIP, CST	2H→R (PmrB) 360A→V (PmrB)
LWGS-4/2-15	*E. coli*	Transport crates	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TET, TGC, FEP, FOX	PIP, CTX, CAZ, CIP, LVX, CHL, CST	–
LWGS-4/2-16	*E. coli*	Transport crates	4	AMP, CST, CTX, SMX, CAZ, TET, TMP, FEP, FOX	PIP, CTX, CAZ, CIP, LVX, CHL, SXT, CST	–
LWGS-4/7-11	*E. coli*	Stunning facilities	8	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TET, FEP, FOX	PIP, CTX, CAZ, C/T, CIP, LVX, CHL, CST	–
LWGS-C-4/3-08	*E. coli*	Scalding water	8	AMP, CST, SMX	PIP, CIP, LVX, SXT, CST	14L→P (PmrB) 44F→x (PmrB)
LWGS-C-4/5-08	*E. coli*	Influent in-house WWTP	8	AMP, CST, SMX, TMP	PIP, SXT, CST	14L→P (PmrB) 44F→x (PmrB)
LWGS-C-4/5-13	*E. coli*	Influent in-house WWTP	8	AMP, CST, SMX	PIP, CST	10L→R (PmrB) 12Q→x (PmrB)
LWGS-C-4/5-15	*E. coli*	Influent in-house WWTP	8	AM, CIP, CST, NAL, TMP	PIP, CIP, LVX	15G→R (PmrA) 85T→A (PmrA) 2H→R (PmrB)
LWGS-C-4/5-17	*E. coli*	Influent in-house WWTP	8	AMP, CIP, CST, NAL	PIP, CIP, LVX, CST	312D→N (PmrB)
LWGS-C-4/7-03	*E. coli*	Stunning facilities	4	AMP, CHL, CIP, CST, CTX, NAL, SMX, CAZ, TET, FEP, FOX	PIP, CST	–
LWGS-C-4/7-06	*E. coli*	Stunning facilities	4	AMP, CST, SMX, TET, TMP	PIP, CIP, LVX, SXT, FOF, CST	29S→x (PmrB)
LWGS-C-4/7-08	*E. coli*	Stunning facilities	4	AMP, CST, SMX, TET, TMP	PIP, CIP, LVX, CHL, SXT, CST	–
Pig slaughterhouses and mWWTPs						
LWSS-C-3/2-02	*E. coli*	Effluent in-house WWTP	8	AMP, CIP, CST, CTX, NAL, SMX, CAZ, TET, TMP, FEP	PP, CTX, CAZ, C/T, CIP, LVX, CHL, CST	204A→x (PmrA) 2H→R (PmrB)
LWSS-C-3/10-10	*E. coli*	Influent municipal WWTP	4	CIP, CST, NAL	CST	80A→V (PmrA) 285A→T (PmrB) 333H→Q (PmrB)
LWSS-C-3/10-14	*E. coli*	Influent municipal WWTP	4	CIP, CST, NAL	CST	80A→V (PmrA) 285A→T (PmrB) 333H→Q (PmrB)
LWSS-3/10-43	*E. coli*	Influent municipal WWTP	16	AMP. CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CAZ, SXT, CST	44F→x (PmrB) 94P→S (PmrB)

Table 4.4.3 (continued)

[a] Antimicrobial Susceptibility Testing Plates of German Federal Institute for Risk Assessment (BfR) containing sulfamethoxazole (SMX), trimethoprim (TMP), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), tigecycline (TGC), ertapenem (ETP), meropenem (MEM), imipenem (IMI), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), cefepime (FEP), colistin (CST), ampicillin (AMP), gentamicin (GEN). MIC were interpreted according to the epidemiological cut off values of EUCAST.

[b] Micronaut-S MDR MRGN-Screening system containing temocillin (TEM), piperacillin (PIP), piperacillin/tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), imipenem (IMI), meropenem (MEM), amikacin (AMK), tigecycline (TGC), chloramphenicol (CHL), fosfomycin (FOF), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LVX) and colistin (CST). MIC were interpreted according to the clinical breakpoints of EUCAST.

[c] Polymorphisms found for coding sequences for PmrA or PmrB.

Table 4.4.4 PmrAB polymorphisms of colistin-resistant *K. pneumoniae* isolates tested negative for *mcr-1* to *mcr-9*.

Strain	Species	Origin	Colistin MIC, mg/L	Resistance phenotype (epidemiological cut-off values of EUCAST)[a]	Resistance phenotype (clinical breakpoints of EUCAST)[b]	PmrAB[c]
Poultry slaughterhouses						
LWGS-4/5-15	*K. pneumonae*	Influent in-house WWTP	32	AMP, CIP, CST, CTX, NAL, SMX, CAZ, TMP, FEP	PIP, CTX, CAZ, CIP, LVX, CHL, SXT, FOF, CST	112T→P (PmrB)
LWGS-4/7-34	*K. pneumonae*	Stunning facilities	16	AMP, CHL, CST, CTX, SMX, CAZ, TET, TMP, FEP	CHL, SXT, FOF, CST	157T→P (PmrB)
LWGS-C-4/2-07	*K. pneumonae*	Transport crates	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, SXT, CST	
LWGS-C-4/2-08	*K. pneumonae*	Transport crates	>64	AMP, CIP, CST, NAL, SMX, CAZ, TET, TMP	PIP, SXT, CST	73P→x (PmrB) 74S→x (PmrB)
LWGS-C-4/3-09	*K. pneumonae*	Scalding water	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, CST	73P→x (PmrB) 74S→x (PmrB)
LWGS-C-4/3-11	*K. pneumonae*	Scalding water	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, FOF, CST	73P→x (PmrB) 74S→x (PmrB)
LWGS-C-4/3-12	*K. pneumonae*	Scalding water	32	AMP, CIP, CST, NAL, SMX, CAZ, TET, TGC, TMP, FEP, FOX	PIP, CIP, LVX, SXT, FOF, CST	73P→x (PmrB) 74S→x (PmrB)
LWGS-C-4/3-13	*K. pneumonae*	Scalding water	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, SXT, CST	73P→x (PmrB) 74S→x (PmrB)
LWGS-C-4/5-01	*K. pneumonae*	Influent in-house WWTP	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, SXT, CST	73P→x (PmrB) 74S→x (PmrB)
LWGS-C-4/5-19	*K. pneumonae*	Influent in-house WWTP	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CIP, LVX, SXT, FOF, CST	73P→x (PmrB) 74S→x (PmrB)
LWGS-C-4/7-29	*K. pneumonae*	Stunning facilities	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, SXT, COL	73P→x (PmrB) 74S→x (PmrB)
LWGS-C-4/7-02	*K. pneumonae*	Stunning facilities	>64	AMP, CIP, CST, NAL, SMX	CST	
LWGS-C-4/7-25	*K. pneumonae*	Stunning facilities	32	AMP, CIP, CST, NAL, SMX, TET, TMP	PIP, CST	73P→x (PmrB) 74S→x (PmrB)
LWGS-C-4/7-28	*K. pneumonae*	Stunning facilities	32	AMP, CIP, CST, CTX, NAL, TET, TGC, FEP, FOX	PIP, CST	73P→x (PmrB) 74S→x (PmrB)
Pig slaughterhouses and mWWTPs						
LWSS-C-3/8-02	*K. pneumonae*	Scalding water	16	AMP, CST, SMX, CAZ, TET, FEP	PIP, TZP, C/T, CST	
LWSS-C-3/10-18	*K. pneumonae*	Influent municipal WWTP	16	AMP, CST	FOF, CST	217A→V (PmrA)
LWSS-C-3/10-19	*K. pneumonae*	Influent municipal WWTP	16	AMP, CST	FOF, CST	217A→V (PmrA)
LWSS-C-3/10-21	*K. pneumonae*	Influent municipal WWTP	32	AMP, CST	PIP, FOF, CST	2A→S (PmrB)
LWSS-C-3/10-22	*K. pneumonae*	Influent municipal WWTP	32	AMP, CST	CST	2A→S (PmrB)
LWSS-C-3/12-01	*K. pneumonae*	On-site preflooder downstream	8	AMP, CIP, CST, NAL, CAZ, FEP	PIP, CTX, CAZ, C/T, CIP, LVX, CHL, CST	"Insertion" of QLQQLARVG between 201E and 202Q
LWSS-C-3/12-02	*K. pneumonae*	On-site preflooder downstream	16	AMP, CIP, CST, NAL, SMX, CAZ, TET, FEP	PIP, CTX, CAZ, C/T, CIP, LVX, CHL, CST	

Table 4.4.4 (continued)

LWSS-C-3/12-06	***K. pneumonae***	**On-site preflooder downstream**	**16**	**AMP, CHL, CIP, CST, NAL, SMX, CAZ, TET, FEP**	**PIP, CTX, CAZ, C/T, CIP, LVX, CHL, CST**	
LWSS-C-3/12-10	*K. pneumonae*	On-site preflooder downstream	>64	AMP, CHL, CIP, CST, NAL	PIP, CTX, CAZ, CIP, LVX, CHL, CST	217A→V (PmrA)
LWSS-C-5/10-15	*K. pneumonae*	Influent municipal WWTP	16	AMP, CST	PIP, CST	147A→E (PmrA) 217A→V (PmrA)
LWSS-C-5/10-16	*K. pneumonae*	Influent municipal WWTP	16	AMP, CST	CST	147A→E (PmrA) 217A→V (PmrA)
LWSS-C-5/10-26	*K. pneumonae*	Influent municipal WWTP	16	AMP, CST	FOF, CST	37A→T (PmrA)
LWSS-5/11-29	*K. pneumonae*	Effluent municipal WWTP	32	AMP, CHL, CIP, CST, CTX, NAL, CAZ, FEP, ETP, FOX, IMI	PIP, TZP, CTX, CAZ, CIP, LVX, CHL, FOF, CST	57E→G (PmrA) 203S→P (PmrB)

[a] Antimicrobial Susceptibility Testing Plates of German Federal Institute for Risk Assessment (BfR) containing sulfamethoxazole (SMX), trimethoprim (TMP), chloramphenicol (CHL), ciprofloxacin (CIP), nalidixic acid (NAL), tetracycline (TET), tigecycline (TGC), ertapenem (ETP), meropenem (MEM), imipenem (IMI), cefotaxime (CTX), ceftazidime (CAZ), cefoxitin (FOX), cefepime (FEP), colistin (CST), ampicillin (AMP), gentamicin (GEN). MIC were interpreted according to the epidemiological cut off values of EUCAST.

[b] Micronaut-S MDR MRGN-Screening system containing temocillin (TEM), piperacillin (PIP), piperacillin/tazobactam (TZP), cefotaxime (CTX), ceftazidime (CAZ), imipenem (IMI), meropenem (MEM), amikacin (AMK), tigecycline (TGC), chloramphenicol (CHL), fosfomycin (FOF), trimethoprim/sulfamethoxazole (SXT), ciprofloxacin (CIP), levofloxacin (LVX) and colistin (CST). MIC were interpreted according to the clinical breakpoints of EUCAST.

[c] Polymorphisms found for coding sequences for PmrA or PmrB.

4.5. Discussion

Our study provides data on the occurrence of colistin resistant Enterobacteriaceae (*E. coli*, *K. pneumoniae*, *E. cloacae* complex) in process water and wastewater along the slaughtering chains in poultry and pig slaughterhouses, their in-house and municipal WWTPs as well as receiving waterbodies.

The highest prevalence of colistin-resistant bacteria was detected in poultry slaughterhouses. This is in consent with other studies indicating frequent occurrence of colistin-resistant *Enterobacteriaceae* in the poultry production chain in Germany (28, 29). Current data on antimicrobial usage in different animal species in Germany are not available. However, the Report of the Federal Ministry of Food and Agriculture on the Evaluation of the Antimicrobials Minimization Concept introduced with the 16th Act to Amend the Medicinal Products Act (16th AMG Amendment) indicates a higher usage of colistin in German poultry production in comparison to other livestock production chains (49). Moreover, between 2014 and 2017 consumption of polypeptide antibiotics in broiler production in Germany slightly increased from 11 tons to 13 tons. Whereas in pig production chain polypeptide antibiotics are mostly used to treat piglets and for the treatment of fattening pigs a decrease from 4 tons in 2014 to 0.5 tons in 2017 was observed (49). Thus, the higher use of colistin in poultry may coincide with the frequent occurrence of colistin-resistant bacteria in this production chain. Furthermore, in comparison to poultry, a longer life span and time gap between administration of antibiotic and slaughtering among pigs may result in a decrease of colistin resistance when selection pressure is absent. Moreover, the kind of antibiotic treatment, e.g. treatment of individual pigs or small groups thereof in comparison to the whole flock treatment, may also be responsible for the lower occurrence of colistin resistance among pigs and accordingly in the pig slaughterhouses (50) . Furthermore, our results are in line with the EU summary report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2017/2018 (51) showing increased colistin resistance in *E. coli* isolates from broilers compared to those from pigs.

