Aus dem Institut für Lebensmittelsicherheit und -hygiene
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Influence of mobile genetic elements on the dissemination of resistance determinants in commensal *Escherichia coli*

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Katharina Anna Juraschek
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II. List of abbreviations

AMP	ampicillin
AmpC	AmpC Beta-Lactamase
AMR	antimicrobial resistance
ARDIG	Antibiotic Resistance Dynamics: the influence of geographic origin and management systems on resistance gene flows within humans, animals, and the environment
AST	antimicrobial susceptibility testing
AZI	azithromycin
BLAST	basic local alignment search tool
BRIG	Blast Ring Image Generator
CA	Kalifornien
CDS	coding sequence
CEF	cefepime
cgMLST	core genome multi locus sequence type
CHL	chloramphenicol
CIA	critically important antimicrobials
CIP	ciprofloxacin
COL	colistin
DHPS	dihydropteroate synthase
DNA	deoxyribonucleic acid
Dtr	DNA transfer and replication
E.	*Escherichia*
EAEC	enteroaggregative *E. coli*
EARS	European Antimicrobial Resistance Surveillance
ECDC	European Centre for Disease Prevention and Control
ECOFF	epidemiological cut-offs
EFSA	European Food Safety Authority
EMA	European Medicines Agency
EPEC	enteropathogenic *E. coli*
ESBL	Extended-Spectrum Beta-Lactamase
ESBLS-EC	Extended-Spectrum-Beta-Lactamase-producing *E. coli*
ESC	extended-spectrum cephalosporin
ETEC	enterotoxigenic *E. coli*
EU	European Union
EUCAST	European Committee on Antimicrobial Susceptibility Testing

EUVSEC	EU Surveillance *Salmonella*/*E. coli*
FOT	cefotaxime
FOX	cefoxitin
FQ	fluoroquinolones
GEN	gentamicin
GÜCCI	Genome-based surveillance of transmissible colistin and carbapenem resistances of gram-negative pathogens
HGT	horizontal gene transfer
HIV	human immunodeficiency virus
HPCI	Highest Priority Critically Important
i.e.	id est
ICE	integrative and conjugative elements
Inc	incompatibility
IS	insertion sequence
JIACRA	joint inter-agency report on integrated analysis of antimicrobial agent consumption and occurrence of antimicrobial resistance in bacteria from humans and food-producing animals
kb	kilo bases
LB	lysogeny broth
MER	meropenem
MFS	major facilitator superfamily
MGE	mobile genetic elements
MIC	minimal inhibitory concentration
MLST	multi-locus sequence typing
MOB	mobility genes
MPF	mating pair formation
NAL	nalidixic acid
NCBI	National Center for Biotechnology Information
NGS	Next Generation Sequencing
NRL	National Reference Laboratory
NRL-AR	National Reference Laboratory for Antimicrobial Resistance
OD	optical density
OHEJP	One Health European Joint Programme
OIE	World Health Organisation for Animal Health
ONT	Oxford Nanopore Technologies
oriT	origin of transfer
PacBio	Pacific Bioscience

PCR	polymerase chain reaction
PCU	population correction unit
pDNA	extrachromosomal DNA
PFGE	pulsed-fiel gel electrophoresis
PMQR	plasmid-mediated quinolone resistances
qac	quaternary ammonium compounds
QRDR	quinolone resistance-determining region
RND	resistance-nodulation-division
SGS	second generation sequencing
SLR	synthetic long-read
SMRT	single-molecule real time
SMX	sulfamethoxazole
SNP	single-nucleotide polymorphism
ST	sequence type
STEC	shiga toxin-producing *E. coli*
T4SS	type 4 secretion system
TAZ	ceftazidime
TE	transposable elements
TET	tetracycline
TGC	tigecycline
TMP	trimethoprim
Tn	Transposon
U	units
UK	United Kingdom
WGS	whole genome sequencing
WHO	World Health Organization
ZoMo	Zoonose Monitoring

1. Introduction

The adaption of bacteria to its environment is as old as the story of life. Bacteria evolve by adapting to the conditions in the prevailing ecosystems. A bacterial habitat, dominated by antimicrobial usage, eventually leads to an adaption of the bacteria by developing respective tolerances or resistances. The increasing development of antimicrobial resistance (AMR), as reported by The World Health Organization (WHO), is a global health and development threat with immense costs to the economy. Besides the human medical and environmental sector, the livestock and food industry also contributed to a global spread of antimicrobial resistant bacteria. Only with comprehensive monitoring programmes, responsible administration and expand of knowledge, the situation can be conveniently managed in the future. A worldwide One Health approach is a reliable option for profoundly understanding the impact and dissemination paths of AMR.

Especially the understanding of the occurrence and the spread of resistance to so-called "Highest Priority Critically Important" (HPCI) antimicrobial agents, i.e., quinolones, is crucial. In the medical sector, HPCI are reserved for the use of human infections, for which extensive evidence of transmission of resistant bacteria is present. Resistance against quinolones and fluoroquinolones occur based on chromosomal point mutations or through the acquisition of plasmid-mediated quinolone resistances (PMQR). Plasmids carrying PMQRs (i.e., conferred by *qnr* genes) are main drivers for the horizontal spread of these resistance determinants and therewith for the potential dissemination to different reservoirs. In general, plasmids are the leading vectors for the spread of resistance determinants. They can be capable for mobilization or conjugative transfer and are one of the main reasons for the rapid adaption of susceptible bacteria to become resistant. The understanding of the phenotypic and genotypic properties encoded by plasmids, especially if they carry determinants against critically important antimicrobial agents, is of great interest. This includes the elucidation of the whole structure, the analysis of main characteristics as well as the understanding of significant associations to other AMR determinants. Moreover, all information needs broader evaluation based on a more general and all-encompassing perspective.

The One Health European Joint Programme (OHEJP) initiative, including the ARDIG (**A**ntibiotic **R**esistance **D**ynamics: the **i**nfluence of **g**eographic origin and management systems on resistance gene flows within humans, animals, and the environment) project, targets the international and integrative examination of the topics evolved around resistance development. This doctoral thesis is part of the OHEJP. Its aim is to tackle the obstacles of AMR development. The focus lies on a broader understanding of the occurrence and diversity of PMQRs and its impact in livestock- and food-associated isolates.

The studies' aim is to understand the prevalence and the characteristics of quinolone- and fluoroquinolone-resistant commensal *Escherichia* (*E*.) *coli* and their mobile genetic elements, especially plasmids. A focal point was set in the determination of potential predominant *qnr*-carrying plasmids prevailing in the German food and livestock sector. Furthermore, we aimed at identifying commonalities and patterns of *qnr*-carrying plasmids to understand their dissemination pathways and persistence mechanisms. Elucidating the structures of the most prevalent plasmids, which carry *qnr* will support an evaluation of associations between these factors and the detection of newly evolved plasmid structures. A major aim is to develop appropriate ways for a comprehensive characterization of plasmid-associated resistance factors with state-of-the-art technologies, resulting in options for reliable prediction of the impact and structure of the most important plasmid types. The study will provide a reference and scaffold for further research approaches implicated in the exploration of the arrangement of important AMR-carrying plasmids.

With this work, we wanted to answer the question of the influence of mobile genetic elements for the dissemination and dynamics of important resistance determinants in commensal *Escherichia coli*.

1.1. *Escherichia coli*

Bacteria of the species *Escherichia coli* are part of the intestinal flora in healthy humans and animals [1]. They represent one of the best studied species and are also well-known zoonotic pathogens [2]. *E. coli* is a fecal indicator [3] and a key species associated with antimicrobial resistances. In the National Reference Laboratory (NRL) for Antimicrobial Resistances hosted at the German Federal Institute for Risk Assessment, *E. coli* was chosen as an indicator organism for monitoring AMR dynamics in commensal isolates from livestock and food [4].

E. coli was assigned to the *Enterobacterales* and belongs to the class of the *Gammaproteobacteria*. It is a rod-shaped, facultative anaerobic and Gram-negative bacterium with a rapid growth as it replicates each 20 minutes under optimal conditions. *E. coli* represents a highly diverse species with various phenotypic and genotypic variants. Traditionally, *E. coli* has been typed based on the somatic (O), capsular (K), and flagellar (H) antigens into more than 700 different serotypes [5]. Currently, these methods were revisited as other promising typing techniques exists, including multi-locus sequence typing (MLST) and phylotyping. To date, two different MLST schemes and one phylotyping scheme by which the isolates can be assigned to seven phylogroups (A, B1, B2, C, D, E, F and *Escherichia* clade I) are available. While the core genome of *E. coli* consists of more than

2,000 genes identified in multiple genomes of this species, the pan-genome can increase to the size of target sequences up to >18.000 loci [6]. Overall, a high diversity of phenotypic characteristics among *E. coli* has been described [7].

E. coli is considered as a commensal bacterium of the gastrointestinal tract of humans and animals, but is also found in water, soil, plants, and vegetables [8, 9]. However, *E. coli* is not just a harmless component of the intestinal microflora, but can cause serious intestinal and extra intestinal infections in immunocompromised and healthy individuals [2]. Based on prevailing virulence factors, patterns of bacterial attachment to host cells, effects of attachment on host cells, production of toxins and invasiveness, *E. coli* is divided into six major pathotypes [10]. These include Shiga toxin-producing *E. coli* (STEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), diffusely adherent *E. coli* and enteroinvasive *E. coli* [3]. These pathogenic isolates are usually associated with gastrointestinal tract infections, bacteraemia, meningitis, peritonitis, septicemia, urinary tract infections and other diseases that require therapeutic intervention [8, 11-13]. Hence, *E. coli* is also a clinically important bacterium causing severe human illnesses [14, 15]. However, the definite categorization of *E. coli* into apathogenic or pathogenic is rather challenging. While many *E. coli* carry some virulence factors, their true virulent potency is only fulfilled in certain conditions or in the presence of different components. Thus, an *E. coli* might be classified as apathogenic although under different conditions it would evolve as harmful. Hence, the impact of potential apathogenic *E. coli* is unknown, but they are frequently detected in livestock and food sources. Multiple studies reported the occurrence of *E. coli* in various food producing animals, such as chickens, cattle and pigs as frequently summarized by EFSA reports [16]. *E. coli* is also found in animal products as cheese and milk, but also in different vegetables like lettuce and sprouts. However, only pathogenic *E. coli* need to be reported to authorities. Because of the ubiquitous existence of this bacterium as well as the importance as a reservoir for AMR, *E. coli* has a high impact as an indicator for AMR in livestock and foods. The World Health Organization recognizes this bacterium as "highly relevant and representative indicator of the magnitude and the leading edge of the global antimicrobial resistance problem". According to the WHO, *E. coli* is one of the most common species, related to foodborne AMR monitoring. The World Organization for Animal Health recommends the monitoring of AMR in commensal *E. coli*, and the European Union uses the data on *E. coli* from farm animals in an annual integrated report on AMR [2, 17]. *E. coli* is known to be susceptible to selection pressures [18] and selection of resistance after using antimicrobial agents has often been reported [8, 19]. Furthermore, it has a high competency to acquire and transfer antimicrobial resistance genes from and to other species [19], which can result in the dissemination of resistance determinants to humans and to the environment. Overall, the resistance to antimicrobial agents in veterinary medicine has been

reported increasingly in *E. coli* and represents a public health concern, worldwide [20]. Thus, *E. coli* is a key species for AMR monitoring, indicating possible public health risk related to antimicrobial resistances. Additionally, as a typical bacterium of the *Enterobacterales*, this species might also reflect changes in the AMR dynamics in other species within this order.

For *E. coli*, resistances against different antimicrobial agents have been increasingly notified in the last decades. Based on its impact, it is now included, along with the rest of the *Enterobacteriaceae*, in the World Health Organization's list of the 12 families of bacteria that pose the greatest threat to human health [21].

1.2. Antimicrobial agents used to combat *E. coli* infections

Antimicrobial agents are used to treat microbial infections in humans and animals. The term antimicrobial agents include all substances that act against microorganisms. The term antibiotics only includes agents that are produced naturally by microorganisms. While antimicrobial agents are beneficial for combating infections, their wide use by humans can contribute to an increased emergence of tolerant and resistant bacteria [22]. Many classes of antimicrobial agents are used in food animals as well as in human medicine. The treatment of livestock with antimicrobial agents has raised concerns, whether it will minimize the effectiveness for treating diseases in humans [23]. Therewith, different antimicrobial agents are assigned to different groups of importance. Most of the antimicrobial agents are classified according to their modes of action. Namely, the mechanisms by which their antibacterial activity inhibits the growth or causes the death of other microorganisms. Generally, the primary action can be divided into the groups of agents: (i) inhibiting the biosynthesis of the bacterial cell wall, (ii) inhibiting the bacterial protein synthesis, (iii) inhibiting the nucleic acid metabolism, and (iv) antimicrobial agents altering the membrane activity. Depending on their range of activity, different antimicrobial agents are considered for different treatments [20].

However, bacteria can also be intrinsically resistant to some antimicrobial agents. Next to the acquisition of genes, resistances can also be species specific and chromosomally associated. *Escherichia coli* is intrinsically susceptible against several antimicrobial agents. Certain intrinsic resistances of *E. coli*, mostly based on outer membrane activity and efflux pump systems, have been described [24]. More than seven efflux systems that can export unrelated antimicrobial agents or other compounds as antiseptics, detergents and dyes, summed up as multidrug resistance efflux pumps were detected in *E. coli* [25].