From nine *mcr* genes tested, *mcr-1* was the most prevalent one, which corroborates the study of (21) that emphasizes the global dissemination and high prevalence of *mcr-1* gene among colistin-resistant bacteria isolated from animals and food products worldwide. With prevalences of 0.04 to 20.3%, *mcr-1* is predominantly detected in Enterobacteriaceae isolates (*E. coli*, *Klebsiella* spp., *Enterobacter* spp., *Salmonella* spp., *Shigella* spp.) from livestock, retail meat (1.4 to 19%) and to a lesser extent in human clinical isolates (0.06 to 2%), worldwide (52–56). In Germany, colistin-resistant isolates from turkey and broilers food chains show the highest

mcr-1 prevalence in comparison to pigs and cattle (29, 38). Thus, livestock and poultry are considered as an origin of *mcr-1* and is its important reservoir for transmission to humans (10). Based on the wide dissemination of *mcr-1*, EMA's Antimicrobial Advice Ad Hoc Expert Group (AMEG) advised to minimize sales of colistin for use in animals EU-wide to achieve a 65% reduction in 2016 (3). Data from Germany indicate a reduction of sales between 2011 and 2016 by 45.7% from 127 to 69 tons.

The genes *mcr-2* to *mcr-9* have not been detected. This could be due to their limited geographical distribution and bacterial host range (38) as well as substantially low prevalence compared with *mcr-1*. Currently in Germany, *mcr-3* was detected in *Aeromonas* spp. isolates of fish origin (57). Furthermore, *mcr-4* has been frequently identified in different *Salmonella* serovars from poultry meat and pork (38) as well as *mcr-5* has been detected in *E. coli* and *Salmonella* isolates of livestock origin (58, 38).

Furthermore, *mcr-2* to *mcr-9* have not been detected in our study. This could be due to their limited geographical distribution and bacterial host range (38). While *mcr-2* to *mcr-8* being detected mostly in *E. coli* and *K. pneumoniae* isolates from pigs and poultry in China and South Europe (12–18), *mcr-9* and *mcr-10* were discovered in clinical strains of *S. enterica* serotype Typhimurium (19) and *Enterobacter roggenkampii*, respectively (20). Currently in Germany, *mcr-3* was detected in *Aeromonas* spp. isolates of fish origin (57). Furthermore, *mcr-4* has been frequently identified in different *Salmonella* serovars from poultry meat and pork (38) as well as *mcr-5* has been detected in *E. coli* and *Salmonella* isolates of livestock origin (58, 38).

E. coli isolates carrying *mcr-1* on transferable IncHI2 plasmids were detected in on-site preflooder downstream the discharge point of mWWTP. Possible entry sources could be run-offs from the fields fertilized with contaminated manure (59) and feces of wild animals (birds) (60). Previously, (61) and (62) detected *mcr-1* harboring *E. coli* in surface water and rivers in Switzerland and Germany, respectively. Moreover, in our study *mcr-1* positive *K. pneumoniae* was recovered from poultry scalding water. This could be a possible source of contamination of carcasses and products and lead to the introduction of *mcr-1* carrying *K. pneumoniae* into the food chain. (63) reported that 24.8% of retail chicken meat in the Netherlands were positive for *mcr-1*, carried mostly by *E. coli* and to a lesser extent by *K. pneumoniae*. Furthermore, 40.6% of poultry meat samples originating from Germany were contaminated with *mcr-1* producing bacteria (28). Some of the *mcr-1* carrying isolates recovered from wastewater used for cleaning of stunning facilities and influents of in-house WWTP from poultry slaughterhouses belonged to ExPEC groups B2 and D, which are known to harbor more virulence factors than commensal strains and pose a zoonotic risk (64). This enables the

transmission of *mcr-1*-positive ExPECs of poultry origin to humans and represents a potential vehicle of *mcr* genes for human diseases, e.g. bloodstream and urinary tract infections (65, 66). Moreover, study of (67) shown that *mcr-1* positive *E. coli* of phylogroups B1 and F also possessed high virulence in rodent models for ExPEC-associated human infections and could therefore pose an elevated risk of infections for humans.

According to the classification of (48), target isolates showed high percentage of multidrug resistance (combined resistance to CST, CIP, TET) with the highest rate of 49.2% for *E. coli*. However, it is important to note that from a human clinical perspective, the antibiotic groups are not considered to be equally clinically relevant (68). Taking into account the KRINKO classification, and employing piperacillin/tazobactam instead of piperacillin, the 3MDRO rates were low with the highest percentage of 5.9% for *K. pneumoniae*. Furthermore, applying clinical breakpoints, isolates were completely susceptible to reserve antibiotics ceftazidime-avibactam and tigecycline as well as carbapenems (IMP, MEM). Moreover, temocillin, which was introduced in 2019 for therapy of ESBL and AmpC producers, and amikacin, classified by WHO as reserve second-line drug, were also effective against all isolates. Thus, these antimicrobials could be still effective in antibiotic therapy in case of infection.

It was already reported that *mcr-1* gene occurs frequently in isolates that are susceptible to most classes of antimicrobials (3). Nevertheless, possible transmission of *mcr-1* gene to highly virulent bacteria carrying other antimicrobial resistance genes, e.g. ESBL and carbapenemases would narrow clinical therapeutic options (69). (70) and (71) reported on *E. coli* isolates from blood stream infections which co-produce NDM-1 and MCR-1.

In our study *mcr-1* gene was detected in a wide range of plasmid types such as IncI1, IncHI2, IncX4, IncF and IncI2, which is in consent with other reports. *mcr-1* located on IncI1 plasmids was detected in *E. coli* recovered from pig manure (59) and chicken feces (72). *E. coli* isolates recovered from pigs in Portugal carried *mcr-1* on IncHI2 and IncX4 plasmids (42). (73) detected *mcr-1* gene on IncX4 and IncHI2 plasmids in *E. coli* from broilers and veal calf in the Netherlands. Furthermore, (74) isolated *E. coli*, *K. pneumoniae* and *C. braakii* from raw meat and liver which harbored *mcr-1* gene on IncX4, IncHI2, and IncI2 plasmids. In addition to livestock and food products, MCR-1 producing *E. coli* which carry the resistance on IncX4, IncHI2, and IncI1 types of plasmids, were isolated from different environmental sources such as surface water in Germany (62) and public seawater beach in Norway (75). The association of *mcr-1* gene with insertion sequence IS*Apl1* might play a major role in its mobilization, its further successful establishment in broad-host plasmids and subsequent dissemination among Enterobacteriaceae (22, 26). On the other hand, without colistin exposure, IS*Apl1* is able to

facilitate the deletion of resistance genes, as described by Zhang and colleagues (2019) for *mcr-1* and *mcr-3.19* (76).

The co-transfer of the decreased susceptibility to fluoroquinolones (MIC of CIP 0.25 mg/L) by the majority of the isolates recovered in the poultry slaughterhouses could be due to plasmid-mediated quinolone resistance (PMQR) genes. They are known to provide only low-level resistance that by itself does not exceed the clinical breakpoint of >0.5 mg/L for susceptibility (77). Furthermore, resistance to tetracyclines was co-transferred by the isolates from pig slaughterhouses, as tetracycline resistance genes are often located on mobile elements such as plasmids, transposons, conjugative transposons, and/or integrons (78). Thus, fluoroquinolones and tetracyclines, which make up 25.7% of the total antimicrobial usage in the veterinary medicine in Germany (79), may impose a selective pressure that could favor the selection of *mcr* genes, even without use of colistin and vice versa. Moreover, Savin and colleagues (2020) reported on antimicrobial residues of ampicillin, ciprofloxacin, and ofloxacin detected in German municipal WWTPs which exceeded their PNECs (Predicted No Effect Concentration) (33, 80). Ofloxacin exceeded its PNEC even after dilution of the treated wastewater with the recipient water. This may contribute to the co-selection of *mcr-1* carrying bacteria in surface water, whereas the residues of ampicillin may promote the dissemination of *mcr*-carrying strains of species with intrinsic resistance to this antimicrobial (e.g. *Klebsiella* spp., *E. cloacae* complex).

The great majority of colistin-resistant *E. coli* and *K. pneumoniae* which were tested negative for known *mcr* genes harbored chromosomal point mutations in the *pmrAB* coding regions. For *E. coli*, a mutation at the position 10 in *pmrB* has been detected by (81) leading to the substitution 10Leu→Pro that confers resistance to colistin. However, in our study, the polymorphisms at this position resulted in leucine to arginine substitution. One *K. pneumoniae* isolate recovered from the wastewater used for cleaning of poultry stunning facilities demonstrated mutation 157T→P (PmrB) that has been previously reported in *K. pneumoniae* from patients and healthy humans (82) as well as in clinical colistin-resistant KPC-producing isolates (83). Furthermore, a substitution 217A→V (PmrA) that has been already described in colistin-resistant isolates from clinical blood cultures (84) was found in isolates recovered from the influent of mWWTPs and on-site preflooders. To determine whether other detected polymorphisms in *E. coli* and *K. pneumoniae* cause resistance to colistin, complementation assays are needed.

We are not aware of other studies in Germany that investigated such environmental samples (i.e. process water and wastewater) which have been taken directly in the slaughterhouses and

their on-site WWTPs that underlines the novelty of our study. In conclusion, our results indicate high prevalence of *E. coli* isolates which carry *mcr-1* on a wide variety of transferable plasmids in process water accruing along the slaughtering process in German poultry slaughterhouses. This may pose an elevated risk of colonization for slaughterhouse employees with occupational exposure to process water and wastewater. Furthermore, despite strict hygiene rules established in German slaughterhouses, *mcr-1* carrying bacteria could be introduced into the food chain through cross-contamination (e.g. scalding water). Moreover, due to insufficient treatment of wastewater, such strains were discharged into the environment. In order to determine the persistence of *mcr-1* carrying *E. coli* isolates in the receiving water bodies, further investigations are needed. Furthermore, besides colistin, overall reduction of the use of antibiotics in livestock is required, as it was shown that *mcr-1* can be also co-selected by fluoroquinolones and tetracyclines. In this way, the input of resistant bacteria into the slaughterhouses can be reduced. Additionally, as *mcr-1* carrying isolates were detected in the effluent of the WWTPs, a broad dissemination to the environment can be expected. Thus, this study supports the necessity of the implementing of advanced wastewater treatment technologies to limit the exposition of the environment with bacteria expressing resistances against last resort antimicrobials.

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5. Phenotypic and molecular characterization of *Klebsiella* spp. recovered from livestock and municipal wastewater

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To be submitted

5.1. Abstract

Klebsiella spec. are ubiquitous bacteria that can colonize animals and humans, and occasionally cause severe infections in both of them. Due to their high stability against environmental and synthetic conditions as well as their potential for efficient acquisition of antimicrobial resistances, *Klebsiella* strains are recognized as an important threat to public health, worldwide. As information on the diversity and impact of *Klebsiella* isolates within the slaughtering process of food-producing animals is lacking, this study aimed to determine phenotypic and genotypic properties of extended-spectrum β-lactamase (ESBL)-producing, and colistin-resistant *K. pneumoniae* and *K. oxytoca* isolates (n=185) from process- and wastewater from poultry and pig slaughterhouses as well as their receiving municipal wastewater treatment plants (mWWTPs). The data were generated to get an overview on specific *Klebsiella* strains which survive the treatment process and are released into the environment via treated wastewater effluents and that might be disseminated through the food-production chain.

Selectively isolated klebsiellae exhibited high heterogeneous antibiotic-resistance patterns. While those originating from poultry slaughterhouses showed the highest rate of colistin resistance (32.4%, 23/71), carbapenem-resistant isolates were only observed in mWWTP samples (n=76; ertapenem 32.9%, meropenem 14.5%, imipinem 9.2%). Overall, the highest diversity of genes (n=77) conferring resistance against ten antimicrobial classes were detected among klebsiellae from mWWTPs, followed by isolates from pig (n=56) and poultry slaughterhouses (n=52). Of note, no cabapenemase encoding genes were detected and the mobile colistin resistance gene *mcr-1* was only identified in isolates from poultry and pig slaughterhouses. In general, a high diversity of clonal lineages along with international high-risk clones causing outbreaks in humans were detected at all sampling sites including effluents and receiving waterbodies of mWWTPs.

Our study confirms that the recovered klebsiellae are highly heterogeneous in their antimicrobial resistance and genetic composition, which affects their pathogenic potential and clinical importance. As they were detected along the slaughtering process as well as in the effluents of mWWTPs and their receiving waterbodies, there is a potential risk of colonization and/or infection of slaughterhouse employees, consumers and humans with exposure to contaminated surface waters. Furthermore, a potential spread of the isolates via food products might be possible.

5.2. Introduction

Klebsiellae (i.e. *K. pneumoniae*, *K. oxytoca*, *K. michiganensis*) are important opportunistic pathogens for humans and cause hospital-acquired infections (1). One of the species belongs to the group of the ESKAPE bacteria (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter* spp.) and is mainly responsible for respiratory, bloodstream, and urinary infections as well as infections in intensive care units among critically ill and immunocompromised patients (2). *Klebsiella* isolates represent up to 7.7% of the most frequently isolated bacteria causing health-care associated infections in the USA and 11.4% in European countries (1, 3). Klebsiellae are also able to cause infections in animals, in particular septicaemia, pneumonia, and mastitis in pigs as well as respiratory infections in broilers (4, 5).

Due to steadily increasing rates of antimicrobial resistance, infections caused by ESKAPE bacteria are becoming hard to treat as demonstrated by elevated morbidity and mortality rates (6). Especially resistances to β-lactam antibiotics (e.g. 3rd generation cephalosporins) by the production of extended spectrum β-lactamases (ESBL) are of great concern, as such strains often harbor further resistances that frequently lead to multidrug (MDR) or pandrug resistance (PDR) with drastically limited therapeutic options (7). The highest public health concern had been assigned to the occurrence of colistin-resistant and/or carbapenemase-producing klebsiellae (8). The resistance genes are often located on mobile genetic elements (i.e. plasmids, transposons), which are horizontally transmissible to other susceptible bacteria. Besides medical settings, carbapenemase-producing *K. pneumoniae* were reported in different environmental compartments, such as wastewater treatment plants (WWTPs) and surface waters, but also in livestock and companion animals (9–11). Nevertheless, their incidence in non-human sources (i.e. wildlife, livestock, pets) is currently low, as carbapenems are not approved for use in veterinary medicine (12). However, a possible introduction from humans to non-human sources has been also reported in several studies (13). The World Health Organization (WHO) had assigned carbapenem-resistant, 3rd generation cephalosporin-resistant *K. pneumoniae* as a "critical pathogen", for which research and development of new antibiotics is mandatory (8).

In contrast to carbapenemase-producing *Klebsiella* spp., ESBL-producing strains are frequently reported worldwide in various ecological niches including surface waters and soil as well as food products and livestock (14–16). Among these, notably high numbers are reported for broilers (17, 18). Daehre and colleagues (2018) reported on SHV-2-producing *K. pneumoniae* ST3128 along the poultry producing chain in parent flocks, hatcheries and fattening farms in

Germany (17). Furthermore, process waters from poultry slaughterhouses including scalding water and wastewater have been reported as a source for ESBL-producing *K. pneumoniae* as well (19). Moreover, ESBL-producing *K. pneumoniae* has been isolated from retail poultry meat in Germany, indicating potential cross-contamination in slaughterhouses despite strict hygiene practices (20). This may lead to colonization or infection of humans upon contact or consumption of contaminated food.

ESBL-producing *K. pneumoniae* have been detected in pigs as well. Founou and colleagues isolated CTX-M-15, and TEM-1B producing *K. pneumoniae* ST14, ST39 and a high-risk clone ST307 in 21.5% of pigs and 11.3% of occupationally exposed workers in slaughterhouses in Cameroon (21). Furthermore, process waters including scalding and dehairing water from German pig slaughterhouses have been recently identified as a reservoir for EBSL-producing *K. pneumoniae* (22). Occupational exposure of farm and slaughterhouse employees to contaminated animals or other contaminated sources (e.g. air, dust, process waters) is related to an increased risk of colonization (23). Moreover, Savin and colleagues (19, 22) reported on in-house WWTPs of poultry and pig slaughterhouses as well as municipal WWTPs as a source of surface water contamination with ESBL-producing *K. pneumoniae* in Germany.

However, there is only limited data on epidemiology of ESBL-producing *Klebsiella* spp. in poultry and pig slaughterhouses as well as municipal WWTPs in Germany. Due to high numbers of processed animals from different fattening farms and folks/herds, slaughterhouses may represent melting points for different livestock-associated bacteria including those of ESKAPE group (22, 19). Thus, process waters and wastewater from poultry and pig slaughterhouses may provide important insights on the epidemiology of ESBL-producing *K. pneumoniae* that is associated with broilers, and pigs, whereas municipal WWTPs constitute an important reservoir for community-associated *Klebsiella* spp. strains.

Thus, the aim of the study was to characterize the population structure (genetic lineages) and antimicrobial resistance genes of ESBL-producing, and colistin-resistant *Klebsiella* spp. strains isolated from process waters and wastewater from German poultry and pig slaughterhouses as well as their municipal WWTPs including receiving waterbodies. The generated data on specific *Klebsiella* strains isolated from process waters as well as from the treated effluents and surface waters, will further help to assess the risk of infection of population groups with possible occupational exposure to contaminated sources (i.e. slaughterhouse and WWTPs employees, farmer workers, consumers).

5.3. Material and Methods

5.3.1. Sampling sites and sample preparation

Water samples (n=82) were collected in delivery and unclean areas during operation and cleaning of producing facilities as well as in in-house WWTPs of the poultry slaughterhouses. Samples were collected at seven sampling sites: transport trucks, transport crates, stunning facilities, scalders, eviscerators, production facilities as well as in-/effluents of the in-house WWTPs (19).

Further water samples (n=67) were collected in the delivery (animal transporters, holding pens) and unclean areas (scalding and dehairing water, aggregate wastewater from production facilities) as well as in the in-house WWTPs (in-/effluent) of the pig slaughterhouses. Their in-house WWTPs and municipal WWTPs receiving pretreated wastewater from the pig slaughterhouses including the on-site preflooders upstream and donwstream (n=36) were sampled as well (22).

The individual samples of 1 L water were collected using sterile Nalgene® Wide Mouth Environmental Sample Bottles (Thermo Fisher Scientific, Waltham, MA, USA). All details regarding sampling and the preparation of process waters and wastewater from the investigated slaughterhouses, their in-house wastewater treatment plants (WWTPs) as well as municipal WWTPs (mWWTPs) including receving waterbodies have been previously described (19, 22).

5.3.2. Isolation, identification and susceptibility testing of *Klebsiella* spp.

ESBL-producing and colistin-resistant klebsiellae were recovered from water samples by selective cultivation on CHROMagar ESBL plates (Mast Diagnostica, Reinfeld, Germany) and SuperPolymyxin agar as previously described (19, 22, 24). To ensure the purity of the isolates, unselective cultivation of individual colonies was performed on Columbia Agar supplemented with 5% sheep blood (v/v) (Mast Diagnostics, Reinfeld, Germany). Species identification for all individual isolates was conducted using MALDI-TOF MS (bioMérieux, Marcy-l´Étoile, France) equipped with the Myla™ software.

Antimicrobial susceptibility-testing was performed by broth microdilution according to CLSI guidelines (M07-A9) following epidemiological cut-off values of European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Overall, 185 klebsiellae (155 *K. pneumoniae*, 30 *K. oxytoca*) were further investigated in detail. Of these, 71 *K. pneumoniae* isolates originated from process waters and wastewater accruing in poultry slaughterhouses during operation and cleaning of facilities: transport crates (n=14),

stunning facilities (n=14), scalding sater (n=8), eviscerators (n=4), influent in-house WWTPs (n=29) and effluent in-house WWTPs (n=2). Further isolates (n=38: 36 *K. pneumoniae*, two *K. oxytoca*) originated from process- and wastewater samples from pig slaughterhouses: transporters (n=8), holding pens (n=2), scalding and dehairing water (n=3), aggregate wastewater from producing facilities (n=8), influent in-house biological WWTPs (n=10), influent in-house chemical-physical WWTPs (n=4) and effluent in-house biological WWTPs (n=3). From water samples taken in municipal WWTPs (mWWTPs) and their receiving waterbodies 48 klebsiellae (20 *K. pneumoniae*, 28 *K. oxytoca*) from influent mWWTPs (n=39), effluent mWWTPs (n=14), on-site preflooder upstream (n=18) and downstream (n=5) the discharging point were further investigated.

5.3.3. Whole-genome sequencing: Dissection of the genetic background of bacteria

A single colony from pre-cultivated Gram-negative bacteria on Columbia agar supplemented with 5% sheep blood (bioMérieux, Nürtingen, Germany) were used for the inoculation of 5 ml lysogeny broth (LB). If necessary, the LB broth was supplemented with antimicrobials as specified (i.e. colistin sulphate at final concentration of 2 mg/L; Sigma Aldrich, Schnelldorf, Germany) and cultivation was conducted under shaking conditions (180-220 rpm) at 37°C for 16-20 h. Genomic DNA (gDNA) from bacterial cells was recovered using liquid cultures with the PureLink® Genomic DNA Mini Kit (Invitrogen, Darmstadt, Germany) as specified by the manufacturers. The purity and quality of 2 µl aliquots of the recovered DNA was determined using the Nanodrop 1000 Spectrophotometer V3.8 (VWR, Darmstadt, Germany). Quantification of the DNA concentration was performed with a Qubit 2.0 fluorometer (Thermo Fisher Scientific, Darmstadt, Germany) as recommended by the manufacturers. DNA samples meeting the requirements of the Illumina Inc. specifications were subjected to DNA sequencing library preparation with the Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Short-read, paired-end sequencing was performed in 2x251 cycles on the Illumina MiSeq benchtop using the MiSeq Reagent v3 600-cycle Kit (Illumina). After sequence determination, the raw read sequences demultiplexed and trimmed using an *in house* pipeline of the German Federal Institute for Risk Assessment (BfR). The trimmed reads were further subjected to *de novo* assembling using the full SPAdes algorithm of the PATRIC database ((25); www.patricbrc.org). Raw read and assembled sequences were further used for individual bioinformatics analysis. Usually, initial bioinformatics analysis was conducted using the tools (i.e. ResFinder v 3.0, MLST v 2.0) of the

Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/) under default values. *In-depth* analyses were carried out using CLC Genomics Workbench 9.5.2 (Qiagen, Hilden, Germany) and DS-Gene (Accelrys Inc. San. Diago, CA, USA). Similarity and identity values of bacterial nucleotide and amino acid sequences were determined using the Blast-suite of the NCBI database. Final annotation of the bacterial genomes as well as of partial genomic sequences was performed using the automated NCBI genome submission interface or the BankIt tool (NCBI).

5.4. Results

5.4.1. Characterization of resistance phenotypes

Recovered klebsiellae exhibited diverse resistance phenotypes including resistances against highly and critically important antibiotics. Overall, *Klebsiella* spp. isolates from poultry and pig slaughterhouses as well as mWWTPs showed highly diverse resistance patterns (Fig. 5.4.1). Particularly, isolates from poultry slaughterhouses and mWWTPs showed higher resistance rates to ciprofloxacin and nalidixic acid in comparison to those from pig slaughterhouses. The highest resistance rate to colistin (32.4%, 23/71) was observed among isolates from poultry slaughterhouses, followed by those from mWWTPs (14.5%, 11/76). From pig slaughterhouses only a single colistin-resistant isolate (2.6%, 1/38) was detected in the wastewater used for cleaning of pig transporters after cultivation on SuperPolymyxin medium.

Klebsiella spp. isolates originating from mWWTPs showed high resistance rates to gentamicin (26.3%, 20/76), whereas only 5.3% (2/38) of those from pig slaughterhouses and none from poultry slaughterhouses were assigned to be resistant against this substance. Furthermore, isolates from mWWTPs exhibited resistance against carbapenems with the highest rate to ertapenem (32.9%, 25/76), followed by meropenem (14.5%, 11/76) and imipinem (9.2%, 7/76). Of note, isolates from poultry and pig slaughterhouses were completely susceptible to imipinem and resistance rates to meropenem were low at 2.8% (2/71) and 2.6% (1/38), respectively. *Klebsiella* spp. isolates originating from poultry (50.7%, 36/71) and pig (47.4%, 18/38) slaughterhouses exhibited higher resistance levels to tetracycline in comparison to those from mWWTPs (22.4%, 17/76). Noteworthy, *Klebsiella* spp. isolates recovered from poultry and pig slaughterhouses as well as mWWTPs, which were taken from CHROMagar ESBL plates (Mast Diagnostica, Reinfeld, Germany) exhibited resitance to cefotaxime at rates of 100% (57/57), 91.9% (34/37) and 98.5% (66/67), respectively.

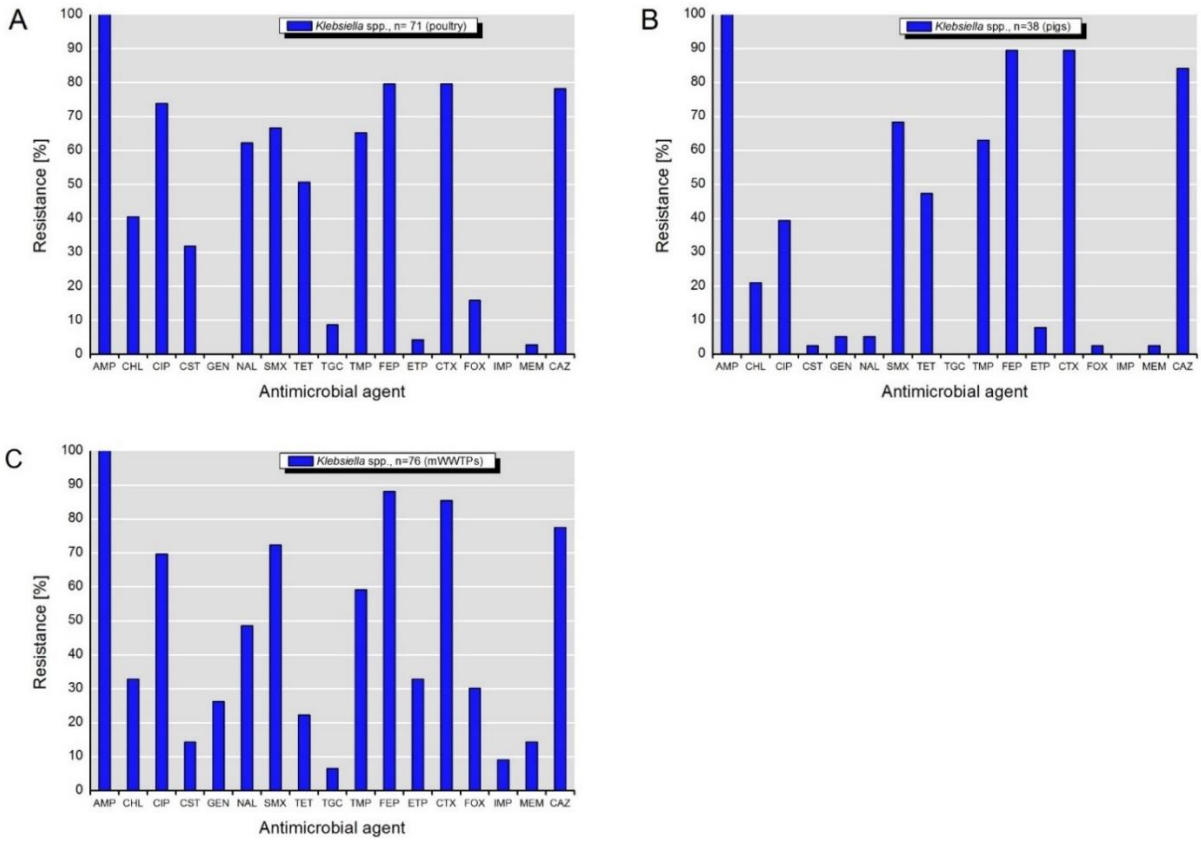

Figure 5.4.1 Resistance to antimicrobial agents detected among isolates of *Klebsiella* spp. isolated from wastewater and process water from (A) poultry slaughterhouses, (B) pig slaughterhouses and (C) municipal WWTPs receiving wastewater from investigated pig slaughterhouses.