To treat infections resulting from pathogenic microorganisms, prescription of antimicrobial agents in the veterinary sector is common. However, the constant use or in some cases

misuse of antimicrobial agents in the veterinary sector as well as in the human medicine sector led to an increasing emergence of AMR, worldwide [26]. The detection of these resistances in foodborne zoonotic bacteria is a concern for public health [27].

To derive reliable management strategies and to give reliable recommendations for risk assessments, EFSA evaluates the AMR situation in the food and animal sector across Europe. In their recently published summary report on antimicrobial resistances in zoonotic and indicator bacteria from humans, animals and food, an overview on the situation of AMR is given for Europe in 2018 and 2019 [28]. In these years, indicator *E. coli* obtained from caecal samples and fresh meat from broilers and fattening turkeys (in 2018) as well as isolates from fattening pigs and cattle under one year of age (in 2019) were analysed.

In this report, a decrease of ESBL-/AmpC-producing *E. coli* in food-producing animals as well as an increase of the complete susceptibility in indicator *E. coli* for some European countries over the period of 2015-2017 compared to the period of 2018-2019 was notified [17, 28, 29]. Furthermore, resistances against third generation cephalosporine and resistance to colistin was uncommon in indicator *E. coli* in 2018 and 2019. The resistance to ampicillin, tetracycline and sulphonamides were frequently detected. In addition, resistance to quinolones and fluoroquinolones was frequently detected among isolates recovered from broilers, fattening turkeys and poultry meat samples. The resistance of *E. coli* to quinolones and fluoroquinolones is of considerable concern, as it is frequently associated with horizontally acquired, mobile resistance transfer mechanisms [30]. This states the importance of monitoring and understanding the resistance mechanisms against quinolones and fluoroquinolones for indicator *E. coli* in a comprehensive manner.

1.3. Quinolones and fluoroquinolones in the European Union (EU)

Quinolones and fluoroquinolones (further referred to as (fluoro)quinolones) were used since 1962, when George Lesher discovered nalidixic acid [31, 32]. This antibacterial agent was the first member of the quinolones used for the treatment of urinary tract infections [33]. Shortly after, the 6-fluoro analogues named fluoroquinolones where discovered, providing a broader spectrum against bacterial infections [34]. Besides their antibacterial activity, fluoroquinolones have been reported to be useful in anti-tumor, anti-tubercular, anti-malaria and anti-HIV treatments [35-40]. In general, they are potent, broad-spectrum antimicrobial agents.

(Fluoro)quinolones are classified regarding their spectrum of activity and pharmacokinetic profile [41, 42]. As shown in Table 1 there are four generations of (fluoro)quinolones, with multiple representatives and different activity spectra and half-life.

Table 1. Classification, representatives, spectrum of activity and area of use for fluoroquinolones and quinolones.

	Selected representatives	Spectrum of activity	Area of use
First generation	nalidixic acid, oxolinic acid, pipemidic acid, rosoxacin	Aerobic Gram-negative bacteria	Treatment of urinary tract infections caused by Gram-negative bacteria
Second generation	ciprofloxacin, enrofloxacin, norfloxacin, enoxacin, pefloxacin, lomefloxacin, nadifloxacin, rufloxacin, ofloxacin	Wider activity against Gram-negative bacteria, activity against some aerobic Gram-positive bacteria	Treatment of tissue-based diseases, pneumonia, skin infections, and urinary tract infections
Third generation	levofloxacin, pazufloxacin, temafloxacin, tosufloxacin, sparfloxacin, grepafloxacin, balofloxacin	Wider broad-spectrum activity against Gram-negative bacteria and activity against Gram-positive bacteria as well as against some anaerobia	Treatment of tissue-based diseases, pneumonia, skin infections, and urinary tract infections
Fourth generation	prulifloxacin, trovafloxacin, alatrofloxacin, delafloxacinm, clinafloxacin, besifloxacin, sitafloxacin, finafloxacin, gatifloxacin, gemifloxacin, moxifloxacin	Activity against Gram-positive organisms, against atypical bacteria, and variable activity against anaerobia	Treatment of tissue-based diseases, pneumonia, skin infections, and urinary tract infections

Nowadays, the newest developed non-fluorinated quinolone antibiotics levonadifloxacin, zabofloxacin and nemonoxacin are gaining more importance in the treatment of infections. In clinical trials, they showed higher potency against Gram-positive bacteria as their antecedents and are still active against Gram-negative bacteria [33, 43]. However, the basic structure of the (fluoro)quinolones are comparable. Figure 1 shows the basic fluoroquinolone molecule, adapted from Domagala [44]. The addition of a fluorine molecule at the carbon atom position 6 was the earliest change, that broad up the second generation of (fluoro)quinolones [45], but other generations of this class where subsequently developed. Modifications included alkylation of the quinolones, addition of 2-methyl group to the C-7 position of the piperazine ring, the addition of an amino group at the C-5 position and the addition of halogen at the C-8 position [46-48].

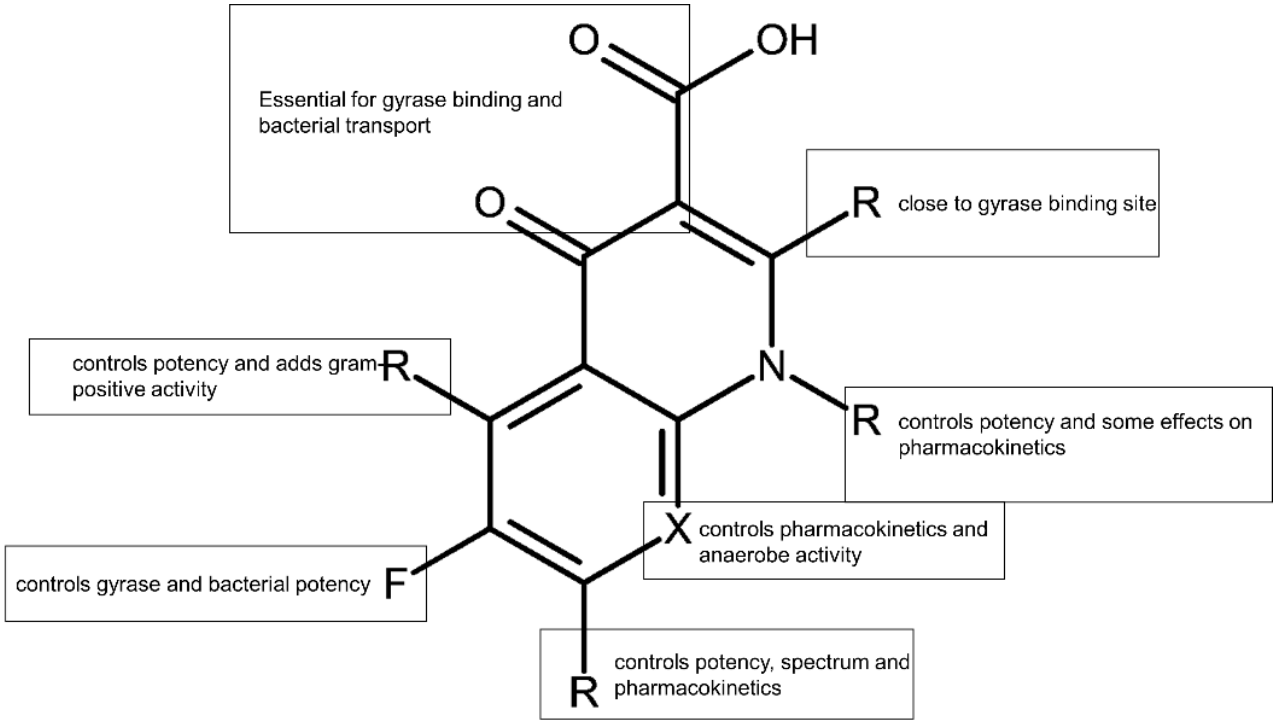

Figure 1. Summary of quinolone antibacterial structure activity relationships, as adapted from Domagala [44].

The mode of action of these (fluoro)quinolones lies in the inhibition of the replication and transcription of the bacterial DNA. Thus, they inhibit the activity of either the DNA gyrase and/or prevent the detachment of the gyrase from the DNA [49]. During the process of replication and transcription, the helicase enzyme uncoils the DNA double helix, which leads to excess supercoiling of the remaining DNA double helix. In *E. coli*, the DNA gyrase (a subclass of the topoisomerase II enzyme in bacteria) allows the relaxation of this supercoil in order to continue the process of replication [50], while topoisomerase IV is responsible for decatenation of the *E. coli* chromosome. Those topoisomerases consist of different subunits: The two monomers of the topoisomerase II, namely GyrA and GyrB, are encoded by the genes *gyrA* and *gyrB*, whereas the corresponding subunits of topoisomerase IV are encoded by the genes *parC* and *parE*, respectively [11]. (Fluoro)quinolones target the DNA gyrase and the topoisomerases IV [51]. Thus, (fluoro)quinolones alter the enzyme-DNA complex to a drug-enzyme-DNA complex, where type II topoisomerase is trapped within this bound [52]. Thus, the topoisomerase IV and the DNA gyrase are unable to re-ligate the DNA substrate. These non-re-ligated substances are referred to as cleaved complexes, leading to cell death. Further, these cleaved complexes block the DNA replication and, therefore, induce a SOS stress response [52]. This leads to an upregulation of multiple stress responses that further enhance the DNA-repair capability, additionally leading to a filamentous cell formation [53].

As fluoroquinolones are antimicrobial agents classified by the World Health Organisation as highest priority, critically important in human medicine, the increase in the prevalence of diverse resistance genes are concerning [54]. Veterinary Surveillance data from 2012 monitored a fluoroquinolone use of 136 tonnes as an active ingredient in food-producing animals in Europe [55]. However, the data published by the Federal Office of Consumer Protection and Food Safety of Germany (BVL) in 2020 monitored a decrease of the delivery amount in the veterinarian sector of Fluoroquinolones from 2011 to 2019. While in 2011 the delivered amount was of 8.2 tonnes and in 2017 even of 9.9 tonnes, this value lessened to 6.0 tonnes in 2019. Therewith, the use of Fluoroquinolones in Germany made up under 1 %, as the overall amount of antimicrobial agents delivered in 2019 was around 670 tonnes. The sales of antimicrobial agents in Europe and the United Kingdom are monitored in mg/PCU (population correction unit). This population correction unit considers the animal population as well as the estimated weight of each animal at the time of treatment with antibiotics and therewith acts as a normalization factor. In Germany, a use of 1.02 mg/PCU was announced in 2016. This measurement differed within European countries. Thus, Norway and Sweden recognized a value of below 0.05 mg/PCU, while Spain, Poland, Hungary, and Portugal identified a value of over 9.00 mg/PCU for fluoroquinolones. Nevertheless, this data should always be analysed in perspective of the size of the livestock farming business in each country. Although mass medication of poultry with fluoroquinolones is permitted in most countries of the EU [56], concerns are present that the use in the veterinary medicine undermines the effectiveness of fluoroquinolones in human medicine [57]. The European Food Safety Authority (EFSA) as well as the European Centre for Disease Prevention and Control (ECDC) [58] stated that the resistance rates in the EU was “high”, “very high” or even “extremely high” regarding fluoroquinolones in the veterinary sector. The resistance in 2018/2019 in EU was considered as ‘very high’ or ‘high’ for isolates from broilers (median: ciprofloxacin 73.5%, nalidixic acid 64.1%), and from turkeys (median ciprofloxacin 56.5%; median nalidixic acid 34.8%). The resistance average in pigs (median 7.7% and 5.7%, respectively) and calves (median 11.2% and 4.1%, respectively) were much lower. However, variations were registered between reporting countries for each animal population. As (fluoro)quinolone resistance is increasing in the EU, the monitoring and analysis of the resistance mechanism is crucial. Especially, the process of gene spread associated with (fluoro)quinolone resistance development needs to be understood [28] to develop sustainable management strategies against emergence and persistence of resistant bacteria.

1.4. Antimicrobial resistances against (fluoro)quinolones

Antimicrobial resistances against (fluoro)quinolones can occur through various mechanisms. Thus, mutations in one or more of the genes related to the type II topoisomerase (*gyrA*, *gyrB*, *parC* and *parE*) are most common for (fluoro)quinolone resistance in *E. coli* [59, 60]. Further, transmissible quinolone resistance mechanisms encoded on plasmids and known as plasmid-mediated quinolone resistance can lead to an altered resistance against (fluoro)quinolones [33].

1.4.1. Chromosomal mutations leading to (fluoro)quinolone resistance

(Fluoro)quinolones target the topoisomerase II and topoisomerase IV enzymes, which are composed of the subunits GyrA and GyrB, or ParC and ParE, respectively [61]. Alterations in specific sequence regions can lead to decreased susceptibility against (fluoro)quinolones and are then referred to as quinolones resistance-determining region (QRDR). This QRDR includes the amino-terminal domains of *gyrA*, *parC*, *gyrB* and *parE* [62]. The most common site of mutation in the QRDR of *gyrA* in *E. coli* is associated with the codons for Ser83 and Asp87 [63]. Mutations at these positions have been notified with a reduced binding of the antimicrobial agent to the gyrase-DNA complex leading to a reduced efficacy of the action of (fluoro)quinolones [64, 65]. Furthermore, mutations at Asp87 lead to a decreased catalytic efficacy of the *E. coli* gyrase [66]. The most common alteration S81P in *parC* causes a selective decrease in the affinity of (fluoro)quinolones for the development of the enzyme-DNA complex [67]. Although mutations in *gyrB* (Asp426 and Lys447) and *parE* (Leu445) are less common, they also have been shown to cause (fluoro)quinolone resistance [68, 69]. Overall, the resistance magnitude due to the QRDR mutation varies depending on the bacterial species affected and on the (fluoro)quinolone used [70]. For some (fluoro)quinolones, single target mutations can result in an 8- to 16-fold increase in the minimal inhibitory concentration (MIC). Consecutive mutations in the QRDR have been reported to yield even higher levels of resistance [71].