Abbreviations for antimicrobial agents: AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; NAL, nalidixic acid; SMX, sulfamethoxazole; TET, tetracycline; TGC, tigecycline; TMP, trimethoprim; FEP, cefepime; ETP, ertapenem; CTX, cefotaxime; FOX, cefoxitin; IMI, imipenem; MEM, meropenem; CAZ, ceftazidime.
**Klebsiella* spp. are intrinsically resistant to ampicillin.

5.4.2. Characterization of antibiotic resistance genes

Recovered klebsiellae isolates constitute a large reservoir of antibiotic resistance genes with isolates from mWWTPs showing the highest diversity. The resistance genes of the analyzed klebsiellae are summarized in Fig. 5.4.2.

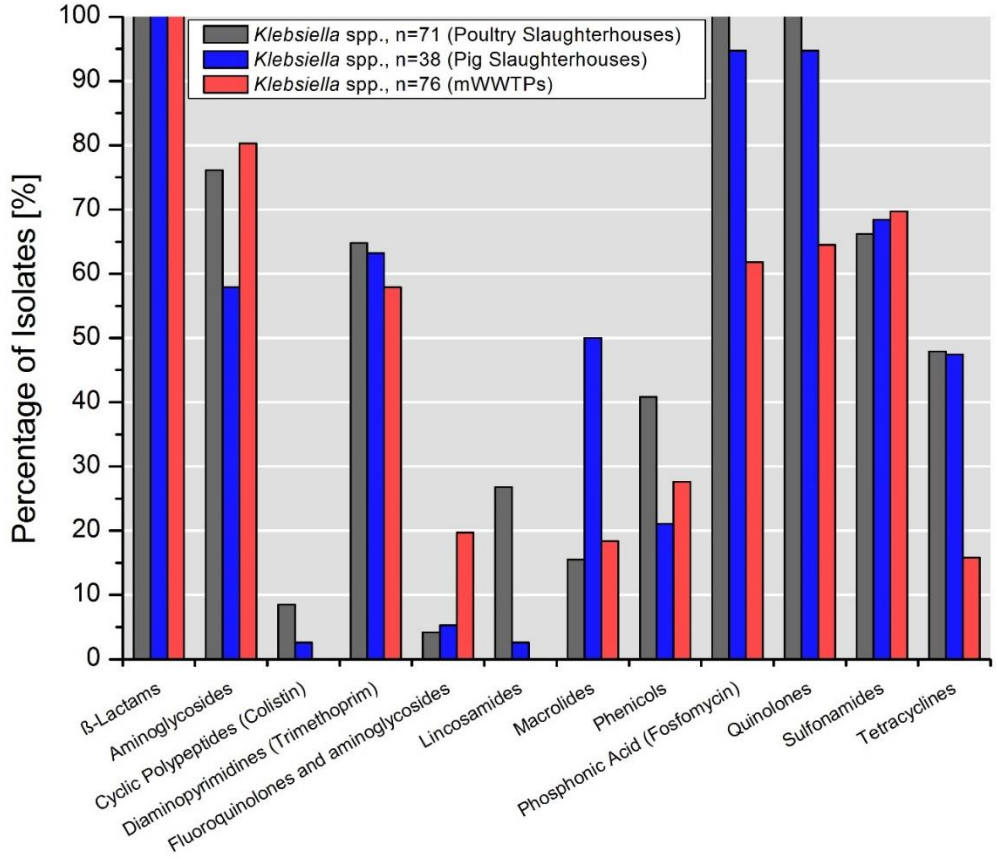

Figure 5.4.2 Percentage of *Klebsiella* spp. isolates recovered from wastewater and process waters from poultry and pig slaughterhouses as well as their receiving municipal WWTPs carrying genes mediating resistance to the specific classes of antimicrobials.

The highest diversity of resistance genes (n=77) was detected among the isolates recovered in the municipal WWTPs, followed by those from the pig (n=56) and poultry (n=52) slaughterhouses (Fig. 5.4.3). Of these, 29 occurred in the isolates from all three compartments. *bla*$_{SHV}$ genes were more predominant in the isolates from poultry slaughterhouses, whereas combinations with *bla*$_{CTM-X-1}$ and *bla*$_{CTX-M-15}$ were more abundant in the isolates from pig slaughterhouses and mWWTPs, respectively. *mcr-1* gene was detected only in isolates recovered from poultry and pig slaughterhouses.

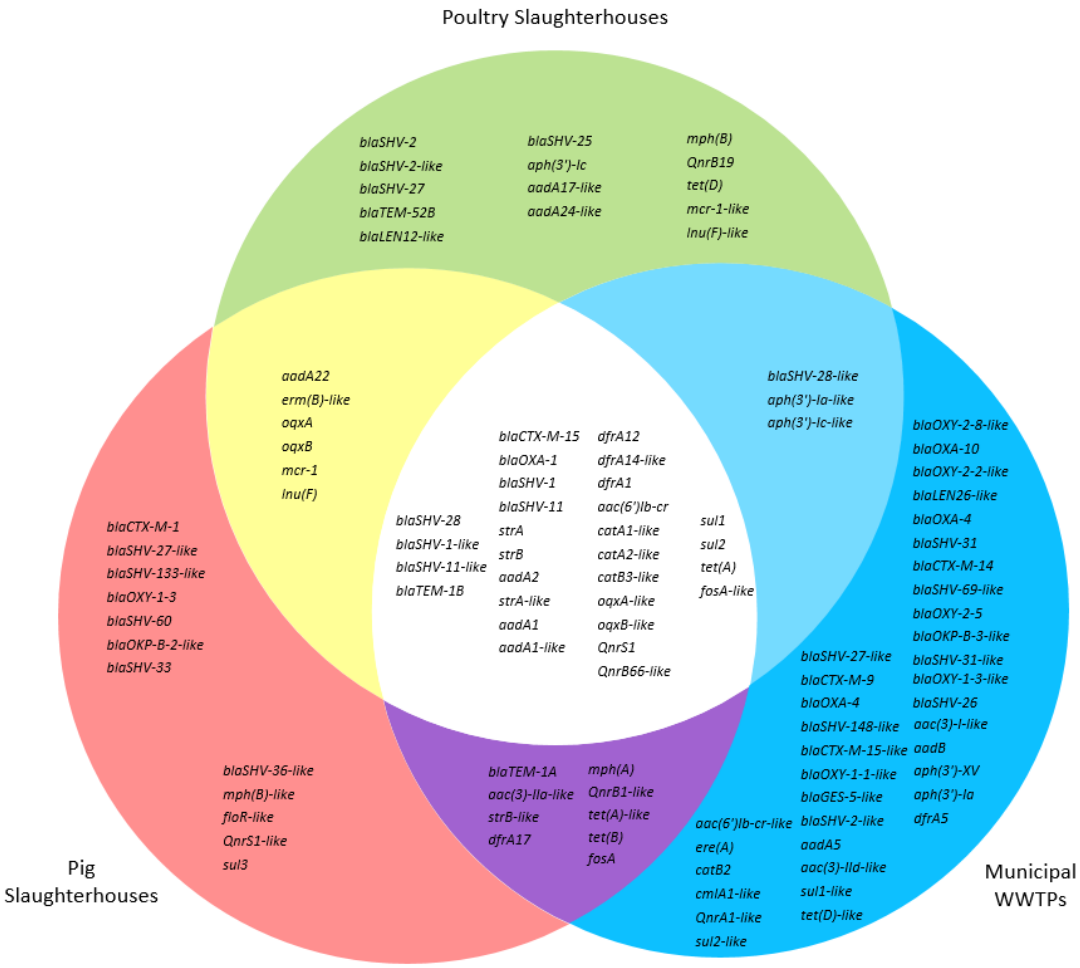

Figure 5.4.3 Antibiotic resistance genes identified in *Klebsiella* spp. isolates from poultry and pig slaughterhouses as well as their receiving municipal WWTPs.

Klebsiella spp. isolates recovered from mWWTPs showed higher abundances of resistance determinants against fluoroquinolones and aminoglycosides (i.e. *aac(6')Ib-cr*). All isolates recovered from poultry slaughterhouses (71/71) and 94.7% (36/38) of those from pig slaughterhouses carried genes mediating resistance to fosfomycin (*fosA-like and fosA*) and quinolones (mostly combinations of *oqxA-like*, *oqxB-like* with *qnrS*, *qnrB*). Whereas, among the isolates from mWWTPs, these rates were lower at 61.8% (47/76) and 64.5% (49/76), respectively. Furthermore, all isolates carried β-lactam resistance genes. Noteworthy, *Klebsiella* spp. isolates from poultry slaughterhouses carried genes conferring resistance to colistin (*mcr-1*), lincosamides (*lnu(F)*) and phenicols (*catA*, *catB*) at higher rates that those from pig slaughterhouses and mWWTPs. Furthermore, isolates from poultry (47.9%, 34/71) and pig (47.4%, 18/38) slaughterhouses showed higher abundances of genes mediating resistance to tetracyclines (*tet(A)*, *tet(B)*, *tet(D)*) than those recovered from mWWTPs (12/76, 15.8%).

Interestingly, 50.0% (19/38) of klebsiellae from pig slaughterhouses carried macrolide resistance genes (*mph(A), mph(B), erm(B)*), whereas such resistant determinants were detected only in 15.5% (11/71) and 18.4% (14/76) of the isolates from poultry slaughterhouses and mWWTPs.

Among genes conferring resistance to β-lactams, aminoglycosides, and quinolones combinations of up to six genes per particular antibiotic class per isolate were detected (Tab. 5.4.1). *bla*$_{\text{OXA}}$ genes were exclusively detected in *K. oxytoca* from mWWTPs. In *Klebsiella* spp. isolates with resistance to imipenem and/or meropenem (n=16), no carbapenemases were detected with exception of one isolate recovered from the effluent of mWWTP that carried *bla*$_{\text{GES-5-like}}$ gene. The rest of the isolates harbored *bla*$_{\text{OXA-1}}$, and *bla*$_{\text{OXA-10}}$ genes in combination with *bla*$_{\text{SHV}}$ and/or *bla*$_{\text{CTX-M}}$.

Table 5.4.1 Antibiotic resistance genes and their combinations detected in isolates of *Klebsiella* spp. recovered from wastewater and process waters from poultry and pig slaughterhouses as well as their receiving municipal WWTPs.

	Poultry Slaughterhouses, n=71		Pig Slaughterhouses, n=38		mWWTPs, n=76	
	Genes	**Percentage**	**Genes**	**Percentage**	**Genes**	**Percentage**
β-Lactams	*bla*$_{SHV-2}$	32.4	*bla*$_{CTX-M-1}$, *bla*$_{SHV-1}$	18.4	*bla*$_{OXY-2-8-like}$	17.1
	bla$_{SHV-28}$	19.7	*bla*$_{CTX-M-1}$, *bla*$_{SHV-1-like}$	13.2	*bla*$_{CTX-M-15}$, *bla*$_{OKP-B-3-like}$, *bla*$_{TEM-1B}$	11.8
	bla$_{SHV-1-like}$	8.5	*bla*$_{CTX-M-1}$, *bla*$_{SHV-11}$	10.5	*bla*$_{LEN26-like}$	6.6
	bla$_{SHV-11-like}$	7.0	*bla*$_{CTX-M-1}$, *bla*$_{SHV-27-like}$	7.9	*bla*$_{OXY-2-2-like}$	6.6
	bla$_{SHV-27}$, *bla*$_{TEM-52B}$	5.6	*bla*$_{CTX-M-1}$, *bla*$_{SHV-27-like}$, *bla*$_{TEM-1B}$	7.9	*bla*$_{CTX-M-15}$, *bla*$_{SHV-1-like}$	5.3
	bla$_{SHV-2-like}$	5.6	*bla*$_{CTX-M-15}$, *bla*$_{SHV-133-like}$	7.9	*bla*$_{CTX-M-15}$, *bla*$_{SHV-11}$, *bla*$_{TEM-1A}$	3.9
	bla$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-11-like}$, *bla*$_{TEM-1B}$	4.2	*bla*$_{CTX-M-15}$, *bla*$_{SHV-11-like}$, *bla*$_{TEM-1A}$	5.3	*bla*$_{OXA-10}$, *bla*$_{SHV-31}$	3.9
	bla$_{SHV-1}$	4.2	*bla*$_{CTX-M-1}$, *bla*$_{OXY-1-3}$	2.6	*bla*$_{OXA-4}$, *bla*$_{OXY-2-8-like}$	3.9
	bla$_{SHV-11}$	2.8	*bla*$_{CTX-M-1}$, *bla*$_{SHV-60}$	2.6	*bla*$_{CTX-M-14}$, *bla*$_{SHV-27-like}$	2.6
	bla$_{SHV-1-like}$, *bla*$_{TEM-1B}$	2.8	*bla*$_{CTX-M-15}$, *bla*$_{OKP-B-2-like}$, *bla*$_{SHV-28}$, *bla*$_{TEM-1B}$	2.6	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-1}$	2.6
	bla$_{SHV-28-like}$, *bla*$_{TEM-1B}$	2.8	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-11-like}$, *bla*$_{TEM-1B}$	2.6	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-11-like}$, *bla*$_{TEM-1B}$	2.6
	bla$_{LEN12-like}$, *bla*$_{SHV-2-like}$	1.4	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-28}$, *bla*$_{TEM-1B}$	2.6	*bla*$_{OXA-10}$, *bla*$_{SHV-69-like}$, *bla*$_{TEM-1B}$	2.6
	bla$_{SHV-25}$	1.4	*bla*$_{CTX-M-15}$, *bla*$_{SHV-11}$	2.6	*bla*$_{OXY-2-5}$	2.6
	bla$_{SHV-28-like}$	1.4	*bla*$_{CTX-M-15}$, *bla*$_{SHV-1-like}$	2.6	*bla*$_{SHV-1}$	2.6
			bla$_{OXY-1-3}$	2.6	*bla*$_{CTX-M-14}$, *bla*$_{SHV-1-like}$	1.3
			bla$_{SHV-28}$	2.6	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-1}$, *bla*$_{SHV-148-like}$	1.3
			bla$_{SHV-33}$	2.6	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-28}$, *bla*$_{TEM-1B}$	1.3
			bla$_{SHV-36-like}$, *bla*$_{TEM-1B}$	2.6	*bla*$_{CTX-M-15}$, *bla*$_{OXA-1}$, *bla*$_{SHV-38-like}$, *bla*$_{TEM-1B}$	1.3
					bla$_{CTX-M-15}$, *bla*$_{OXY-2-2-like}$, *bla*$_{TEM-1B}$	1.3
					bla$_{CTX-M-15}$, *bla*$_{OXY-2-5}$	1.3
					bla$_{CTX-M-15}$, *bla*$_{SHV-1}$	1.3
					bla$_{CTX-M-15}$, *bla*$_{SHV-11}$	1.3

Table 5.4.1 (continued)

					***bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{SHV-28}}$, *bla*$_{\text{TEM-1B}}$**	**1.3**
					bla$_{\text{CTX-M-15-like}}$, *bla*$_{\text{SHV-11}}$, *bla*$_{\text{TEM-1A}}$	1.3
					bla$_{\text{CTX-M-9}}$, *bla*$_{\text{OXA-4}}$, *bla*$_{\text{OXY-2-8-like}}$	1.3
					bla$_{\text{CTX-M-9}}$, *bla*$_{\text{OXY-1-1-like}}$	1.3
					bla$_{\text{GES-5-like}}$, *bla*$_{\text{SHV-2-like}}$	1.3
					bla$_{\text{OKP-B-3-like}}$	1.3
					bla$_{\text{OXA-10}}$, *bla*$_{\text{SHV-31-like}}$	1.3
					bla$_{\text{OXA-10}}$, *bla*$_{\text{SHV-69-like}}$	1.3
					bla$_{\text{OXY-1-3-like}}$, *bla*$_{\text{SHV-12}}$	1.3
					bla$_{\text{SHV-26}}$	1.3
					bla$_{\text{SHV-28-like}}$	1.3
	Overall	100		100		100
Aminoglycosides	*aadA2-like, strA, strB*	12.7	*aadA5*	15.8	*aadA1*	14.5
	aadA2, aadA22, strA, strB	11.3	*strA, strB*	13.2	*strA, strB*	13.2
	aadA2	7.0	*aadA1, aadA5, strA, strB*	7.9	*aadA5, strA, strB*	6.6
	aadA2, aph(3')-Ia-like, strA, strB	7.0	*aac(3)-IIa-like, aadA1-like, strA, strB*	2.6	*aac(3)-IId-like, strA-like, strB-like*	5.3
	aadA2-like, aph(3')-Ic, strA, strB	5.6	*aac(3)-IIa-like, strA, strB*	2.6	*aac(3)-I-like, aadA2, aadB*	5.3
	strA,strB	5.6	*aadA1, strA, strB*	2.6	*aadA1, aadA5, aadB, aph(3')-XV, strA, strB*	5.3
	aadA17-like, strA-like	4.2	*aadA2, strA, strB*	2.6	*aadA1-like*	5.3
	aadA22	4.2	*aadA2, strA-like, strB*	2.6	*aadA2, strA-like, strB-like*	5.3
	aadA2, aph(3')-Ic, strA, strB	2.8	*aadA2, strA-like, strB-like*	2.6	*aac(3)-IIa-like, strA, strB*	3.9
	aadA2, strA, strB	2.8	*aadA22*	2.6	*aac(3)-IId-like*	3.9
	aadA24-like	2.8	*strB*	2.6	*aac(3)-I-like*	3.9
	aadA1, aadA2, strA, strB	1.4			*aadA1, aph(3')-Ia-like*	2.6
	aadA17-like	1.4			*aadA1, strA,strB*	1.3
	aadA1-like	1.4			*aadA2, aadB,aph(3')-Ic-like*	1.3
	aadA2, aadA24-like, aph(3')-Ic, strA, strB	1.4			*aadA2, aph(3')-Ia, strA-like, strB-like*	1.3

Table 5.4.1 (continued)

	aadA2, aph(3')-Ic-like, strA, strB	**1.4**			***aph(3')-Ia***	**1.3**
	aadA22, strB	1.4				
	aph(3')-Ic	1.4				
	Overall	76.1		57.9		80.1
Cyclic Polypeptides (Colistin)	*mcr-1*	7.0	*mcr-1*	2.6	-	-
	mcr-1-like	1.4				
	Overall	8.4		2.6		-
Diaminopyrimidines (Trimethoprim)	*dfrA12*	52.1	*dfrA1*	15.8	*dfrA14-like*	26.3
	dfrA14-like	8.5	*dfrA14-like*	15.8	*dfrA1*	14.5
	dfrA1	4.2	*dfrA17*	15.8	*dfrA17*	9.2
			dfrA1,dfrA17	7.9	*dfrA12*	6.6
			dfrA12	7.9	*dfrA5*	1.3
	Overall	64.8		63.2		57.9
Fluoroquinolones and aminoglycosides	*aac(6')Ib-cr*	4.2	*aac(6')Ib-cr*	5.3	*aac(6')Ib-cr*	10.5
					aac(6')Ib-cr-like	9.2
	Overall	4.2		5.3		19.7
Lincosamides	*lnu(F)*	22.5	*lnu(F)*	2.6	-	-
	lnu(F)-like	4.2				
	Overall	26.8		2.6		-
Macrolides	*erm(B)-like*	14.1	*mph(A)*	26.3	*mph(A)*	17.1
	mph(B)	1.4	*mph(B)-like*	15.8	*ere(A)*	1.3
			erm(B)-like, mph(A)	7.9		
	Overall	15.5		50.0		18.4
Phenicols	*catA1-like*	32.4	*floR-like*	13.2	*catB3-like*	14.5
	catA2-like	4.2	*catA1-like, catB3-like*	2.6	*catA1-like*	7.9
	catB3-like	4.2	*catA2-like*	2.6	*catB2*	2.6
			catB3-like	2.6	*catA2-like*	1.3
					cmlA1-like	1.3
	Overall	40.8		21.1		27.6
Phosphonic Acid (Fosfomycin)[a]	*fosA-like*	100	*fosA-like*	76.3	*fosA-like*	50.0
			fosA	18.4	*fosA*	11.8
	Overall	100		94.7		61.8
Quinolones[b]	*oqxA-like, oqxB-like*	87.3	*oqxA-like, oqxB-like*	57.9	*oqxA-like, oqxB-like*	23.7
	oqxA-like, oqxB-like, QnrS1	5.6	*oqxA-like, oqxB-like, QnrS1*	23.7	*oqxA-like, oqxB-like, QnrS1*	18.4

Table 5.4.1 (continued)

	oqxA-like, oqxB-like, QnrB66-like	4.2	*oqxA-like, oqxB-like, QnrS1-like*	5.3	*oqxA-like, oqxB-like, QnrB66-like*	6.6
	oqxA, oqxB	1.4	*oqxA, oqxB, QnrB1-like*	2.6	*oqxA-like, oqxB-like, QnrA1-like*	5.3
	oqxA-like, oqxB-like, QnrB19	1.4	*oqxA-like, oqxB-like, QnrB66-like*	2.6	*oqxA-like, oqxB-like, QnrB1*	5.3
			QnrB66-like, QnrS1	2.6	*QnrA1-like*	2.6
					QnrS1	2.6
	Overall	100		94.7		64.5
Sulfonamides	*sul1*	47.9	*sul1, sul2*	31.6	*sul1*	27.6
	sul1,sul2	9.9	*sul2*	28.9	*sul2*	23.7
	sul2	8.5	*sul3*	5.3	*sul1, sul2-like*	9.2
			sul1	2.6	*sul1, sul2*	6.6
					sul1-like	2.6
	Overall	66.2		68.4		69.7
Tetracyclines	*tet(D)*	25.4	*tet(A)*	28.9	*tet(A)*	7.9
	tet(A)	22.5	*tet(A)-like*	10.5	*tet(A)-like*	5.3
			tet(B)	7.9	*tet(B), tet(D)-like*	2.6
	Overall	47.9		47.4		15.8

[a] *fosA* intrinsic in *K. pneumoniae*
[b] *oqxA, oqxB* intrinsic in *K. pneumoniae*

5.4.3. MLST (multilocus sequence typing) distribution

Phylogenetic analyses revealed a high genetic diversity between the individual populations of *K. pneumoniae* and *K. oxytoca*. Overall, 129 *K. pneumoniae* isolates were assigned to 34 previously described STs, whereas 26 isolates exhibited yet unassigned STs (Tab. 5.4.2). Among the *K. pneumoniae* isolates recovered from poultry slaughterhouses, ST15 was the most predominant clone (54.9%, 39/71), that was also detected in mWWTPs in the on-site preflooder upstream. One *K. pneumoniae* clone of ST219 recovered from the aggregate wastewater from producing facilities of the pig slaughterhouse was detected in mWWTPs in influent, effluent and on-site preflooder upstream. In addition to *K. pneumoniae* ST15 and ST219, clones of ST268 and ST2459 were detected in the preflooders upstream.

Of note, *K. pneumoniae* isolates (n=14) which survived the treatment process by mWWTPs were assigned to ST16, ST132, ST219 and ST307, whereas in the effluents of the in-house WWTPs from pig slaughterhouses *K. pneumoniae* isolates of ST15 (n=2) were detected. Furthermore, *K. pneumoniae* ST15 and ST412 was detected in scalding, and dehairing water in poultry and pig slaughterhouses, respectively.

Interestingly, most isolates that exhibited allele variants resulting in STs that up to now have not been described within the prevailing MLST scheme, were recovered in mWWTPs (37.5%, 18/48) followed by pig slaughterhouse (22.2%, 8/36).

Table 5.4.2 MLST distribution of *K. pneumoniae* isolates recovered from poultry and pig slaughterhouses as well as their receiving municipal WWTPs.

***K. pneumoniae*, n=71 Poultry Slaughterhouses**			***K. pneumoniae*, n=36 Pig Slaughterhouses**			***K. pneumoniae*, n=48 municipal WWTPs**		
Sequence type	n	%	Sequence type	n	%	Sequence type	n	%
ST15	39	54.9	ST412	6	16.7	ST268	5	10.4
ST896	17	23.9	ST873	6	16.7	ST219	4	8.3
ST280	3	4.2	ST17	4	11.1	ST252	4	8.3
ST37	3	4.2	ST3113	3	8.3	ST2459	3	6.3
ST392	3	4.2	ST1307	2	5.6	ST503	3	6.3
ST458	2	2.8	ST1867	1	2.8	ST15	2	4.2
ST107	1	1.4	ST1948	1	2.8	ST753	2	4.2
ST147	1	1.4	ST219	1	2.8	ST132	1	2.1
ST611	1	1.4	ST307	1	2.8	ST14	1	2.1
ST789	1	1.4	ST37	1	2.8	ST16	1	2.1
			ST48	1	2.8	ST307	1	2.1
			ST54	1	2.8	ST34	1	2.1
						ST359	1	2.1
						ST441	1	2.1
			Unknown ST	8	22.2	Unknown ST	18	37.5

Of *K. oxytoca*, only two isolates recovered from mWWTPs (7.1%, 2/28) were assigned to ST13 (influent) and ST107 (effluent).

5.5. Discussion

The present study provides novel important insights in the diversity of antimicrobial resistances, as well as genetic lineages of ESBL-producing, and colistin-resistant klebsiellae from process waters and wastewater from German poultry and pig slaughterhouses as well as their municipal WWTPs.