As the (fluoro)quinolones need to cross the bacterial envelope, to reach their target, mutations hindering this procedure can alter resistance. In general, this alteration is attained by hindered entrance and reduced uptake (especially for Gram-negative bacteria), or active transport out of the cell (efflux) of the antimicrobial agent. Thus, the reduction of outer membrane porin diffusion channel can hinder the entrance of the (fluoro)quinolones to the periplasmic space [72, 73]. Further, alterations in genes encoding regulatory proteins could control the effectiveness of efflux pumps [74]. However, mutations in efflux pump structural

genes are rather uncommon [75]. The latter mechanism can alter the susceptibility against several antimicrobial agent and does not necessarily only change resistances against (fluoro)quinolones.

1.4.2. Plasmid-mediated quinolone resistances (PMQRs)

The main mechanism of quinolone resistance is due to the presence of multiple mutations in the QRDR region. However, another mechanism is the presence of plasmid-mediated quinolone resistances (PMQRs) [76]. PMQRs are usually involved in a reduction of the susceptibility to (fluoro)quinolones without assured alteration to resistance. Nevertheless, the accumulations of mutations in the QRDR and the presence of multiple PMQRs can lead to a non-susceptible phenotype [77, 78]. Moreover, the facilitating for the selection of additional (fluoro)quinolones resistance-mediating mutations has been described before [79]. Nowadays, three mechanisms of PMQR are known. This, includes the first reported pentapeptide repeat encoding protein *qnr*, the (fluoro)quinolone-modifying aminoglycoside acetyltransferase encoding gene ***aac(6')-Ib-cr*** and the plasmid-mediated quinolone efflux pumps QepA and OqxAB [80].

The PMQR protein Qnr alters the susceptibility against (fluoro)quinolones by binding to the gyrase or topoisomerase IV and resulting in an inhibition of the gyrase-DNA interaction. Thus, it minimizes the chance for the (fluoro)quinolones to stabilize the lethal gyrase-DNA-quinolone complex [81]. The pentapeptide repeat protein is made of tandemly repeated 5-amino acid sequences followed by a consensus sequence [82]. Today, six different members of the *qnr* family are known. The first, *qnrA*, was identified in 1998 in a clinical *Klebsiella* (*K*) *pneumoniae* strain [83] followed by the discovery of *qnrS*, detected in *Shigella flexneri* [84], *qnrB* in *K*. *pneumoniae* [85], *qnrC* in *Proteus mirabilis* [86] and *qnrD* in *Salmonella enterica* [87]. The latest gene is *qnrVC* and was detected on a plasmid of *Aeromonas punctate* and *Vibrio fluvialis* [88]. In general, these *qnr* genes differ in sequence by approximately 35 % or more to each other and further contain allelic variants differing by 10 % or less [76]. While *qnr* is categorized as plasmid-mediated, those genes were also found in the chromosomal DNA of Gram-positive and -negative bacteria.

The second detected PMQR gene, ***aac(6')-Ib-cr*** codes for the aminoglycoside acetyltransferase AAC(6')-Ib-cr. Compared to other ***aac(6')-Ib*** genes, the -cr variant is unique due to two codon changes, Trp102Arg and Asp179Tyr. These mutations are responsible for the (fluoro)quinolone susceptibility-alterations compared to the wildtype ***aac(6')-Ib***. Therewith, the enzyme N-acetylates the (fluoro)quinolone substance at the amino-nitrogen on its

piperazinyl substituent. This reduces the effectiveness of the (fluoro)quinolone and leads to a higher MIC [76, 89].

The third type of PMQRs are the plasmid-mediated efflux pumps encoded by *qepA* or *oqxAB*. Those efflux-pumps are decreasing the susceptibility to hydrophilic (fluoro)quinolones and are known to be multidrug efflux pumps. While *qepA* belongs to the major facilitator (MFS) family [90, 91], *oqxAB* is categorized to the resistance-nodulation-division (RND) family [92]. The efflux pumps encoding PMQRs are rather rare. Also, some other plasmid-mediated efflux pumps active on quinolones have been reported and need to be studied in more depth [79].

Only a few surveys were conducted to analyse the prevalences of *qepA* and *oqxAB*. Most epidemiological studies of *qepA* and *oqxAB* from human and animal sources, report a prevalence below 1 % [76]. Whenever (fluoro)quinolone-resistant strains were analysed, *aac(6')-Ib-cr* was frequently detected (up to 51 %) [93]. Overall, *qnr* had been identified in a multitude of *Enterobacterales* globally, as in America, Europe and Asia [94]. When found in Gram-positive organisms, *qnr* was mostly chromosomally encoded [95, 96]. Regarding hospital isolates, the most prevalent *qnr* gene variants were *qnrB*, *qnrA* and *qnrS* [86]. In isolates from livestock and food, *qnrS* was detected more frequently than *qnrB* [97-101]. Therewith, *qnr* has been detected in all kinds of food animals (cattle, chicken, ducks, fish, pigs, sheep, turkey, etc.) as well as in domestic animals (birds, dogs, cats, rabbits, etc.) [79]. Further, *qnr* is frequently detected in samples, preselected for ESBL in *E. coli* [102, 103]. Overall, the spread of *qnr* over a variety of bacteria and sources is quite excessive.

1.5. Spread of resistance genes

The spread of resistance genes among different bacteria, matrices and geographical areas is one of the most problematic challenges in preventing the dissemination of AMR. New resistance genes evolve and are transferred and disseminated within different sources by horizontal gene transfer (HGT) mechanisms. While vertical gene transfer, the inheritance of genes from mother to daughter cell during cell division, is limited to one bacterial species and slow in evolution, HGT is a major driver in the spread of genetic information. Horizontal gene transfer is defined as transfer of genetic material uncoupled from cell division [104]. The mechanisms of HGT delivers further possibilities for gene exchange than vertical exchange and is a huge contributor for the rapid spread of resistance genes and the development of resistances [105]. Horizontal gene transfer includes the spread of genes through (i) natural transformation, (ii) transduction and (iii) conjugation/mobilization.

i) Transformation of extracellular DNA

Transformation in bacteria is defined as the uptake of extracellular DNA from the surroundings to the recipient cell [106]. Here, the recipient needs to be naturally competent [107]. Most naturally competent bacteria have a time-limited potential for the uptake of DNA caused by some environmental conditions as changed growth conditions, nutrient availability or cell density [108]. Natural transformation is the main explanation for bacteria acquiring DNA from outside of the host range of mobile genetic elements (MGE) or bacteriophages [108]. It contributes to the genetic variability as the resistance gene uptake and allows the adaption to different environments.

ii) Transduction by bacteriophages

Transduction is the transfer of bacterial genes via bacteriophages. Bacteria-specific viruses, the bacteriophage, can carry DNA in their capsids, bind to the recipient cell and inject the foreign DNA. During this transduction process the recombination of this foreign DNA into the recipient's genome is possible [105]. Transduction has been observed in the transfer of certain AMR genes between different species from the same or different sources [109]. Despite transduction and transformation being less prominent than conjugation, both mechanisms are quite important, as they are able to transfer chromosomally encoded as well as plasmid-borne AMR genes [105].

iii) Conjugation by plasmids

The process of conjugation is the transfer of genetic material from a donor to a recipient by direct cell-cell contact. A donor cell forms a F-pilus and gets in contact with the recipient cell, forming a conjugation tube. This step includes the transferosome (type IV secretion (T4SS)) known as F-factor, which is necessary for the synthesis of the pilus. The respective double-stranded DNA is separated at the origin of transfer (*oriT*) into single-stranded DNA molecules. One strand enters the recipient's cell, and the complementary strand will be synthesized. For this, the relaxosome is needed, as it creates a nick at the *oriT* and allows the transfer of the ssDNA (T-strand) into the donor cell. This conjugation system needs essential components working together. In self-transmissible plasmids, the conjugation is dependent on *tra* genes and the *oriT* site. Therewith the *tra* consists of the components Dtr (DNA transfer and replication) also named MOB (set of mobility genes) and a membrane-associated mating pair formation (MPF) complex, which is a form of the T4SS. The Dtr prepares the plasmid for the

transfer and includes the relaxases, the relaxosome complex and the primase. The MPF components consists of the pilus-encoding region, the information for channel formation and the area for coupling proteins. It is responsible for cell contact, channel formation and transfer initiation signals. A plasmid with these set of MPF and MOBs is self-transmissible or conjugative. Mobilization describes the process of small non-conjugative plasmids, which are present in the same bacterial cell, using the transfer apparatus provided by the conjugative element. Mobilizable plasmids need to use a compatible MPF of another genetic element of the donor cell to enable the process of conjugation. Non-mobilizable plasmids, without MOB and MPF region including *oriT*, can still be transmitted by transformation or transduction. The process of conjugation often provides genetic advantages. Many MGE are known to transfer AMR genes. As conjugation boosts the genetic exchange it helps bacteria to adapt to different environmental conditions. Thus, conjugation facilitates the spread of resistance genes and therewith the spread of resistant bacteria.

1.6. Mobile genetic elements

Mobile genetic elements (MGEs) are divided into two major groups. Those include the ones transmissible from cell to cell as plasmids, conjugative transposons, or integrative and conjugative elements. Furthermore, MGE exists, which cannot be transferred by themselves, but i.e., by integration into plasmid genomes. Intracellular DNA mobility and intercellular DNA exchange are the major causes for dissemination of different genes as for example resistance genes.

i) Gene cassettes

A gene cassette is one of the smallest mobile elements (0.5 to 1 kb), usually consisting of one gene or open reading frame. In class 1 integrons, the main characteristic of a gene cassette is the *attC* recombination site. This guarantees the recognition by the IntI and is essential for its mobility [110]. However, gene cassettes rather rarely include a promoter and, therefore, the expression of their gene(s) depends on promoter regions in the vicinity. They mostly do exist incorporated within an integron, but are also able to remain freely as circular DNA for a short term [111]. Thus, gene cassettes can move within a genome or be transferred by another MGE via HGT. Multiple gene cassettes can be build up as a “cassette array” within the same integron and thus be responsible for multidrug resistance.

ii) Transposon and insertion sequence (IS) elements

The transposon (Tn) is a DNA sequence that is able to move itself. It is referred to as “jumping gene”. A Tn can transfer itself from one plasmid to another, as well as from a chromosome to a plasmid and vice versa. This transposition is due the transposase, located on the Tn. Transposons do belong to a group of MGEs called transposable elements (TEs) as do IS elements, composite Tns, non-composite Tns (Tn3 family) and transposable phage Mu [112]. A Tn differs from IS elements by encoding a function, that is able to change the phenotype. The IS are the smallest, transposable genetic elements among bacteria [113]. They do encode the transposase independently. Composite Tns and non-composites Tns can be the carrier of antibiotic resistance genes. The difference between them is that composites Tns are flanked by the same or closely related IS element on both sites (with the IS elements located in opposite orientations), while non-composites Tns are not [114]. Thus IS, and non-composite Tns do play an important role in the transmission of resistance genes. In general, Tns can be located in the chromosome or on a plasmid.

iii) Integrative and conjugative elements (ICEs)

ICEs are modular mobile genetic elements integrated into a host genome. They are mobile between cells and further contain genes needed for integration and excision. The conjugation process of ICEs relies on the type IV secretion system. As they are integrated in the host chromosome, they do passively replicate during replication [115]. However, when ICE gene expression is induced, it excises from the chromosome and forms a circular DNA molecule. From this state, it can be transferred to a recipient cell and recombines to the new host chromosome, through ICE-encoded recombinase. They can be inserted into a host chromosome and are driving the HGT of e.g. resistance genes [116].

iv) Plasmids

Plasmids are extrachromosomal mobile genetic elements. Some plasmids do have the ability to spread horizontally via conjugation. Most plasmids are present in circular, double-stranded DNA molecules within the bacterial cell. They are a very important vehicle for carrying other MGEs and transferring them do different hosts. Mainly, they promote their own transfer and replicate independently from the bacterial chromosome.

Plasmids vary in sizes from less than 1 kb to up to 200 kb as well as in their copy numbers. They can vary in their host range broadness as well as in their incompatibility (Inc) groups. The Inc group of the plasmids is the basis for their classification. Usually, plasmids from the same Inc group are not able to exist stably in the same cell. Many resistance plasmids are known for *Enterobacterales* as *E. coli*. Those include larger, often conjugative plasmids as well as smaller, mobilizable or non-mobilizable ones [114]. Plasmids are able to confer a considerable amount of genes as resistance genes or virulence genes [117]. Thus, they are the key vectors of horizontal gene transfer.

1.7. AMR plasmids carrying *qnr* genes in *E. coli*

As plasmids are categorized into different Inc groups, each replicon type can provide different characteristics. Thus, some Inc groups are associated with the carriage of multiple resistance determinants or the harbouring of some specific ones. More than 27 major Inc groups were shown to be associated with antimicrobial resistance genes in *Enterobacterales* [118]. The most prevalent is IncF [119]. However, *E. coli* is known to harbour a broad range of different plasmid replicons and is therefore a high contributor in the dissemination of different plasmids and their resistance determinants [119]. The (fluoro)quinolone resistance-altering *qnr* gene is described on various plasmid types of different Inc groups, which are often associated with specific *qnr* gene variants. Thus, *qnrS1* has frequently been described on IncN [120] and IncX plasmids [121]. Those IncX plasmids are, next to *qnrS*, associated with β-lactam (*bla*) genes. In general, *qnr* genes have been frequently reported in the combined presence of *bla* genes on the same plasmid, independently of the Inc group. Further, they are widely disseminated in Europe [121]. While IncX is assigned to be a narrow host range plasmid type, IncN counts as a broad range one and is, therefore, important in the dissemination of *qnrS* genes. In addition, IncX plasmids were described to form cointegrates with some plasmids carrying virulence genes, which altered their ability into a broader host range plasmid. IncX plasmids are mainly isolated from *E. coli* and *Salmonella* strains and primarily encode antimicrobial resistance genes, including those for resistance to extended-spectrum β-lactams and quinolones. Genes encoding carbapenemases had been detected on IncX plasmids, too [122]. Thus, they are important contributors in the mutual spread of resistance against (fluoro)quinolones and extended-β-lactamases in *E. coli*. Other *qnr* variants as *qnrB* have also been detected on IncN plasmids. However, especially *qnrB19* is associated with a small Col440I plasmid type, sometimes also categorized as ColE(-like) plasmid. Those types of plasmids are recognized as colicinogenic plasmids. The type I Col-plasmids, which are especially associated with *qnrB19*, are small and mobilizable plasmids of 6-10 kb in size. As they are usually not self-transmissible, they rely on outer conditions for

their dissemination. They are detected in many areas of the world, as well as in different sources [123, 124]. Further, the Col plasmids can coexist in the same bacterial host, bearing different and multiple resistance determinants [125]. Thus, they play an important role in the spread of different resistance genes.

To detect and characterize these plasmids correctly and categorize them into the respective Inc group is crucial for a reliable assessment. In addition, to determine the complete nucleotide sequence of a plasmid and to be able to elucidate the whole structure of it is greatly important.

1.8. Next Generation Sequencing (NGS) and bioinformatics analysis for determining characteristics of AMR gene carrying *E. coli*

The widely adapted method of NGS has provided the foundation for truly understanding microbial communities and their properties. It allows for the detection of different characteristics of a strain as its species identification, the composition of its genome as well as the detection of other additional genetic structures, as the mobilome of a strain. Overall, nucleic acid sequencing has improved the capacity to characterize bacterial genomes in detail and revolutionized genetic investigations. Whole genome sequencing (WGS) evolved from first generation sequencing, also known as shotgun sequencing to high throughput next generation sequencing and the technology of long-read, real-time sequencing, known as third generation sequencing. Currently, this evolution found a junction in synthetic long-read sequencing. Second generation sequencing platforms, with Illumina being the most prominent one, are known for relatively low costs, high throughput and shorter read lengths. The Illumina platform uses a “sequencing by synthesis” approach and generates read templates by bridge amplification on a solid phase surface. Although revolutionizing, second generation sequencing has its limitations, as it works with a PCR amplification step, which introduces a bias as well as possible nucleotide alterations during the synthesis of DNA. The short read lengths make it insufficient for some biological tasks, as the assembly of complex genomic regions. Complex repeat regions in DNA fragments are especially challenging for a reliable assembly. Another challenge for short-read sequencing technique consists in obtaining complete sequences of large extrachromosomal elements (i.e. plasmids), which are often highly diverse, do contain numerous repeated sequences and are modular in structure. Especially mobile genetic elements can be very complex in their composition. This makes it difficult to detect, locate and evaluate them correctly [126]. Third generation sequencing was developed to address those limitations. Nanopore sequencing by Oxford Nanopore Technologies (ONT) and single-molecule real-time (SMRT) sequencing,

developed by Pacific Bioscience (PacBio) are the dominant methods in this area [127]. By using these third-generation technologies, outstanding long reads are achievable. The average read length is higher than 10 kb, with an N50 of over 20 kb and a maximum read length of >60 kb. Hence, the precise structure of repetitive regions can easily be resolved within a single read. The drawbacks of long-read sequences are a lower throughput as well as a significantly higher error rate [128]. The combination of these two predominant methods for sequencing, as in a hybrid approach, is another advantageous technique. It uses the high accuracy and high throughput short-read data with the error prone long reads generated by third generation sequencing. This combination of long reads providing information about the genome structure with short reads promoting the detailed assembly has emerged as an advantageous application [128].

Next to the use of the proper sequencing platform, the bioinformatics analysis after generating the reads is a quite important step. The choice of the filter parameters, the assembly approach and the error-correction can alter the outcome of the predicted genome immensely. This represents a task that should be supervised by experienced bioinformaticians. While this needs to be kept in mind when it comes to data interpretation, a thorough analysis of these different available and used methods would be out of the scope of the herein discussed approaches.

However, downstream analysis of the generated assemblies is another important factor in characterizing features of the genome. These include procedures as reference based alignments, variant calling or the general visualization of the data. In addition, the annotation, the detection of certain genes, IS elements, plasmid types or estimation of the phenotype belongs to the bioinformatics analysis, conducted after the generation of the fasta file.

Overall, NGS combined with bioinformatics analysis allows for a comprehensive comparison between strains as well as for a detailed characterization. The therewith-attained discriminatory power is crucial for a thorough surveillance in different sectors [129]. In the context of resistance gene determination and phenotype estimation, reliable plasmid detection is key.

1.8.1. NGS for the estimation of resistance genes and resistance phenotypes in *E. coli*

As already discussed, resistance in otherwise susceptible bacteria can occur by chromosomal point mutations or through the acquisition of resistance determinants [130]. For a long time, methods like PCR, for detecting resistance genes, or minimum inhibitory concentration measurements, for estimating the phenotypic resistance profile, were the gold standard. Nowadays, whole-genome sequencing is a valuable tool for predicting these

attributes. AMR genes and point mutations leading to resistances can be identified by screening the generated nucleotide sequences or comparing it against a curated database [131]. Herewith, the AMR genes are mainly detected by assembly-based or less often by read-based approaches. The assembly-based approach requires the preliminary assembly of the reads and the hereinafter local alignment (e.g. through BLAST) to identify the respective resistance genes [132]. Thus, relying on a reference database. Due to the alignment approach, this method is able to detect acquired resistance genes as well as point mutations within a genome. As the determination deeply relies on a reference, the reference database needs to be curated and updated in a frequent manner. Thus, novel or remote homologous AMR genes can be missed, what needs to be taken into account when analysing a phenotype or comparing results with laboratory-generated data [130]. As bacterial strains continually evolve and form new resistance determinants, the solely prediction of phenotypes or estimation of resistance determinants with WGS methods is still not recommended on its own [133]. Therefore, WGS should be recognized as valuable tool for confirming and adding information to gold-standard methods as the mentioned PCR-analysis or MIC estimation.

1.8.2. NGS as application for plasmid detection

Plasmids are one of the main carriers for AMR genes. Furthermore, they are transmitting IS elements as well as transposon. These MGEs can interact with the chromosome or other prevailing genetic elements. A correct linking of genetic areas to the respective carrier is a hurdle. To understand the mechanism of transmission and the evolution of certain plasmids, the whole structure should be known. Great efforts have been made to establish reliable typing techniques for plasmids [133]. While plasmid typing was done through incompatibility studies, PCR analysis and DNA-DNA Hybridization, WGS is the new standard to detect these characteristics of a plasmid. While tools for typing plasmids *in-silico* do exist and are established to this point, the indisputable correct overall plasmid reconstruction through WGS is still questionable. As many plasmids do change in a modular manner or interact with the chromosome, the predominant alignment-based prediction of plasmids remains uncertain. Repetitive regions, complex structures or rearrangements can be overlooked or wrongly assigned. The laborious Sanger-sequencing is still the main method, if a reliable plasmid structure should be generated [134]. Hybrid sequencing, or long-read sequencing with a thorough sequencing depth, is a common way to close a plasmid sequence. It allows for correct size estimation, reliable Inc group and AMR gene assignment and detection of smaller and larger MGE to the same extent. Furthermore, many tools have been developed, allowing for binning of contigs for estimating the plasmid affiliation. Another possibility is the synthetic long-read approach. It uses the benefits of short-reads, namely their high accuracy,

with a chemical linking of the pre-shredded DNA. At this point, this approach seems to be a promising way for plasmid characterisation. However, the task of plasmid detection and correct assembling is still in the making and crucial for correct plasmid detection and understanding of AMR transmission and evolution.

1.9. The ARDIG project

The ARDIG project is an EU initiated project within the work frame One Health. It is an acronym for “Antibiotic Resistance Dynamics: the influence of geographic origin and management systems on resistance gene flows within humans, animals and the environment”. The main achievements are aimed in the area of characterizing plasmids as well as their important gene transfer entities. This should be achieved through investigations of antimicrobial resistance mechanisms and the fitness of multidrug resistant isolates in different environments. Especially the mechanisms of ESBL/AmpC/Carbapenemase/*mcr*-1 and -2/PMQR production was aimed to understand. Mobile elements at the molecular level in *Enterobacterales* isolates from different epidemiological units/ecological niches across geographic regions were considered for this. Detailed characterization of isolates collected from different compartments was performed through WGS using Illumina, PacBio and MinION sequencing techniques. Lastly, the adaptions of bioinformatics analysis for assembling of the sequencing data of a mobile genetic element were planned. The aim of the ARDIG project is to provide a better understanding in the differences of patterns for resistance determinants in a European wide approach. In addition, possible factors that are influencing the transmission of AMR between different environments and sources should be elucidated. Especially the strategy of in-depth molecular characterization will add crucial information. The outcomes can be included in following strategies and can lead to the prediction of emergency and spread of prevalent and novel AMR determinants. Overall, ARDIG will provide a basis for the development of AMR risk and transmission models in future control measures and mitigation of risk outgoing from AMR.

2. Published Articles

2.1 Publication I

Juraschek K, Borowiak M, Tausch SH, Malorny B, Käsbohrer A, Otani S, Schwarz S, Meemken D, Deneke C, Hammerl JA.

Outcome of Different Sequencing and Assembly Approaches on the Detection of Plasmids and Localization of Antimicrobial Resistance Genes in Commensal *Escherichia coli*

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Microorganisms (Basel). 2021; 14; 9(3)

		contribution
1.	Design of the project including design of individual experiments	60%
2.	Performing the experimental part of the study	75%
3.	Analysis of the experiments	85%
4.	Presentation and discussion of the study in article form	80%

microorganisms

MDPI

Article

Outcome of Different Sequencing and Assembly Approaches on the Detection of Plasmids and Localization of Antimicrobial Resistance Genes in Commensal *Escherichia coli*

Katharina Juraschek [1,*], Maria Borowiak [2], Simon H. Tausch [2], Burkhard Malorny [2], Annemarie Käsbohrer [1,3], Saria Otani [4], Stefan Schwarz [5], Diana Meemken [6], Carlus Deneke [2,†] and Jens Andre Hammerl [1,*,†]

1 Epidemiology, Zoonoses and Antimicrobial Resistance, German Federal Institute for Risk Assessment (BfR), Max-Dohrn Str. 8-10, 10589 Berlin, Germany; annemarie.kaesbohrer@bfr.bund.de
2 Study Centre for Genome Sequencing and Analysis, German Federal Institute for Risk Assessment (BfR), Max-Dohrn Str. 8-10, 10589 Berlin, Germany; Maria.borowiak@bfr.bund.de (M.B.); simon.tausch@bfr.bund.de (S.H.T.); Burkhard.Malorny@bfr.bund.de (B.M.); Carlus.Deneke@bfr.bund.de (C.D.)
3 Unit for Veterinary Public Health and Epidemiology, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria
4 DTU Food, National Food Institute, Technical University of Denmark, Kemitorvet, Building 204, 2800 Kgs Lyngby, Denmark; saot@food.dtu.dk
5 Institute of Microbiology and Epizootics, Department of Veterinary Medicine, Freie Universität Berlin, 14163 Berlin, Germany; Stefan.Schwarz@fu-berlin.de
6 Institute of Food Safety and Food Hygiene, Working Group Meat Hygiene, Department of Veterinary Medicine, Freie Universität Berlin, 14163 Berlin, Germany; diana.meemken@fu-berlin.de
* Correspondence: Katharina.Juraschek@bfr.bund.de (K.J.); Jens-Andre.Hammerl@bfr.bund.de (J.A.H.)
† Shared senior researcher authorship.