The high rate of ciprofloxacin resistance in klebsiellae from poultry underlines the urgent need for a complete ban of (fluoro)quinolones in veterinary medicine, as the antimicrobials of this class were considered to be critically important for humans. Since 2005, the USA had disclaimed the use of enrofloxacin in poultry (26), which seems to prevent the further rise of fluoroquinolone resistances in human *Campylobacter* spp. isolates. In contrast to the USA, the EU countries continuously report on increasing resistance rates for fluoroquinolones among zoonotic and indicator bacteria from humans and livestock (13), which might be associated with the fact that antmicrobials of this class are still used for treatment applications. Particular in *Campylobacter coli*, *Salmonella* Infantis and *S.* Kentucky isolates from humans, as well as from poultry and derived meat, the resistance rates range from high to extremely high (13). *Klebsiella* spp. might serve as a vehicle involved in the spread of resistance genes to susceptible bacteria (27). This hypothesis fits well with our observation that *Klebsiella* spp. isolates from our study frequently carry *qnrB* or *qnrS*, which are designated as transmissible plasmid-mediated quinolone resistance determinants (PMQR).

The resistance phenotype of the isolates from pig slaughterhouses, which exhibited resistance to ciprofloxacin and were susceptible to nalidixic acid may be due to the occurrence of PMQR mechanisms (*qnrBS*, *oqxAB*, *aac(60)Ib-cr* genes) (28). Whereas the resistance phenotype of the isolates from poultry slaughterhouses and mWWTPs (CIP and NAL) indicate additional mechanisms such as point mutations within the DNA gyrase (*gyrA*, *gyrB*) and topoisomerase IV (*parC*, *parE*) (28). However, in contrast to the isolates from mWWTPS, there was a difference between the percentages of the isolates carrying PMQR and exhibiting reduced susceptibility to fluoroquinolones, indicating that PMQR genes might be silent in ESBL-producing *K. pneumoniae* from poultry and pigs and could be triggered in response to the antibiotic use.

The high abundance of tetracycline resistance and *tet* resistance genes among isolates from poultry and pig slaughterhouses might be explained with an overuse of the antimicrobial in

livestock, mostly in pigs. In 2017, 49 tons of tetracyclines were sold to the veterinerians in Germany, ot these 68.8% (33.7 tons) were used to treat piglets (11.6 tons) and pigs (22.1 tons) (7). Despite the fact that tetracyclines are almost not used in Germany to treat infections in poultry, the resistance rate to this substance and the abundance of resistance determinants (*tet(A), tet(D)*) among the isolates from poultry and pig slaughterhouses were almost similar. This may emphasize the co-selection of antibiotic-resistance genes (ARGs), in particular *tet* determinants, by using antimicrobials of other classes, desinfectants and heavy metals added to the poultry feed (29–31). Moreover, this may highlight the widespread distribution of *tet* genes in bacteria from various ecosystems associated with poultry production which act as a reservoir for its transfer (32). Some isolates from poultry slaughterhouses and mWWTPs expressed resistance to tigecycline, which is also a reserve antibiotic that is effective against rapidly emerging multidrug-resistant Gram-negative and Gram-positive pathogens. Acquired mutations in *tetA* lead to increased MICs of tigecycline and could be a possible resistance mechanism (33).

Low incidence of carbapenem resistance among the isolates from poultry and pig slaughterhouses could be related to the fact that carbapenems are not approved for therapeutical use in veterinary medicine but are reserved for humans (12). These findings correlate well with other reports (13) indicating absence or rare occurrence of carbapenem-resistant Enterbacteriaceae (CRE) in European livestock. Nevertheless, some studies report on single cases of CRE in livestock in Europe, e.g. VIM-producing *E.coli*, *S. enterica* subsp. *enterica* and OXA-48 expressing *E. coli* in pigs and broilers in Germany (34–38). *Klebsiella* spp. isolates from mWWTPs yielded a higher percentage of carbapenem-resistance, indicating its wider dissemination in general population and the influence of wastewater from clinics (39). Furthermore, among all isolates no carbapenemase genes were detected. However, ESBL-production in combination with decreased membrane permeability may be responsible for reduced susceptibility to carbapenems (40).

Abundance of phenicol resistance genes (*catAB, floR*) correlates well with resistance phenotypes of the isolates (lead substance chloramphenicol). However, isolates from poultry slaughterhouses showed the highest percentage of resistance genes to phenicols conferred mostly by *catA1*, whereas isolates from mWWTPs carried mainly *catB* genes. This is in accordance with other studies which revealed that resistance to chloramphenicol in Enterobacteriaceae strains associated with food animals is mediated mostly by the genes *catA1* and *floR* (41). Interestingly, antimicrobial substances belonging to this class (e.g. florfenicol, thiamphenicol) are not approved for use in poultry in Germany but in Poland and China.

However, *catA1* is often integrated within a resistance gene cluster that ensures the maintenance of corresponding resistance even without drug administration (41).

The highest abundance of macrolides resistance genes (*mphA*) among *Klebsiella* spp. isolates from pig slaughterhouses can be explained by the high use of macolides in pigs. Antibiotics of thi sclass, especially tylosin, are the 3rd most frequently applied antimicrobials after tetracyclines and penicillins among fattening pigs (42). In 2017, approximately 40% (7.2 tons) of macrolides sold to veterinarians were used to treat infections in fattening pigs in Germany (7). However, they are mostly used to treat mycoplasma infections as well as haemorrhagic digestive disease and are not relevant for the treatment of diseases caused by Gram-negative bacteria (12). Furthermore, a high percentage of *Klebsiella* spp. isolates from poultry slaughterhouses carried genes conferring resistance to lincosamides (*lnuF*). In Germany only lincomycin is approved for treatment of poultry. Like macrolides, this substance is active against Gram-positive bacteria, and is essential in the treatment of *Mycoplasma pneumoniae*, infectious arthritis and hemorrhagic enteritis (12). Lincosamide resistance can lead to cross-resistance to macrolides (e.g. tylosin in veterinary medicine and erythromycin in human medicine) and other lincosamides like highly important antimicrobial (HIA) for human medicine clindamycin. Interestingly, isolates from poultry slaughterhouses and to a lesser extent from pig slaughterhouses carried *ermB* genes. The transfer of *ermB* gene to Gram-positive pathogens (e.g. staphylococci, enterococci, streptococci) may result in MLS_B (macrolide, lincosamide, streptogramin B) cross-resistance (43). In this way, critically important antimicrobials for human medicine which are used to treat infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) and by vancomycin-resistant *Enterococcus faecium* (VRE) (i.e. erythromycin, clindamycin, Synercid) are greatly compromised in their efficiency. Thus, *Klebsiella* spp. is an important pool of antimicrobial resistance genes and may serve as a reservoir of ARGs that can be transffered, inter alia, to Gram-positive pathogens (27) narrowing the treatment options with potential consequences for human medicine.

The highest rate of resistance to colistin was observed among the isolates from poultry slaughterhouses. This may be attributed to the fact that colistin is broadly used in German poultry for treating infections with Gram-negative enterobacteria (7, 12). In 2017, 56.5% (13 tons) of polypeptid antibiotics sold to veterinarians were used to treat infections in poultry (7). Interestingly, *mcr-1* was detected only among the isolates from poultry and pigs indicating its higher prevalence in livestock compared to the humans. This finding is in consent with other studies (44, 45), which had reported on low prevalence of *mcr-1* gene in German municipal

WWTPs. Furthermore, Sib and colleagues (2020) highlighted a lower prevalence of *mcr-1* gene in hospital sewage in comparison to communal wastewater (46). Other mechanisms might also be involved into the acquisition of colistin resistant phenotype, as there was a difference between the abundance of detected mobile resistance determinants (i.e. *mcr-1* gene) and the percentage of colistin resistant isolates recovered from slaughterhouses and mWWTPs. Chromosomal mutations in genes (esp. *pmrA/B, phoP/Q, mgrB*) encoding proteins that regulate the transcription of enzymes which modify the lipopolysaccharide (47, 48) may play an important role. Furthermore, other mobile resistance genes that have not yet been discovered are conceivable as well.

In our study *K. pneumoniae* ST15 was the most prevalent clone among isolates from poultry slaughterhouses and occurred along the whole process chain including scalding water and the effluent of the in-house WWTPs. Members of this clonal lineage often express resistance to β-lactams and are mostly CTX-M-15 producer but also encodes all types of carbapenemase genes *bla*$_{\text{KPC}}$, *bla*$_{\text{OXA-48 like}}$, *bla*$_{\text{NDM}}$, *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$ (49). Furthermore, they are frequently resistant to fluoroquinolones and are also associated with mobilizable *mcr-1* gene (50, 51). However, isolates from our study carried almost exclusively *bla*$_{\text{SHV-1/-2/-28}}$ genes and possessed no other acquired PMQR genes except of intrinsic *oqxAB*, but carried *mcr-1* genes. These findings reinforce the theory that resistance genes encoding SHV β-lactamases are ubiquitous in ESBL-producing *K. pneumoniae* strains (52). Recently, a pan-resistant isolate of *K. pneumoniae* ST15 recovered from a U.S. patient was reported (53). It was resistant to all 26 drugs tested, icluding β-lactams, colistin, and tigecycline. This demonstrates the ability of this clone to successful develop resistance against a large range of antimicrobials through acquiring of mobile elements and accumulation of chromosomal mutations. If no changes in antimicrobial use patterns in veterinary medicine are taken, there will be a probability of livestock associated *K. pneumoniae* ST15 developing multidrug resistance to antimicrobials critically important for human medicine. This would severely narrow the therapeutic options for patients.

One of the most predominant clones detected in poultry and pig slaughterhouses belonged to ST896, ST412 and ST873. *K. pneumoniae* ST896 and ST412 has been already reported in China from clinical specimens (54, 55). Furthermore, *K. pneumoniae* ST412 was frequently associated with hypervirulent pathotype (55). Moreover, *K. pneumoniae* ST873 detected in pig transporters, holding pens and in-house WWTPs was reported in outbreaks in Dutch hospitals. The clone was producing NDM-1 and was able to transfer the *bla*$_{\text{NDM-1}}$ carrying plasmid between different species (56). *K. pneumoniae* ST17 was also one of the most prevalent type in pig slaughterhouses. It is an international clone reported in clinical facilities worldwide that

often expresses an MDR phenotype and carries genes encoding ESBL and carbapenemases (57–59). *K. pneumoniae* ST37, ST48, ST147, ST307 which were recovered in poultry and pig slaughterhouses as well as ST14, ST16 from mWWTPs belong to important international outbreaks clones which tend to carry carbapenemases (49). Moreover, *K. pneumoniae* ST15, ST17, ST37, ST147 detected in poultry and pig slaughterhouses as well as ST14 from mWWTPS have already been described as high-risk XDR clones which cause worldwide outbreaks in humans (49). *Klebsiella pneumoniae* ST15, ST147, ST280 recovered from poultry slaughterhouses as well as ST48 from pig slaughterhouses and ST16 from mWWTPs were already reported in clinical/urban wastewater system in Germany and carried carbapenemases (39).

The most abundant *K. pneumoniae* clones from mWWTPs are ST268 and ST307, which have been already reported as bacteria of the German surface waters (60). These clones has been previously reported in clinical specimens from humans (61, 62). *Klebsiella pneumoniae* ST268 is strongly associated with hypervirulence causing liver abscesses, sepsis and invasive infections (61). Furthermore, *K. pneumoniae* ST307 was supposed to posses a high transmission potential enabeling its fast distribution between different countries (62). One of the major clones from mWWTPs *K. pneumoniae* ST252 and ST219 has been already isolated from clinical specimens of the U.S. patients (63) and from wastewater in Rumania (64), respectively. Moreover, *K. pneumoniae* ST252 with hypermucoviscous phenotype causing community-acquired infections was previously reported in Mexico (65).

5.6. Conclusions

We have found for the first time that ESBL-producing, colistin-resistant *K. pneumoniae* isolates from German poultry and pig slaugtherhouses are represented by a wide spectrum of clonal lineages including those of clinical relevance. Furthermore, they carried a high diversity of antibiotic-resistance genes and expressed diverse antibiotic-resistance patterns, compromising critically and highly important antimicrobials for human medicine.

Presence of *K. pneumoniae* clones of such international high-risk lineages in slaughterhouses may pose a threat of colonisation and infection of employees with occupational exposure to contaminated reservoirs as well as consumers through possible contamination of carcassess. As these high-risk *K. pneumoniae* clones were further detected in the effluent of in-house and municipal WWTPs, a broad dissemination to the environment can be expected. This may have a negative impact on environmental health, as some of them are greatly adaptable to stress and unfavorable conditions such as nutrient limitation and low temperatures. The risk of

colonization of humans or, depending on the individual health conditions and the intensity of exposure, even infection via direct contact with insufficiently treated wastewater, contaminated surface water or meat cannot be excluded as well.

Furthermore, currently there are no monitoring programms to assess its occurrence in wastewater and food products, although it cannot be ruled out that contaminated surface waters and meat could serve as a possible route for dissemination of klebsiellae in the community. *Klebsiella* spp. may persist in the gut and act as a reservoir for mobile resistances transferring these determinants to other commensal enteric bacteria. This may narrow the therapeutic options in case of antibiotic treatment.

In general, there is necessity to clarify persistance of such clones and their extracellular DNA in surface water. Furthermore, as such clinically-relevant ESBL-producing, and colistin-resistant *K. pneumoniae* clones were detected along the slaughtering process, additional investigations to determine the colonization rates of the employees are highly needed. Moreover, this study supports the necessity for the development and implementing of novel wastewater treatment processes to prevent the dissemination of ESBL-producing, and colistin-resistant *K. pneumoniae* of clinically relevant clonal lineages and their resistance genes into surface water and further ecological niches.