Abstract: Antimicrobial resistance (AMR) is a major threat to public health worldwide. Currently, AMR typing changes from phenotypic testing to whole-genome sequence (WGS)-based detection of resistance determinants for a better understanding of the isolate diversity and elements involved in gene transmission (e.g., plasmids, bacteriophages, transposons). However, the use of WGS data in monitoring purposes requires suitable techniques, standardized parameters and approved guidelines for reliable AMR gene detection and prediction of their association with mobile genetic elements (plasmids). In this study, different sequencing and assembly strategies were tested for their suitability in AMR monitoring in *Escherichia coli* in the routines of the German National Reference Laboratory for Antimicrobial Resistances. To assess the outcomes of the different approaches, results from in silico predictions were compared with conventional phenotypic- and genotypic-typing data. With the focus on (fluoro)quinolone-resistant *E. coli*, five *qnrS*-positive isolates with multiple extrachromosomal elements were subjected to WGS with NextSeq (Illumina), PacBio (Pacific BioSciences) and ONT (Oxford Nanopore) for in depth characterization of the *qnrS1*-carrying plasmids. Raw reads from short- and long-read sequencing were assembled individually by Unicycler or Flye or a combination of both (hybrid assembly). The generated contigs were subjected to bioinformatics analysis. Based on the generated data, assembly of long-read sequences are error prone and can yield in a loss of small plasmid genomes. In contrast, short-read sequencing was shown to be insufficient for the prediction of a linkage of AMR genes (e.g., *qnrS1*) to specific plasmid sequences. Furthermore, short-read sequencing failed to detect certain duplications and was unsuitable for genome finishing. Overall, the hybrid assembly led to the most comprehensive typing results, especially in predicting associations of AMR genes and mobile genetic elements. Thus, the use of different sequencing technologies and hybrid assemblies currently represents the best approach for reliable AMR typing and risk assessment.

Keywords: AMR; mobile genetic elements; *qnrS*; hybrid assembly; long-read sequencing; short-read sequencing

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1. Introduction

Antimicrobial resistance (AMR) in food- and livestock-associated bacteria can represent an important threat to public health and needs to be monitored [1,2]. Currently, the mandated AMR monitoring in European countries generates broad datasets on minimal inhibitory concentrations (MICs) of country- and matrix-specific isolates against selected antimicrobial agents [3]. For this, commensal *Escherichia* (*E.*) *coli* were chosen as indicator organisms, as they belong to the common intestinal microbiota of livestock and thus reflect trends in the development of antimicrobial resistances associated with the lifestyle of animals [4]. Up to now, the use of phenotypic data represents the gold standard for AMR monitoring [5]. However, due to the broad diversity of determinants associated with decreased susceptibilities of isolates against specific antimicrobial classes, whole-genome sequencing (WGS) provides deeper insight into the genetic basis of antimicrobial resistances, possible routes of transmissions and important clonal lineages, which are useful for risk assessment [6]. Therefore, the European Food Safety Authority (EFSA) strongly advocates the implementation of WGS into the AMR monitoring of the national reference laboratories [7]. A WGS-based monitoring will prospectively provide a uniform basis for the identification of dissemination paths of genetic elements, supporting the fight against resistance development in livestock-associated and foodborne commensals and pathogens [8]. Furthermore, sequencing data will be available for retrospective analyses of novel resistance or virulence determinants [9]. However, to ensure the high quality WGS data, the prevailing techniques need to be standardized, and minimum quality parameters for the multisite use of datasets need to be specified. For reliable AMR prediction and correct plasmid detection, high throughput sequencing with high accuracy and reasonable cost is required. The selection of sequencing and assembly approaches can significantly influence the results of resistance gene detection and localization [10]. As plasmids are commonly implicated in the dissemination of AMR, it is important to correctly determine whether resistance genes are fixed on the chromosome or located on mobile genetic elements (MGEs) [11].

WGS evolved from first generation sequencing to high throughput next generation sequencing (NGS) up to long-read, real-time sequencing, known as third generation sequencing [9]. Second generation sequencing (SGS) platforms are known for relatively low costs, high throughput and shorter read lengths and are usually the first choice in routine diagnostics [12,13]. However, second generation sequencing has its limitations, as it usually includes a PCR amplification step, which can introduce a bias and nucleotide alterations during DNA synthesis. In addition, its short-read lengths make it unfavorable for some biological tasks [14,15]. Especially mobile genetic elements can be complex in their composition, making it difficult to determine them correctly [16]. Single-molecule real-time (SMRT) sequencing (Pacific Bioscience: PacBio) and Nanopore sequencing (Oxford Nanopore Technologies: ONT) are the dominant methods of third generation sequencing [17]. Although both techniques offer longer reads compared to SGS, their drawbacks include a lower throughput and a significantly higher error rate [14], making them rather disadvantageous for routine and outbreak diagnostics. According to the strengths and weaknesses, a combination of short- and long-read sequencing seems to be promising for the determination of complex genomic regions [14] or complete plasmid sequences.

Due to the importance of (fluoro)quinolones in human medicine [18], the steadily increasing number of *E. coli* developing resistances against substances of these classes represents an emerging risk to public health [19]. Decreased susceptibility against (fluoro)quinolones is based on diverse genetic determinants, as chromosomal alterations of the DNA gyrase/topoisomerase genes and plasmid-acquired determinants lead to modified aminoglycoside acetyltransferases (AAC(6′)-Ib-cr) [20], specific efflux pumps (QepA, OqxAB) [21] and pentapeptide repeat proteins (Qnr). However, the acquisition of some plasmid-associated genes in *E. coli* is not necessarily linked to the development of a phenotypical resistance, according to epidemiological or clinical interpretation guidelines [22]. Thus, determinants affecting the susceptibility of isolates to (fluoro)quinolones might

spread unnoticed in *Enterobacteriaceae* by horizontal gene transfer. It has been hypothesized that some of the acquired determinants can also force chromosomal alterations in the DNA gyrase/topoisomerase, ultimately leading to strong phenotypic resistance of the isolates [23]. Thus, there is an urgent need for the characterization of transmissible (fluoro)quinolone resistance determinants to get deeper insights into the diversity of their hosting plasmids, the potential transmission pathways and their impact on resistance development.

In this study, the short- and long-read sequencing data were compared with results from molecular (i.e., pulsed-field gel electrophoresis (PFGE) macro-restriction and plasmid profiling) and microbiological analyses (i.e., minimum inhibitory concentration (MIC) determination) to investigate the impact of different sequencing and assembly strategies on the detection of resistance genes and the characterization of plasmids. This study has a particular focus on the detection of *qnrS1*, as this gene represents the most frequently found transmissible determinant associated with (fluoro)quinolone resistance in German livestock and food [24]. However, considering all available sequencing approaches and assembly pipelines published thus far, this work does not aim to represent an exhaustive comparison of all methods. Nevertheless, the provided raw and assembled sequencing data can be used by other groups to assess and evaluate their established assembly and annotation pipelines. The generated data and analysis of this study will support (i) the improvement of AMR monitoring for commensal *E. coli*, by implementing WGS as a gold standard for AMR prediction and (ii) an improved determination of MGEs associated with AMR gene prediction in the terms of risk assessments.

2. Materials and Methods

2.1. Selection of Bacterial Isolates

Five pre-selected *qnrS1*-positive *E. coli* from different sources with individual resistance profiles and multiple extrachromosomal elements were included in this study. These isolates originated from the German annual AMR monitoring program in 2016/2017 and were obtained from different sources, including poultry (n = 2), pig cecum (n = 1), calf cecum (n = 1) and bovine meat (n = 1). The five isolates represent common plasmid types of (fluoro)quinolone-resistant and *qnrS1*-positive *E. coli* from German livestock and food.

2.1.1. Antimicrobial Susceptibility Testing

For determination of the minimum inhibitory concentration (MIC), the isolates were subjected to broth microdilution according to EUCAST (European Committee on Antimicrobial Susceptibility Testing) recommendations on a universal European antimicrobial test panel (Sensititre™, TREK Diagnostic Systems, East Grinstead, UK). The tested antimicrobial agents (Supplement S5) were used according to the European Commission Implementing Decision No. 2013/652/EU [25] for the monitoring and reporting of antimicrobial resistance in zoonotic and commensal bacteria [25]. The *E. coli* strain ATCC 25922 was included in MIC determination as quality control. MIC values were interpreted according to EUCAST epidemiological cut-off (ECOFF) values [26].

2.1.2. PFGE Profiling and Plasmid Prediction

Pulsed-field gel electrophoresis (PFGE) was performed according to the PulsNet protocol [27]. Macro-restriction of genomic DNA was conducted using the restriction endonuclease XbaI (Thermo Fisher Scientific, Darmstadt, Germany). In addition, S1 nuclease (Thermo Fisher Scientific) PFGE was used to determine the presence and size of plasmids. Enzymatically treated agarose plugs were embedded in 1% agarose gels and separated in a CHEF-DR III system (Bio-Rad Laboratories, Madrid, Spain). For size determination, the *Salmonella* Braenderup strain H9812 was used.

For the detection of plasmids <20 kb, extrachromosomal DNA (pDNA) was isolated with the CosMCprep "Mini prep of plasmids" kit (Beckman, Krefeld, Germany) according

to the manufacturer's protocol. Plasmid visualization was performed in 0.8% agarose gels (Biozym Gold Agarose, Biozym, Vienna, Austria) separated for 1.5 h at 90 V.

For localization of *qnrS1*, Southern blotting and DNA-DNA hybridization of S1-PFGE gels were conducted using a digoxigenin-labelled (Roche Diagnostics, Mannheim-Penzberg, Germany) *qnrS1* probe, as previously described [28].

2.1.3. Gene Prediction with PCR

For estimation of resistance genes, PCR-based detection of *qnrS1* was conducted on a Bio-Rad CFX system, as previously described [29]. For detecting bla_{TEM}, primers and conditions were used as described elsewhere [30].

2.1.4. In Vitro Filter Mating Experiments

For in vitro filter mating experiments, the sodium azide tolerant *E. coli* strain J53 was used as a recipient. All strains were grown in LB (lysogeny broth) to an OD_{600} of 0.8. A 500 µL aliquot of the donor was mixed with 1 mL of the recipient bacteria. The bacterial suspension was centrifuged at 5000× *g* for 10 min and the supernatant was discarded. Sedimented bacteria were applied onto a Millipore filter membrane (0.45 µm pore-size) on LB agar. After an incubation of 12–16 h at 37 °C, bacteria were removed from the filter by suspension in 5 mL of 0.7% (*w/v*) saline solution. An aliquot of 100 µL was applied onto LB agar supplemented with nalidixic acid (0.15 mg/L) and sodium azide (100 mg/L). The plates were incubated at 37 °C for 20–24 h. Upcoming colonies were stored in glycerol at −80 °C and subjected to S1-PFGE for determination of the plasmid transfer.

2.2. Genomic DNA Extraction for Whole-Genome Sequencing (WGS)

A single colony was cultivated in 12 mL LB and incubated for 14–16 h at 37 °C. After incubation, the culture was centrifuged at 4500× *g* for 10 min. The pellet was resuspended in 300 µL phosphate-buffered saline (PBS), and 10 mL of the extraction buffer (100 mM NaCl, 10 mM Tris-Cl, pH 8.0, 25 mM EDTA, pH 8.0, 0.5% (*w/v*) SDS, 20 µg/mL RNase A (Qiagen, Hilden, Germany)) (modified from Sambrook & Russell, 2001 [31]) was added. The solution was mixed and incubated for 1.5 h at 37 °C. Thereafter, Proteinase K (20 mg/mL; Qiagen) was added, and the mixture was incubated for another 1.5 h at 50 °C. The lysate was then separated into two 15 mL tubes (Thermo Fisher Scientific), and 5 mL of saturated phenol (Sigma-Aldrich, Taufkirchen, Germany) was added to each tube. The mixture was rotated for 20 min on a PTR-35 tube rotator (Grant-instruments, Cambridgeshire, Great-Britain) at 20 rpm. Afterwards, it was centrifuged at 4500× *g*. The aqueous phase was transferred into a new 15 mL tube. Again, 2.5 mL saturated phenol (~73%) and 2.5 mL phenol-chloroform-isoamyl alcohol (25:24:1) (Sigma-Aldrich) were added. The tubes were rotated at 20 rpm on the tube rotator and centrifuged at 4500× *g*. The clean aqueous phases of each tube were transferred into a new 15 mL tube, 5 M ammonium acetate (Sigma-Aldrich) was added, followed by 25 mL of ice-cold ethanol (>99.5%, Sigma-Aldrich). After 5 min incubation at room temperature, clouds of DNA threads were collected with an inoculation loop and transferred into 1 mL of 70% ethanol in a 2 mL tube. The tube was centrifuged for 10 min at 10,000× *g* and washed with 1 mL 70% ethanol (*v/v*). The obtained pellet was dissolved in 500 µL elution buffer and incubated overnight at 5 °C. The extracted high molecular weight DNA was stored at 5 °C until further use.

2.3. Whole-Genome Sequencing

To ensure the use of high-quality DNA for sequencing on the different platforms, quantification with the Qubit fluorometer and quality assessment with the fragment analyzer, according to their protocols, was conducted (Supplement S1). For short-read sequencing on the Illumina NextSeq 500, DNA libraries prepared with the Nextera DNA Flex Library Preparation Kit (Illumina) according to the manufacturer's protocol were used. NextSeq sequencing was performed in 2 × 151 cycles with the Illumina NextSeq 500/550 Mid Output Kit v2.5 (300 Cycles) [32]. For long-read sequencing with the PacBio SMRT sequencing tech-

nology, DNA library preparation was conducted according to the standard manufacturer's conditions in the protocol "Preparing Multiplexed Microbial Libraries Using SMRTbell Express Template Prep Kit 2.0" (Part Number 101-696-100, Version 02, February 2019). This protocol includes a size selection step, conducted with AMPure beats, removing SMRTbell templates < 3 kb. For long-read sequencing with the Oxford Nanopore technology (ONT), sequencing libraries were prepared using the Rapid Barcoding Kit (SQK-RBK004, ONT) and sequenced on an ONT MinION sequencer connected to an ONT MinIT v19.12.5 device (including Guppy base caller v3.2.10) using a FLO-MIN106 R9 flow cell.

2.4. De Novo Assembly Strategies and Genome Characterization

After sequencing, all short-reads were pre-processed and filtered with fastp under default parameters [33]. For the assembly of NextSeq short-read sequencing data, Unicycler v0.4.8 [34] was used. Reads generated with PacBio and MinION (ONT) were assembled using Flye v2.8.1 [35]. Hybrid assemblies were generated with Unicycler v0.4.8 under default parameters.

Assembled contigs were analyzed with abricate Version 1.0.1 [36–43] and platon 1.4.0 [42,44,45]. Results from abricate and platon were used to locate AMR genes. Annotation of plasmids was conducted with the PATRIC RASTk-enabled Genome Annotation service [46]. Visualization of *qnrS*-carrying plasmids was done with Unicycler assembled PacBio reads with the Blast Ring Image Generator (BRIG; v0.95) [47]. The transmissibility of plasmid genomes was assessed using the mob-suite tool [48]. The closest related plasmid was detected with a blastn search [49].

2.5. Accession Numbers

The complete datasets (raw reads) from different sequencing approaches were deposited in GenBank under the BioProject ID PRJNA589028. Accession numbers of the individual datasets are given in Supplement S2. Genome assemblies of the individual datasets are given in Supplement S7.

3. Results and Discussion

3.1. Impact of Different Long- and Short-Read Sequencing Approaches

To estimate the impact of different sequencing and assembly strategies for reliable in silico prediction of *qnrS1*-carrying plasmids in *E. coli*, five isolates representing multiple extrachromosomal elements and *qnrS1*-carrying plasmids of different size ranges (Table 1) were chosen for in depth characterization. De novo assembly from the data of NextSeq, ONT and PacBio sequencing (Supplement S7) varied according to the expectations for the outcome of short- and long-read sequencing. Overall, all sequencing data met the recommended requirements for accuracy, coverage, read length and the range of read counts (data not shown) for Illumina, Oxford Nanopore and Pacific Biosciences, respectively. This suggests the use of high-quality sequencing runs for bioinformatics analyses. In addition to the recommended quality parameter, the use of specific extraction methods can influence results obtained by whole-genome sequencing. However, the impact of different extraction systems seemed to have a lesser influence on the outcome of WGS [50].

We analyzed how the different sequencing and assembly approaches per sample affect the outcome of in silico typing for the number and size of predicted plasmids and the AMR associated with them. First, the different approaches were assessed for their suitability for bacterial chromosome finishing (Table 2). By using NextSeq data, none of the chromosomes of any isolate could be finished. In contrast, ONT data alone or in combination with NextSeq sequences allowed closing of the longest contig of every sample. Chromosome finishing using PacBio sequences failed for two of the five samples. However, the Unicycler-PacBio/-NextSeq hybrid assembly resulted in closed chromosomes for four isolates. Although long-read assembly (Flye) approaches frequently generated finished chromosomes, the result of the hybrid approach led to slightly longer closed chromosomal contigs with a higher accuracy, as wrongly predicted deletions were corrected. This leads to

the presumption that the addition of the short-reads in the hybrid approach can replenish the chromosome with data otherwise missed in the long-read-only approach, although stated as already closed. Thus, our data are in good agreement with prevailing reports indicating that WGS approaches aiming for a full reconstruction of all genomic elements of an isolate will benefit from long-read or hybrid sequencing data [8,51,52].

Table 1. Overview on basic information of the *E. coli* isolates, including their antimicrobial resistance profile and the size of extrachromosomal elements.

Isolate ID	Matrix	Date of Isolation	AMR Profile	Sizes of Identified Plasmids [+]
17-AB00050	cecum, broiler	22 November 2016	AMP, CEF, CIP, FOT, GEN, SMX, TAZ	174 kb; 100 kb; 90 kb; 45 kb *; <20.5 kb
17-AB00090	feces, turkey	14 December 2016	AMP, CIP, NAL, TET	100 kb *; 65 kb; 53 kb; <20.5 kb
17-AB00432	cecum, calf	21 February 2017	AMP, CEF, CIP, FOT, NAL, TAZ, TET, TMP, SMX	95 kb *
17-AB00587	meat, bovine	23 March 2017	AMP, CEF, CIP, FOT, TAZ	115 kb; 100 kb *; 30 kb
17-AB00639	cecum, pig	24 April 2017	AMP, CEF, CIP, FOT, GEN, SMX, TAZ, TMP	140 kb; 100 kb; 95 kb; 45 kb *; <20.5 kb

Abbreviations: AMP: ampicillin, AZI: azithromycin, CEF: cefepime, FOT: cefotaxime, FOX: cefoxitin, TAZ: ceftazidime, CHL: chloramphenicol, CIP: ciprofloxacin, COL: colistin, GEN: gentamicin, NAL: nalidixic acid, SMX: sulfamethoxazole, TET: tetracycline, TGC: tigecycline, TMP: trimethoprim; *: plasmid carrying *qnrS*, identified by S1-PFGE analysis; [+]: data was obtained from S1-PFGE analysis and DNA-DNA hybridization.

While short-read sequencing applications yielded a high sequence accuracy, the technology is known to be insufficient for closing whole genome structures. Reliable estimation of MGEs within an organism might be challenging without additional information [14,53–56]. In contrast, long-read sequencing applications are more reliable in detection and closing of, e.g., extrachromosomal elements, but are assumed to be error prone for the prediction of specific genes under some circumstances [34,57,58].

3.2. Small Plasmids Are Difficult to Detect

To get an overview on transmissible extrachromosomal elements, the number and size of plasmids detected by S1-PFGE were compared to circularized contigs per sample identified in silico (Table 1, Figure 1, Supplement S3, Supplement S4). As S1-PFGE is unsuitable for reliable size estimation of small plasmids (<20 kb), agarose gel electrophoresis of plasmid DNA was conducted for confirming their presence (data not shown).

For isolate 17-AB00050, all assembly methods detected a 6.7 kb plasmid, which was assigned to the Col156 Inc-group. The MinION long-read-only approach (Flye-ONT) generated a genome of 13 kb, which is represented by a duplication of the 6.7 kb Col156 plasmid. Except for the Col156 plasmid, short-read (NextSeq) sequencing was insufficient to yield any further closed plasmid genomes for this isolate. All long-read-only (Flye-ONT, Flye-PacBio) and hybrid approaches (Unicycler-PacBio/Nextseq, Unicycler ONT/NextSeq) correctly recognized the 46 kb IncX3 plasmid. The Flye assembler generated a 62 kb plasmid that could be linked to the p0111 Inc-group. However, no other assembly method identified this plasmid, and the S1-PFGE also showed no evidence for its biological presence (Supplement S3). Both hybrid approaches, as well as Flye-assembled ONT and PacBio sequences, resulted in a 93 kb plasmid. However, no method was able to link this plasmid to a known Inc group. Only Flye-ONT and Flye-PacBio as well as the Unicycler-PacBio hybrid assembly led to the detection of the ~103 kb IncFIB plasmid (Figure 1). Finally, no in silico based prediction was able to detect the 174 kb plasmid, recognized by the S1-PFGE analysis of this isolate (Table 1, Supplement S3). For *E. coli* 17-AB00090, all long-read and hybrid assembly approaches reliably detected a 50 kb IncX1, a 71 kb IncFII and a 107 kb IncI-α plasmid. Furthermore, both hybrid assembly approaches recognized the same small plasmids (a 1551 bp Col(MG828), a 4018 bp ColRNAI and a 5873 bp ColRNAI plasmid) as the Unicycler-NextSeq assembly. Both Flye-ONT and Flye-PacBio resulted in double-sized plasmid genomes of 8 and 11 kb, where the complete sequence and the Inc ColRNAI sequence were duplicated.

Table 2. Characteristics of contigs detected with different sequencing and assembly strategies in five *E. coli* isolates. Circularized contigs are presented in bold.

	17-AB00050			17-AB00090			17-AB00432			17-AB00587			17-AB00639		
	Number of Contigs (Plasmidal Content Relative to Total Contig Length)	Number of Circular Contigs/All Contigs < 10 kb	Longest Contig [bp]	Number of Contigs (Plasmidal Content Relative to Total Contig Length)	Number of Circular Contigs/Contigs < 10 kb	Longest Contig [bp]	Number of Contigs (Plasmidal Content Relative to Total Contig Length)	Number of Circular Contigs/Contigs < 10 kb	Longest Contig [bp]	Number of Contigs (Plasmidal Content Relative to Total Contig Length)	Number of Circular Contigs/Contigs < 10 kb	Longest Contig [bp]	Number of Contigs (Plasmidal Content Relative to Total Contig Length)	Number of Circular Contigs/Contigs < 10 kb	Longest Contig [bp]
Unicycler-NextSeq	466 (5.5%)	2/2	341,211	186 (4.3%)	3/3	502,527	159 (1.6%)	0/0	314,433	201 (4.2%)	0/0	485,939	183 (7.6%)	2/2	340,946
Flye-PacBio	13 (4.6%)	4/1	4094,393	6 (4.6%)	6/2	**5004,742**	2 (2.1%)	2/0	**4,736,227**	3 (4.4%)	3/0	**4,938,765**	7 (7.9%)	4/1	3,693,252
Flye-ONT	8 (5.3%)	7/0	**5,524,427**	5 (4.6%)	5/0	**4,970,722**	3 (2.7%)	3/0	**4,728,917**	3 (4.4%)	3/0	**4,931,189**	7 (8.3%)	7/4	**4,756,147**
Unicycler-PacBio/NextSeq	27 (4.2%)	6/2	4,341,057	7 (4.6%)	7/3	**5,004,751**	3 (2.4%)	3/0	**4,736,229**	3 (4.4%)	3/0	**4,938,758**	13 (8.1%)	7/2	**4,763,387**
Unicycler-ONT/NextSeq	22 (4.0%)	6/2	**5,533,851**	7 (4.6%)	7/3	**5,004,751**	3 (2.4%)	3/0	**4,736,229**	3 (4.4%)	3/0	**4,938,758**	9 (8.2%)	9/2	**4,763,387**

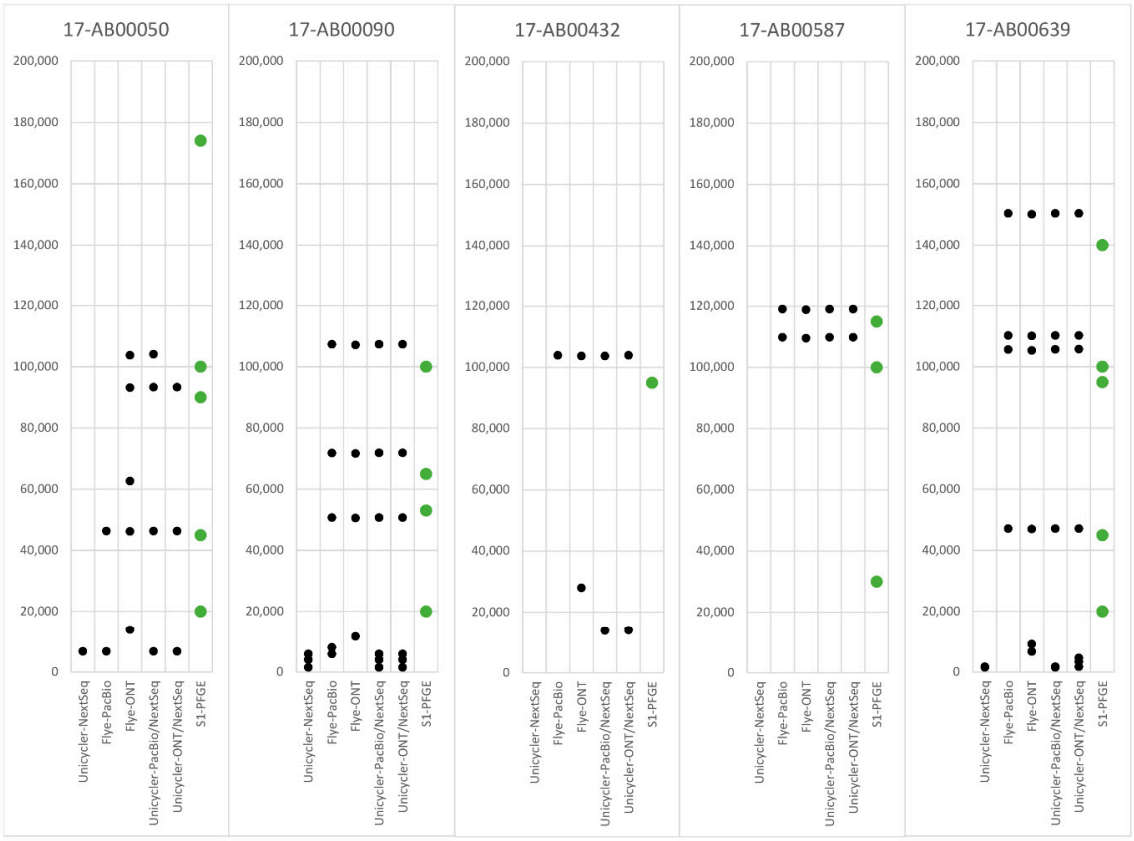

Figure 1. Distribution of plasmid size, detected with different sequencing and assembly approaches. A black dot represents a determined closed plasmid at the respective size for the given method. A green dot represents the size of the plasmid, detected in the laboratory with S1-PFGE.