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6. General conclusion

Colonized livestock is the most important source for the introduction of antibiotic-resistant strains of several facultative pathogens (e.g. MRSA, ESBL-producing *E. coli*) into the slaughtering process. Together with a high amount of contaminated organic matter (e.g. feces, bristles) they are execrated into process waters and wastewater at different processing steps. However, data on the occurrence, phenotypic and genotypic properties of ESKAPE bacteria, and ESBL-producing *E. coli* in process waters and wastewater from German pig and poultry slaughterhouses are lacking. Furthermore, their dissemination into receiving water bodies is not investigated as well. Thus, the main objective of the thesis was the investigation of clinically-relevant antibiotic-resistant bacteria in process waters and wastewater from poultry and pig slaughterhouses and the assessment of the bacterial dissemination into surface waters.

The first question was aimed at the investigation of the occurrence and diversity of ESKAPE-bacteria and ESBL-producing *E. coli* in German poultry and pig slaughterhouses. For this purpose, different process waters and wastewater were screened for target bacteria. The recovered isolates were characterized for their antimicrobial resistance phenotypes and were further subjected to different molecular typing approaches. Furthermore, genes encoding extended-spectrum-β-lactamases and carbapenemases as well as mobilizable colistin resistance genes in *Enterobacteriaceae* and non-fermenters were determined.

Generally, the results showed a high incidence of the target bacteria along the sampling sites. Process waters and wastewater from poultry slaughterhouses were important reservoirs for antibiotic resistant bacteria with clinical relevance. *E. coli* of the phylogroups B2, D and F, implicated as extraintestinal pathogens (ExPEC), were detected at all sampling points of both slaughterhouses. Some of the determined clonal lineages were attributed as high risk clones (i.e. ST69, ST10, ST648 and ST117) which are involved in human infections worldwide. The majority of the ESBL-producing *E. coli* exhibited genes that are coding for CTX-M-1, TEM-116, TEM-52 and SHV-12 β-lactamases. No carbapenemases were detected. The detected MRSA lineages mostly belonged to the CC9 (t1430, t13177) and CC398 (t8588, t011, t034), which are the most common LA-MRSA in Europe. The occurrence of VRE was rare, as only one VRE isolate of ST1249, previously isolated in chicken products from the United Kingdom, was detected.

Furthermore, process waters and wastewater accruing in delivery and unclean production areas in pig slaughterhouses constituted an important reservoir for ESKAPE bacteria and ESBL-producing *E. coli*. Only a minor percentage of ESBL-producing *E. coli* was allocated to the

virulence-associated groups B2 and D. However, detected clones of ST10, ST117, ST101, ST354, ST453, ST1170, ST1284 and ST1431 have already been described in clinical infections in Germany. Recovered LA-MRSA mostly belonged to CC398 (t011, t034, t2011, t2576) and were distributed at all sampling points. Noteworthy, ESKAPE bacteria, which bear the highest potential risk to humans, such as *E. coli* of clinically relevant clonal lineages (ST10, ST69, ST95, ST131, ST167, ST405, ST648), CPE, VRE as well as HA-MRSA of CC5 and CC22 were mainly detected in municipal wastewater. Nevertheless, the abundance of CPE in untreated wastewater from mWWTPs was low.

For the second question, the emergence and characteristics of colistin-resistant *Enterobacteriaceae* (*E. coli*, *Klebsiella* spp., *Enterobacter cloacae* complex) as well as ESBL-producing, and colistin-resistant isolates of *Klebsiella* spp. in water samples from slaughterhouses and their in-house WWTPs were investigated. The recovered isolates were characterized regarding their population structure, antimicrobial resistance and their ability to transfer colistin-resistance mediated by *mcr-1* gene.

A high prevalence of colistin-resistant *Enterobacteriaceae* (*E. coli*, *Klebsiella* spp. and *E. cloacae* complex) was identified in the screened samples. However, only a low percentage (<6%) of those expressed MDR phenotype (combined resistance to TZP, CTX and CIP). Nevertheless, a large proportion of *E. coli* and *K. pneumoniae* isolates carried *mcr-1* on a variety of transferable plasmids belonging to the IncI1, IncHI2, IncX4, IncF and IncI2 groups that ranged between 30 kb and 360 kb. Furthermore, the majority of *E. coli* and *K. pneumoniae* isolates tested negative for *mcr-1* to *mcr-9* revealed non-synonymous polymorphisms in *pmrAB* genes, which might be involved in the acquisition of the colistin-resistance phenotype.

In addition, a wide variety of bacterial lineages including clinically relevant clones (e.g. ST15, ST17, ST37, ST147, ST412, ST873, ST896) were detected among ESBL-producing, and colistin-resistant *K. pneumoniae* isolates. Furthermore, they carried a high diversity of antibiotic-resistance genes with the highest number of 77 among the isolates from municipal WWTPs conferring resistance against ten classes of antimicrobials. They expressed diverse antibiotic-resistance patterns, compromising critically and highly important antimicrobials for human medicine. Isolates originating from poultry slaughterhouses showed the highest resistance rate to colistin. Whereas, the highest percentage of resistance to carbapenems was observed among isolates from mWWTPs. Of note, no cabapenemases were detected.

The third question focused on the analysis of possible risks arising from livestock-associated ESKAPE-bacteria, ESBL-producing *E. coli* and colistin-resistant *Enterobacteriaceae* (*E. coli*, *Klebsiella* spp. and *E. cloacae* complex) for human health.

A high percentage of ESBL-producing *E. coli* isolates recovered in the samples from the poultry slaughterhouses and to a lesser extend from the pig slaughterhouses was allocated to the virulence-associated phylogroups B2, D and F. Furthermore, high risk *E. coli* clones which cause urinary and bloodstream infections and were already isolated in German patients and hospitals, were detected in livestock process waters and wastewater. Also, despite strict hygiene rules established in German slaughterhouses, *mcr-1* carrying bacteria could be introduced into the food chain through cross-contamination (e.g. scalding water). Moreover, clinically relevant clones of ESBL-producing *K. pneumoniae* and LA-MRSA were detected along the slaughtering process and in in-house WWTPs as well. Thus, there is a certain probability of slaughterhouses and in-house WWTPs employees of becoming colonized and/or infected during occupational exposure to such contaminated matrices. However, to accurately assess such a risk, further studies are needed, as multiple parameters need to be determined, e.g. pathogens concentration, exposure time, employees' health status.

However, it is worthy of note that the most potentially harmful bacteria for humans (i.e. CPE, VRE, HA-MRSA) were almost exclusively isolated from wastewater of municipal origin and from the treated effluent. Furthermore, ESBL-producing *K. oxytoca* which pose an elevated risk of colonization for such vulnerable groups as children and neonates, was also increasingly recovered in samples from mWWTPs. This poses a potential risk of becoming colonized and/or infected while being exposed to contaminated process waters and wastewater.

The final research question was aimed at the assessement of the dissemination of the target bacteria from poultry and pig slaughterhouses into surface waters and the associated risk for human health.

The target clinically-relevant antibiotic-resistant bacteria were detected in the effluents from the in-house WWTPs of poultry slaughterhouses and municipal WWTPs, underlying their inefficacy on reducing the microbial loads. Furthermore, in-house WWTPs of pig slaughterhouses were a significant input source of livestock-associated bacteria with zoonotic potential into the municipal WWTPs. Such an insufficient treatment of wastewater by direct dischargers and municipal WWTPs enabled their release into receiving water bodies, so that the broad dissemination to the environment can be expected and their further dissemination into the general population cannot be excluded. The fact that the strains of *A. baumannii*, VRE and MRSA which survived wastewater treatment can persist in the environment for varying periods of time, supports this assumption. Moreover, ExPEC and *mcr-1* carrying strains of *E. coli* and *K. pneumoniae* were discharged into the environment as well. As these bacteria are greatly adaptable to stress and unfavorable conditions and may persist in the environment for a long

time, there is a risk for humans of becoming colonized or infected, e.g. through interaction with polluted surface waters. However, detection of selected ESKAPE-bacteria (i.e. VRE, species of ACB- and *E. cloacae* complexes, *K. pneumoniae*) and ESBL-producing *E. coli* in the preflooders of the municipal WWTPs upstream of the discharge point suggests that bacteria with such clinically relevant antimicrobial resistance phenotypes are already ubiquitous in German surface waters. In addition to WWTPs located further upstream, the potential aqueous input sources for the bacterial pollution could be runoffs and drainage water from fields fertilized with manure, especially during the manure application. Furthermore, urban runoffs and spillover of untreated wastewater from the combined sewer systems during heavy rainfall or snowmelt may also play an important role. This underlines the importance of optimization and modernization of the sewer systems in Germany, e.g. by building of retentions basins, constructed treatment wetlands or underground storage basins.

The overall results of this thesis support the hypothesis that prescription and consumption patterns of antibiotics in livestock production need to be reconsidered. This would reduce the input of clinically relevant resistant bacteria into the slaughterhouses and their consequent excretion into process waters, wastewater and subsequently into the municipal WWTPs or directly into receiving water bodies. The incidence of such bacteria in surface waters suggests that conventional biological treatment is insufficient to achieve full elimination. This highlights the importance of advanced treatment technologies in terms of prevention of possible environmental dissemination of such potentially dangerous bacteria.

Nevertheless, municipal and on-site WWTPs of the slaughterhouses play an important role regarding reduction of the organic pollutants from the wastewater. Furthermore, they simultaneously represent targets for intervention and mitigation measurements, where additional measurements could be employed to avoid further environmental contamination and transmission of ESKAPE pathogens. In order to reduce environmental pollution with antibiotic resistance genes and facultative pathogenic bacteria (i.e. ESKAPE bacteria), the use of combinations of oxidative, adsorptive, and membrane based technologies should be considered. In particular, to protect the (ground)water bodies used for drinking water production, bathing and recreational water or in case of reclaiming and reusing of wastewater for irrigation. Taking the above mentioned into account, the use of innovative state-of-the-art wastewater treatment technologies needs to be encouraged, especially for direct dischargers.

Appendix

Appendix for Chapter 2

Table A1 Number of positive samples per target bacteria and sampling point. Numbers of positive samples and total numbers of samples at each sampling point are stated

	E. coli	ACB complex	MRSA	*K. pneumoniae*	*E. cloacae* complex	*Citrobacter spp.*	*P. aeruginosa*	VRE
Slaughterhouse S1[a]								
Poultry Transport Crates	3/5	2/5	0/5	0/5	0/5	0/5	0/5	1/5
Stunning Facilities	5/5	1/8	0/5	1/5	0/5	0/5	0/5	0/5
Scalding water	1/5	1/5	1/5	0/5	0/5	0/5	0/5	0/5
Eviscerators	4/5	5/5	2/5	1/5	0/5	0/5	0/5	0/5
Production Facilities	5/5	4/5	1/5	0/5	2/5	0/5	0/5	0/5
Influent in-house[b] WWTP	7/8	7/8	2/8	1/8	1/8	0/8	0/8	0/8
Effluent in-house[b] WWTP	2/8	6/8	1/8	0/8	0/8	0/8	0/8	0/8
Slaughterhouse S2[c]								
Poultry Transport Trucks	2/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Poultry Transport Crates	4/5	4/5	0/5	3/5	1/5	2/5	1/5	0/5
Stunning Facilities	5/5	3/5	2/5	4/5	1/5	0/5	0/5	0/5
Scalding water	2/5	5/5	1/5	1/5	2/5	0/5	0/5	0/5
Eviscerators	4/5	5/5	2/5	1/5	0/5	0/5	0/5	0/5
Influent in-house[d] WWTP	6/8	7/8	0/5	6/8	2/8	0/5	0/5	0/5
Effluent in-house[d] WWTP	3/8	6/8	5/8	2/8	1/8	0/5	0/5	0/5

Table A1 (continued)

a – sampling campaigns in December 2016, July 2017, August 2017, December 2017, February 2018
b – additional sampling campaigns in the S1 in-house WWTP in September 2017, November 2017, March 2018
c – sampling campaigns in October 2017, November 2017, March 2018, April 2018, Mai 2018
d – additional sampling campaigns in the S2 in-house WWTP in July 2018, September 2018, October 2018

Table A2 Results of the MLST analysis for *E. coli* strains with new STs[a].