All long-read and hybrid assembly approaches recognized the 103 kb IncY plasmid within the strain 17-AB00432. Furthermore, the hybrid approaches as well as Flye assembled ONT and PacBio sequences recognized another 13 kb IncR plasmid, which was not observed by S1-PFGE (Supplement S3). However, Flye again generated a doubled sized plasmid with a full duplication of the sequence, including the IncR marker. For *E. coli* 17-AB00587, all Flye and hybrid assembly approaches were able to recognize a 109 kb IncI-α and a 119 kb IncFIB plasmid. However, no assembly approach detected the 30 kb plasmid, which was reliably detectable by S1-PFGE (Supplement S3).

For *E. coli* 17-AB00639, all Flye and hybrid assemblies identified a 150 kb IncFII plasmid and a 105 kb IncI-α plasmid. In these assemblies, a 110 kb plasmid was further detected but could not be linked to a known incompatibility group. A 47 kb plasmid was also detected by these assembly methods. However, both hybrid assemblies assigned an IncX1, while both Flye approaches further assigned an IncX3 marker to this sequence. The NextSeq assembly and the hybrid assemblies detected a 1552 bp Col(MG828) and a 1748 bp ColpVC plasmid. Furthermore, the Unicycler-ONT assembly yielded a 3374 bp plasmid, which could not be linked to any known Inc group, and a 4593 bp ColRNAI plasmid. These plasmids were found in duplicated size for the Flye output. Overall, all plasmids of this isolate could be reliably detected by S1-PFGE (Supplement S3), while the sizes between in vitro and in silico investigations differed (Figure 1).

Overall, the NextSeq approach resulted in the highest discrepancy for plasmid prediction. Although it always detected circularized plasmids below 10 kb, when they were detected by gel electrophoresis, the NextSeq assembly did not result in closing of any larger

plasmid. However, the detection of the Inc group with NextSeq sequencing was reliable and is therefore useful as a reference. In summary, the hybrid assembly with Unicycler was assessed as the most reliable approach to detect the number of extrachromosomal DNA correctly, linking them to a certain Inc group and closing these elements. The hybrid approach combines the benefits of short- and long-read sequencing for the detection of small plasmids and the genome finishing of large plasmids and chromosomes. Furthermore, Flye-ONT and Flye-PacBio assemblies sometimes resulted in the detection of plasmids that were not detectable by PFGE. In addition, duplicated Inc sequences or duplication of the complete plasmid sequence was observed, potentially leading to misinterpretations. It is known that long-read assembly can exclude short extrachromosomal DNA elements [34,59] due to size-selection or bead clean-up steps. This can lead to an exclusion of small plasmids, e.g., harbouring resistance and virulence genes [10], which might affect the assessment of the isolate. As plasmids are important vectors for transmitting resistance determinants, the correct determination of their presence is important [60–62]. It is of high importance for a correct risk assessment to recognize whether a gene is located on a MGE or fixed on the chromosome [63,64]. Based on our data, we propose the use of long-read sequencing for chromosome finishing; the use of PacBio or ONT did not affect the outcome. However, the data needs to be handled with care during estimation of the exact number of chromosomal elements in a sample. Hybrid assembly represents the most useful and powerful tool for plasmid counting as well as for reliable size and Inc-group prediction of smaller and larger plasmids. Besides methodological influences, the use of specific algorithms and pipelines also influences the detection of plasmid associated sequences [65]. While some of these are based only on the detection of plasmid replicon sequences [42], others use similarity and identity values for experimentally confirmed plasmid databases.

3.3. Hybrid Assembly Allows a Deep Insight into the Plasmid Structure

To determine the diversity of AMR-carrying plasmids and to understand the impact of resistance determinants and other plasmidal features (e.g., transposon sequences, transfer genes) for the spread of the genes, a deep knowledge of the composition of resistance plasmids is needed. With a focus on (fluoro)quinolone resistance, we aimed to dissect the *qnrS1*-carrying plasmids of the individual *E. coli* of livestock and food (Figure 2).

The *qnrS1* gene was detected with a 100% sequence identity in all isolates. However, for NextSeq sequences, the linkage of *qnrS1* to a plasmid incompatibility marker was only possible for 17-AB00050. In contrast, all datasets based on assemblies using long reads successfully led to a prediction of an incompatibility marker for *qnrS1*-carrying plasmids (Table 3). Furthermore, the use of NextSeq assemblies provided no evidence for the linkage of any further resistance gene to the *qnrS1*-carrying plasmid.

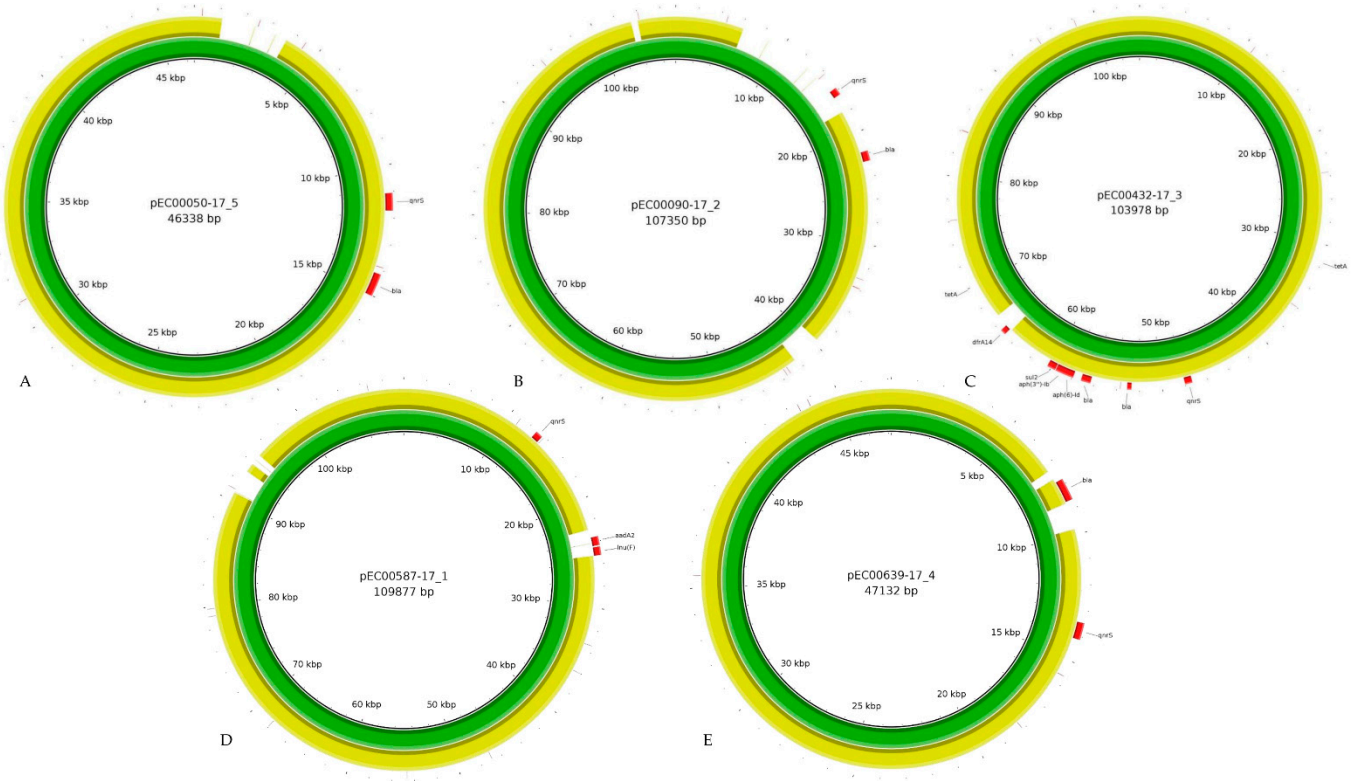

Figure 2. Plasmid composition of extrachromosomal elements carrying the *qnrS1* gene. (**A**): 17-AB00050 (**B**): 17-AB00090 (**C**): 17-AB00432 (**D**): 17-AB00587 (**E**): 17-AB00639. Illustrations were generated from hybrid assembly with BRIG (v0.95). Red elements represent the resistance genes. Green colored rings represent the isolates plasmid. Yellow colored rings represent the reference plasmid (annotation of plasmids are available in Supplement S6).

The *qnrS1*-carrying plasmid pEC00050-17_5 (isolate 17-AB00050) was 46.3 kb in size and belonged to the IncX3 group. Besides *qnrS1*, the plasmid also carried the Extended Spectrum β-Lactam (ESBL) resistance $bla_{\text{SHV-12}}$, which was flanked by two IS*6* elements, while the *qnrS1* gene was associated with an IS*Kra4* element. Overall, pEC00050-17_5 was closely related (query coverage: 94%, identity: 99.95%) to the *Citrobacter freundii* plasmid pCF12 (accession number: MT441556.1). In contrast to pCF12, pEC00050-17_5 additionally carried a 3 kb sequence encoding the IS*Sso4* insertion sequence of the IS*21* family. When investigated with the in silico mob-suite tool, pEC00050-17_5 was predicted to be conjugative, as it identified the MOB_{P} relaxase type and the MPF_{T} type. Despite the in silico prediction, the plasmid was not transmitted by in vitro filter mating studies in *E. coli* J53.

The 107 kb *qnrS1*-carrying plasmid pEC00090-17_2 (*E. coli* 17-AB00090) was found to be related to the *Salmonella enterica* plasmid pCE-R2-11-0435_92 (query coverage: 86%, identity: 99.42%) (accession number: CP016520.1) recovered from retail chicken in Canada. pEC00090-17_2 belongs to the IncI-α group and carried a $bla_{\text{TEM-1}}$ β-lactam resistance gene in close proximity to *qnrS1*. The transmissibility of the plasmid was shown by the in silico mob-suite tool as well as by in vitro filter mating studies. Comparative sequence analysis revealed that pEC00090-17_2 carried additional sequences (~15 kb) encoding transposases, DNA invertases, hypothetical proteins and *qnrS1*, which were absent in pCE-R2-11-0435_92. The presence of *qnrS1* on pEC00090-17_2 indicates an evolution step due to the acquisition of additional resistance markers. The new region included the IS*26* insertion sequence and the cn_6346_IS26 and cn_6346_IS26 composite transposon, all from the IS*6* family, as well as the transposon Tn2.

Table 3. Characteristics of contigs detected with different sequencing and assembly strategies in five *E. coli* isolates harboring the *qnrS1* gene. Circularized contigs are presented in bold.

	17-AB00050			**17-AB00090**			**17-AB00432**			**17-AB00587**			**17-AB00639**		
	Contig Size [bp]	**Plasmid Marker**	**Other AMR Genes**	**Contig Size [bp]**	**lPlasmid Marker**	**Other AMR Genes**	**Contig Size [bp]**	**Plasmid Marker**	**Other AMR Genes**	**Contig Size [bp]**	**Plasmid Marker**	**Other AMR Genes**	**Contig Size [bp]**	**Plasmid Marker**	**Other AMR Genes**
Unicycler-NextSeq	42,601	IncX3	-	5348	-	-	13,373	-	-	1762	-	-	8821	-	-
Flye-PacBio	**46,338**	IncX3	bla_{SHV-12}	**107,341**	IncI1_1_α	bla_{TEM-1}	**103,789**	IncY	*tet*(A), bla_{CTX_M-15}, bla_{TEM-1}, *aph*(3″)-ib, *sul*2, *dfr*A14, *tet*(A), *aph*(6)-Id	**109,877**	IncI1_1α	*aad*A2, *lnu*(F)	**47,133**	IncX1, IncX3	bla_{TEM-1}
Flye-ONT	**46,207**	IncX3	bla_{SHV-12}	**107,104**	IncI1_1_α	bla_{TEM-1}	**103,779**	IncY	*tet*(A), bla_{CTX_M-15}, bla_{TEM-1}, *aph*(3″)-ib, *sul*2, *dfr*A14, *tet*(A), *aph*(6)-Id	**118,872**	IncI1_1α	*aad*A2, *lnu*(F)	**46,996**	IncX1, IncX3	bla_{TEM-1}
Unicycler-PacBio/NextSeq	**46,338**	IncX3	bla_{SHV-12}	**107,350**	IncI1_1_α	bla_{TEM-1}	**103,978**	IncY	*tet*(A), bla_{CTX_M-15}, bla_{TEM-1}, *aph*(3″)-ib, *sul*2, *dfr*A14, *tet*(A)	**109,877**	IncI1_1α	*aad*A2, *lnu*(F)	**47,132**	IncX1	bla_{TEM-1}
Unicycler-ONT/NextSeq	**46,338**	IncX3	bla_{SHV-12}	**107,350**	IncI1_1_ α	bla_{TEM-1}	**103,975**	IncY	*tet*(A), bla_{CTX_M-15}, bla_{TEM-1}, *aph*(3″)-ib, *sul*2, *dfr*A14, *tet*(A), *aph*(6)-Id	**109,876**	IncI1_1α	*aad*A2, *lnu*(F)	**47,132**	IncX1	bla_{TEM-1}
PFGE determined size [bp]		45,000			100,000			95,000			100,000			45,000	
in vitro conjugational transfer		no			yes			no			yes			yes	

Abbreviation: AMR, antimicrobial resistance.