Isolat ID	Sampling point	Phylogenetic group	*bla*-genes	Resistance profile[b]	Allelic profile							Nearest matches
					adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	
Slaughterhouse S1												
LWGS-1/5-62	Influent in-house WWTP	B1	$bla_{CTX-M-1}$	PIP, CTX, CAZ, CIP		4	33	16	24	8	14	ST5686
LWGS-1/7-32	Wastewater from Stunning Facilities	B1	$bla_{CTX-M-1}$	PIP, CTX, CAZ, CIP, LVX, CHL, SXT	429	4	375	16	11	8	6	ST224, ST906, ST2186
LWGS-1/5-71	Influent in-house WWTP	B1	$bla_{CTX-M-1}$	PIP, CTX, CAZ, CIP, CHL	429	4	375	8	24	8	14	ST889, ST892, ST3995
LWGS-1/6-10	Effluent in-house WWTP	B1	$bla_{CTX-M-1}$	PIP, CTX, CAZ, CIP	429	4	375	16	24	8	14	ST3995, ST5686
LWGS-1/6-03	Effluent in-house WWTP	B1	$bla_{CTX-M-1}$	PIP, CTX, CAZ	429	4	375	16	24	8	14	ST3995, ST5686
LWGS-1/6-21	Effluent in-house WWTP	B1	$bla_{CTX-M-1}$	PIP, CTX, CAZ	429	4	375	16	24	8	14	ST3995, ST5686
LWGS-1/3-04	Scalding water	B1	$bla_{CTX-M-1}$	PIP, CTX, CAZ, CIP	429	4	375	16	24	8	14	ST3995, ST5686
LWGS-1/8-05	Aggregate Wastewater from Production Facilities	B2	$bla_{TEM-52c}$	PIP, CTX, CAZ	429	31	5	28	1	1	2	ST57, ST5860, ST8144
LWGS-1/6-22	Effluent in-house WWTP	B1	$bla_{TEM-52c}$	PIP, CTX, CAZ	457	6	15	131	24	7	7	ST711
Slaughterhouse S2												
LWGS-4/7-22	Wastewater from Stunning Facilities	B1	$bla_{CTX-M-1}$	TEM, PIP, CTX, CAZ, C/T, CIP, LVX, CHL, SXT	429	29	33	16	11	7	2	ST1844
LWGS-4/6-40	Effluent in-house WWTP	B1	bla_{SHV-12}	PIP, CTX, CAZ, CHL, SXT	429	556	5	18	11	8	6	ST4663
LWGS-4/6-41	Effluent in-house WWTP	B1	bla_{SHV-12}	PIP, CTX, CAZ, CHL	429	556	5	18	11	8	6	ST4663
LWGS-4/1-06	Wastewater from Poultry Transport Trucks	E	$bla_{CTX-M-1}$	PIP, CTX, CAZ, CIP, LVX, SXT	52	116	55	16	113	31	38	ST4994
LWGS-4/2-17	Wastewater from Poultry Transport Cages	B1	$bla_{CTX-M-1}$	PIP, CTX, CAZ, CIP, LVX, CHL, SXT, CST	429	19	4	16	9	38	6	ST5203
LWGS-4/7-23	Wastewater from Stunning Facilities	B1	$bla_{CTX-M-15}$	PIP, CTX, CAZ, CIP, LVX	457	19	32	1	9	8	6	ST1723, ST3365
LWGS-4/2-16	Wastewater from Poultry Transport Cages	B1	$bla_{CTX-M-1}$	PIP, CTX, CAZ, CIP, LVX, CHL, SXT, CST	429	4	4	18	24	8	14	ST223, ST465, ST2120
LWGS-4/2-20	Wastewater from Poultry Transport Cages	B1	$bla_{CTX-M-1}$	PIP, CTX, CAZ, CIP, LVX, SXT	429	4	14	16	358	8	14	ST5686, ST7329
LWGS-4/6-18	Effluent in-house WWTP	E	$bla_{CTX-M-1}$	PIP, CTX, CAZ	429	31	5	28	1	1	2	ST57, ST5860, ST8144

[a]The ST was not assigned numerical designations by the *E. coli* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).
[b]Abbreviations for antimicrobial agents: TEM, temocillin; PIP, piperacillin; CTX, cefotaxime; CAZ, ceftazidime; C/T, ceftolozane-tazobactam; CIP, ciprofloxacin; LVX, levofloxacin; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; CST, colistin

Appendix for Chapter 3

Table A1 List of all investigated antibiotics and metabolites (limit of quantification, µg/L)

β-lactams				
Penicillins				
Amoxicillin (0.05)	Ampicillin (0.20)	Penicillin G (0.05)	Cloxacillin (0.02)	Dicloxacillin (0.02)
Flucloxacillin (0.02)	Methicillin (0.01)	Mezlocillin (0.02)	Nafcillin (0.02)	Oxacillin (0.01)
Penicilin V (0.02)	Piperacillin (0.10)			
Carbapenems				
Meropenem (0.20)				
Cephalosporines				
Cefaclor (0.05)	Cefotaxime (0.05)	Ceftazidime (0.10)		
Macrolides and lincosamides				
Azithromycin (0.05)	Clarithromycin (0.05)	Clindamycin (0.02)	Erythromycin (0.02)	Anhydroerythromycin (0.02)
Roxithromycin (0.05)	Spiramycin (0.10)	Tylosin (0.05)		
Tetracyclines				
Chlortetracycline (0.20)	Doxycycline (0.20)	Oxytetracycline (0.20)	Tetracycline (0.20)	
Fluoroquinolones				
Ciprofloxacin (0.20)	Enrofloxacin (0.20)	Moxifloxacin (0.20)	Ofloxacin (0.20)	
Sulfonamides				
Sulfachlorpyridazine (0.05)	Sulfadiazine (0.10)	Sulfadimethoxine (0.05)	Sulfadimidine (0.02)	Sulfadoxine (0.05)
Sulfaethoxypyridazine (0.05)	Sulfamerazine (0.05)	Sulfamethoxazole (0.02)	N4-Acetylsulfamethoxazole (0.10)	
Sulfamethoxypyridazine (0.01)	Sulfathiazole (0.10)	Trimethoprim (0.02)		
Others				
Linezolid (0.10)	Vancomycin (0.10)	Metronidazole (0.10)		

Table A2 Number of positive samples per target bacteria and sampling point. Numbers of positive samples and total numbers of samples at each sampling point are stated.

	E. coli	ACB complex	MRSA	*K. pneumoniae*	*E. cloacae* complex	*Citrobacter spp.*	*P. aeruginosa*	VRE	*K. oxytoca*
Pig slaughterhouses S1/S2[a]									
Wastewater from animal transporters	9/10	7/10	9/10	2/10	2/10	1/10	0/10	0/10	1/10
Wastewater from holding pens	5/7	1/7	4/7	2/7	0/7	1/7	2/7	0/7	0/7
Scalding and dehairing water	5/10	4/10	6/10	2/10	2/10	0/10	1/10	0/10	0/10
Aggregate wastewater from producing facilities	9/10	9/10	9/10	5/10	1/10	0/10	0/10	0/10	0/10
Influent biological WWTPs	10/10	10/10	10/10	7/10	0/10	0/10	0/10	0/10	0/10
Influent chemical-physical WWTP	4/5	5/5	5/5	4/5	0/5	1/5	1/5	0/5	0/5
Effluent biological WWTPs	9/10	7/10	7/10	4/10	3/10	1/10	0/10	0/10	0/10
Effluent chemical-physical WWTP	3/5	5/5	1/5	0/5	0/5	0/5	0/5	0/5	0/5
Municipal WWTPs of S1/S2[b]									
Influent municipal WWTPs	9/9	9/9	4/9	7/9	4/9	2/9	0/9	9/9	7/9
Effluent municipal WWTPs	9/9	3/9	0/9	8/9	3/9	0/9	0/9	8/9	6/9
On-site preflooder upstream	8/9	4/9	0/9	2/9	1/9	0/9	0/9	2/9	3/9
On-site preflooder downstream	9/9	7/9	2/9	3/9	3/9	1/9	0/9	2/9	6/9

a – sampling campaigns in S1: March 2017, September 2017, October 2017, January 2018, February 2018 and S2: March 2018, April 2018, May 2018, June 2018, July 2018.
b – sampling campaigns in mWWTP-S1: January 2018, February 2018, March 2018 and mWWTPs-S2: March 2018, April 2018, May 2018, June 2018, July 2018.

Table A3 Results of MLST analysis of *E. coli* strains with new STs[a]

Isolat ID	Sampling point	Phylo-genetic group	*bla*-genes	Resistance profile[b]	Allelic profile							Nearest matches*
					adk	*fumC*	*gyrB*	*icd*	*mdh*	*purA*	*recA*	
Pig Slaughterhouses S1/S2												
LWSS-3/6-40	Wastewater from holding pens	C	bla_{TEM-1}	PIP, CTX, CAZ, CIP, LVX, CHL, SXT	10	11	4	1	8	13	73	ST617, ST2228, ST2444
LWSS-5/1-22	Influent biological WWTP	B1	bla_{TEM-1}	PIP, TZP, CTX, CIP, LVX, CHL, SXT, COL	43	41	15	90	11	8	30	ST359, ST4461, ST6388
LWSS-5/6-15	Aggregate wastewater from producing facility	A	$bla_{CTX-M-15}$	PIP, TZP, CTX, CAZ, CIP, LVX,	429	4	12	1	20	18	7	ST410, ST1574, ST1837
LWSS-5/5-02	Scalding and dehairing water	B1	$bla_{CTX-M-1}$	TEM, PIP, CTX, CAZ, C/A, C/T, CIP, LVX, SXT, COL	429	4	4	16	24	8	14	ST58, ST572, ST3094
LWSS-5/3-03	Wastewater from animal transporters	B1	$bla_{CTX-M-1}$	PIP, CTX, CAZ, SXT	429	4	4	16	24	8	14	ST58, ST572, ST3094
LWSS-5/2-04	Effluent biological WWTP	B1	$bla_{CTX-M-1}$	TEM, PIP, CTX, CAZ	429	4	4	16	24	8	14	ST58, ST572, ST3094
LWSS-5/1-27	Influent biological WWTP	B1	$bla_{CTX-M-137}/bla_{TEM-1}$	PIP, CTX, CAZ, CIP, LVX, SXT,	429	4	4	16	24	8	14	ST58, ST572, ST3094
LWSS-5/6-61	Aggregate wastewater from producing facility	A	$bla_{CTX-M-55}$	PIP, CTX, CAZ, CIP, LVX, CHL, SXT	864	11	135	8	8	8	2	ST744, ST8900
LWSS-5/2-16	Effluent biological WWTP	C	$bla_{CTX-M-1}$	TEM, PIP, CTX, CAZ, CHL, SXT	429	4	12	1	20	12	7	ST88, ST806, ST1279
Municipal WWTPs of S1/S2												
LWSS-3/12-19	On-site preflooder upstream	B2	$bla_{CTX-M-15}$	PIP, CTX, CAZ, CHL, SXT	53	40	47	323	36	28	29	ST131, ST1410, ST2581
LWSS-3/13-18	On-site preflooder downstream	B1	$bla_{CTX-M-15}$	PIP, CTX, CAZ, CIP, SXT	457	65	5	1	9	13	6	ST162, ST469, ST1298
LWSS-5/10-47	Influent municipal WWTP	A	$bla_{CTX-M-15}$	TEM, PIP, TZP, CTX, CAZ, C/T, CIP, LVX, SXT	864	11	4	8	8	13	2	ST167, ST693, ST2266
LWSS-5/10-101	Influent municipal WWTP	B1	$bla_{OXA-48}/bla_{CTX-M-15}$	TEM, PIP, TZP, CTX, CAZ, C/T, CIP, LVX	429	4	58	1	9	2	7	ST295, ST433, ST841
LWSS-3/13-01	On-site preflooder downstream	E	bla_{TEM-1}	PIP, CTX, CAZ, CIP, SXT	569	26	2	363	5	5	19	ST3268, ST6471
LWSS-3/10-56	Influent municipal WWTP	A	$bla_{CTX-M-1}$	PIP, CTX, CAZ, C/T, CIP, LVX, CHL, SXT	864	99	5	91	8	7	2	ST361, ST3286, ST3481
LWSS-5/12-07	On-site preflooder upstream	D	-	PIP, CTX, CIP, LVX	569	26	2	25	5	5	19	ST38, ST1966, ST3472
LWSS-5/12-06	On-site preflooder upstream	D	$bla_{CTX-M-14}$	TEM, PIP, CTX, CAZ, CIP, LVX, CHL, FOF	569	26	2	25	5	5	19	ST38, ST1966, ST3472
LWSS-3/11-34	Effluent municipal WWTP	D	bla_{TEM-1}	PIP, TZP, CTX, CAZ, CIP, LVX, SXT	569	26	2	25	5	5	19	ST38, ST1966, ST3472
LWSS-3/11-31	Effluent municipal WWTP	C	$bla_{CTX-M-15}$	PIP, TZP, CTX, CAZ, CIP, LVX, CHL	429	4	12	1	20	18	7	ST410, ST1574, ST1837

Table A3 (continued)

LWSS-3/10-47	**Influent municipal WWTP**	**C**	***bla*$_{CTX-M-15}$**	**PIP, TZP, CTX, CAZ, C/T, CIP, LVX, CHL**	**429**	**4**	**12**	**1**	**20**	**18**	**7**	**ST410, ST1574, ST1837**
LWSS-3/12-11	On-site preflooder upstream	C	*bla*$_{CTX-M-15}$	PIP, TZP, CTX, CAZ, C/T, CIP, LVX, SXT	39	4	12	1	20	18	7	ST410, ST1574, ST1837
LWSS-5/13-29	On-site preflooder downstream	B1	*bla*$_{CTX-M-15}$	PIP, CTX, CAZ, CIP	429	4	14	16	24	8	6	ST949, ST5686
LWSS-5/13-19	On-site preflooder downstream	B1	*bla*$_{CTX-M-1}$	PIP, CTX, CAZ, CIP, SXT	92	4	87	96	24	8	7	ST2011, ST3738

[a]The ST was not assigned numerical designations by the E. coli MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

[b]Abbreviations for antimicrobial agents: PIP, piperacillin; TZP, piperacillin-tazobactam; CTX, cefotaxime; CAZ, ceftazidime; C/T, ceftolozane-tazobactam; IMP; CIP, ciprofloxacin; LVX, levofloxacin; CHL, chloramphenicol; SXT, sulfamethoxazole-trimethoprim; FOF, fosfomycin

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Savin M, Bierbaum G, Hammerl JA, Heinemann C, Parcina M, Sib E, Voigt A, Kreyenschmidt J. 2020. ESKAPE bacteria and extended-spectrum-β-lactamase-producing *Escherichia coli* isolated from wastewater and process water from German poultry slaughterhouses. *Appl Environ Microbiol* 86:e02748-19. doi.org/10.1128/AEM.02748-19.

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