The *qnrS1*-carrying plasmid pEC00431-17_3 (*E. coli* 17-AB00432) exhibited an IncY incompatibility sequence and closely resembled (query coverage: 98%, identity: 99.98%) the *E. coli* plasmid tig00003056 (accession number: CP021681.1). pEC00431-17_3 exhibited a size of 103 kb and carried several resistance genes, including *tet*(A), *qnrS1*, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, *aph(6)-Id*, *aph(3")-Ib*, *sul*2 and *dfrA2*, and might thus pose a risk in spreading multiple resistance genes. $bla_{\text{CTX-M-15}}$ and *qnrS1* are in proximity to each other, located on an IS*Kpn19* insertion sequence of the IS*Kra4* family. As shown in Figure 2 (Supplement S6), pEC00431-17_3 differed from tig00003056 in the acquisition of an integrase as well as the trimethoprim resistance-mediating dihydrofolate reductase gene *dfrA14*, located on the cn_3458_IS26 composite transposon, belonging to the IS*6* family.

Bioinformatically, the 109 kb *qnrS1*-carrying plasmid pEC00587-17_1 (*E. coli* 17-AB00-587) was assigned to the IncI-1-α group. Besides *qnrS1*, the plasmid exhibited *aadA2*, an *lnu*(F) resistance gene. Similar to pEQ2, *qnrS1* of pEC00587-17_1 was found to be located on the cn_4905_ISKpn19 composite transposon from the IS*Kra4* family. The closest relative of pEC00587-17_1 was pEQ2 (query coverage: 94%, identity: 100%), from an *E. coli* isolate recovered from pig feces in the UK. Hence, 4 kb were additionally located on pEC00587-17_1, which are represented by genes encoding hypothetical proteins and the resistance genes *aadA2* and *lnu*(F), located on the cn_4072_IS26 composite transposon of the IS*6* family. Plasmid pEQ2 was described as a fusion of pEQ1 and a *qnrS1*-carrying IncX1 plasmid, encoding replication, maintenance, and conjugative transfer [66]. Here, we noted another adaption by the acquisition of additional resistance genes. pEC00587-17_1 was shown to be transmissible by both in silico prediction using mob-suite and filter mating studies.

The 47 kb *qnrS1*-carrying IncX plasmid pEC00639-17_4 (isolate 17-AB00639) resembled the similar sized *E. coli* plasmid pNVI2422 (query coverage: 96%, identity: 99.99%), recovered from turkey meat in Norway. Nucleotide differences between the plasmids are based on the presence and absence of gene coding for hypothetical proteins. Besides *qnrS1*, pEC00639-17_4 also carried the β-lactam resistance $bla_{\text{TEM-1}}$, which was flanked by an IS*26* insertion sequence belonging to the IS6 family. The adjacent *qnrS1* gene was located near the IS*Kpn19* insertion sequence belonging to the IS*Kra4* family. The in silico predicted plasmid transmissibility could be experimentally confirmed.

Except for the NextSeq approach, all other assemblies resulted in the same plasmid genome prediction for all five strains. In general, any linkage between *qnrS1*, the plasmid type and its associated characteristics (e.g., broad/narrow host plasmid type, mobilization, etc.) could only be made by using Flye-ONT or Flye-PacBio. Thus, we were able to detect certain insertion elements leading to the acquisition of, e.g., resistance determinants as well as other components. We were able to detect the *qnrS* element frequently in proximity to certain *bla* genes, as described previously [67]. Furthermore, we linked the presence of *qnrS* to the presence of the IS*26* and IS*Kra4* family. These elements had been recognized before as important for the transmission of *qnr* genes [68]. However, these important observations regarding the plasmid structure and *qnrS* characteristic are not possible using NextSeq sequencing alone.

3.4. Common Mistakes in Resistance Gene Detection and Phenotype Evaluation

For a reliable assessment of a resistance transfer probability within and beyond species, the understanding of the genetic determinant and the surrounding environment is essential. Thus, the *E. coli* isolates were screened for the presence of specific antimicrobial resistance genes (Table 4) from data derived in silico and compared to respective MIC data. Therewith, different sequencing and assembly strategies resulted in wrong prediction due to a disparity in gene assignment as well as a lack of information about duplicated genes. For isolate 17-AB00090, the Flye-ONT assembly resulted in detection of the β-lactamase gene $bla_{\text{TEM-135}}$. Other assembly strategies revealed $bla_{\text{TEM-1}}$ instead of $bla_{\text{TEM-135}}$ at the same position. As both genes exhibit 99.8% nucleotide identity, misinterpretation will affect only the prediction of the gene variant. However, for other resistance genes, the prediction of the wrong variant could alter the in silico prediction of the phenotype. Thus, mistakes of this

kind could lead to wrong conclusions [69]. One major drawback of using WGS-based antimicrobial resistance prediction is that only known genes associated with resistance development can be reliably interpreted. However, some machine-learning algorithms for reliable prediction of novel antimicrobial resistance determinants have been developed and successively optimized [70,71].

Table 4. Antimicrobial resistance phenotype and resistance determinants predicted with various assembly and sequencing techniques in five *E. coli* isolates.

17-AB00050				
Class of In Silico Type Phenotype	**Subclass of In Silico Type Phenotype**	**Determined Resistance Gene(s)**	**Determined Phenotype**	**Class/Subclass of Determined Phenotype**
Quinolone	Quinolone	*qnrS1*	Ciprofloxacin	Fluoroquinolone
β-Lactam	Penicillin, Cephalosporin	$bla_{\text{EC-8}}$, $bla_{\text{SHV-12}}$	Ampicillin	β-Lactam
			Cefepime	Cephalosporin
			Cefotaxime	Cephalosporin
Aminoglycoside	Gentamicin	*aac(3)-VIa*	Gentamicin	Aminoglycoside
Sulfonamide	Sulfonamid	*sul1*	Sulphamethoxazole	Sulfonamide
Aminoglycoside	Streptomycin	*aadA1*	Not within the test panel	Not within the test panel
17-AB00090				
Class of In Silico Type Phenotype	**Subclass of In Silico Type Phenotype**	**Determined Resistance Gene(s)**	**Determined Phenotype**	**Class/Subclass of Determined Phenotype**
β-Lactam	Penicillin	$bla_{\text{TEM-1}}$, $bla_{\text{TEM-1}}$*[1], $bla_{\text{TEM-135}}$*[2]	Ampicillin	β-Lactam
β-Lactam	Penicillin, Cephalosporine	$bla_{\text{EC-18}}$		
Quinolone	Quinolone	*qnrS1*	Ciprofloxacin	Fluoroquinolone
			Nalidixic acid	Quinolone
Tetracycline	Tetracycline	*tet*(A)	Tetracycline	Tetracycline
17-AB00432				
Class of In Silico Type Phenotype	**Subclass of In Silico Type Phenotype**	**Determined Resistance Gene(s)**	**Determined Phenotype**	**Class/Subclass of Determined Phenotype**
β-Lactam	Penicillin	$bla_{\text{TEM-1}}$	Ampicillin	β-Lactam
β-Lactam	Penicillin, Cephalosporin	$bla_{\text{CTX-M-15}}$, bla_{EC}	Cefepime	Cephalosporin
			Cefotaxime	Cephalosporin
			Ceftazidime	Cephalosporin
Aminoglycoside	Kanamycin	*aph(3′)-Ia*	Not within the test panel	Not within the test panel
Quinolone	Quinolone	*qnrS1*	Ciprofloxacin	Fluoroquinolone
			Nalidixic acid	Quinolone
Aminoglycoside	Streptomycin	*aph(3″)-Ib*, *aph(3″)-Ib**	Not within the test panel	Not within the test panel
		aph(3′)-Ia,		
		aph(6)-Id, *aph(6)-Id**		
Sulfonamide	Sulfonamide	*sul*2, *sul*2*	Sulphamethoxazole	Sulfonamide
Tetracycline	Tetracycline	*tet*(A), *tet*(A)*[3], *tet*(B)	Tetracycline	Tetracycline

Table 4. *Cont.*

17-AB00587				
Class of In Silico Type Phenotype	**Subclass of In Silico Type Phenotype**	**Determined Resistance Gene**	**Determined Phenotype**	**Class/Subclass of Determined Phenotype**
β-Lactam	Cephalosporin	$bla_{\text{CTX-M-1}}$, $bla_{\text{EC-15}}$	Cefepime	Cephalosporin
			Cefotaxime	Cephalosporin
			Ceftazidime	Cephalosporin
Lincosamide	Lincosamide	*lnu*(F)	Not within the test panel	Not within the test panel
Macrolide	Macrolide	*mph*(A)		
Quinolone	Fluoroquinolone	*qnrS1*	Ciprofloxacin	Fluoroquinolone
Aminoglycoside	Streptomycin	*aadA*2	Not within the test panel	Not within the test panel
17-AB00639				
Class of In Silico Type Phenotype	**Subclass of In Silico Type Phenotype**	**Determined Resistance Gene(s)**	**Determined Phenotype**	**Class/Subclass of Determined Phenotype**
β-Lactam	Penicillin	$bla_{\text{TEM-1}}$	Ampicillin	β-Lactam
β-Lactam	Penicillin, Cephalosporin[*3]	$bla_{\text{CTX-M-1}}$[*4], $bla_{\text{EC,}}$	Cefepime	Cephalosporin
		$bla_{\text{TEM-1}}$[*4], $bla_{\text{TEM-1}}$	Cefotaxime	Cephalosporine
			Ceftazidime	Cephalosporine
Aminoglycoside	Gentamicin	*aac*(3)-*Iva*	Gentamicin	Aminoglycoside
Aminoglycoside	Hygromicin	*aph*(4)-*Ia*	Not within the test panel	Not within the test panel
Macrolide	Macrolide	*mph*(A)		
Quinolone	Fluoroquinolone	*qnrS1*	Ciprofloxacin	Fluoroquinolon
Aminoglycoside	Streptomycin	*aph*(3″)-*Ib*, *aph*(3″)-*Ib*[*4],	Not within the test panel	Not within the test panel
		aph(6)-Id[*5]		
Sulfonamide	Sulfonamide	*sul2*	Sulphamethoxazole	Sulfonamide
Diaminopyrimidine	Trimethoprim	*dfrA5*	Trimethoprim	Diaminopyrimidine

[*1]: duplication detected in all long-read and hybrid assemblies but not in NextSeq assemblies. [*2]: gene only determined with data Flye-ONT assemblies. [*3]: duplication detected in all long-read and hybrid assemblies but not in short-read-only assemblies. [*4]: detected in all long-read and hybrid assemblies but not in NextSeq assemblies. [*5]: not detected in short-read-only assemblies and Flye-ONT sequences.

NextSeq sequencing detected all occurring resistance genes only once for every sample. In contrast, all long-read and hybrid assembly approaches resulted in multiple duplications of certain resistance genes (Table 4), which does not necessarily influence the in silico based prediction of the resistance phenotype, but leads to a different organization of the affected plasmids. Further dissection using NextSeq data showed a slightly higher sequencing depth of the respective resistance gene regions, indicating that a duplication of the genes might exist.

3.5. Evaluation of the Phenotype

We further assessed the reliability of the different sequencing and assembly approaches for accurate resistance phenotype prediction. Antimicrobial susceptibility testing (AST) of the individual isolates was conducted in triplicate (Supplement S5) and compared to the respective in silico outcome.

Obviously, the failure of detecting certain resistance genes can lead to discordance in estimating the right phenotype (Table 4). Sequencing of strain 17-AB00090 resulted in the detection of the $bla_{\text{EC-18}}$ gene (Accession: A0A244BQ89), which led to the prediction of cephalosporin resistance. However, AST provided no evidence for a non-wildtype phenotype to cephalosporins. We made similar observations for the predicted macrolide resistance phenotype of 17-AB00587 and 17-AB000639, based on the presence of *mph*(A). As most *E. coli* isolates are intrinsically resistant to macrolides, a change in the macrolide

resistance phenotype will not be detectable, regardless of the presence or absence of *mph*(A). This incorrect classification underlines the current difficulties of extrapolating from WGS data to resistance phenotypes [72,73].

Here, all sequence approaches resulted in congruent estimation of resistance genes. Besides the quality of the sequence data used, there are also other reasons that led to differences in the results when genotypes and phenotypes were compared. First, the observed phenotype is rarely traceable to only one single resistance gene. Most often, co-occurrence of different resistance genes can account for the same resistance property, or resistance phenotypes may result from complex gene networks that cannot be determined by occurrence of single genes [73]. Furthermore, some resistance genes do not confer resistance, but only slightly increase the MIC value for the respective antimicrobial agent [74,75]. Genotypic approaches can misinterpret gene silencing or generally only determine known resistance genes. This means that resistant isolates carrying a novel resistance gene or a mutation can be incorrectly classified as susceptible. Thus, in silico phenotype estimation remains a complex task, only solvable in mutual approaches of bioinformatics and laboratory work [9]. All currently used WGS-based methods are generally appropriate for reliable antimicrobial resistance prediction. Nevertheless, the use of long-read data alone can lead to a wrong prediction of individual chromosomal alterations involved in the development of antimicrobial resistances, i.e., to quinolones or rifampicin [73]. However, further sequencing quality parameters as well as the used of harmonized antimicrobial resistance databases will improve their comparability.

4. Conclusions

Long-read sequencing is an essential approach for reliable genome finishing. However, long-read assembly alone can lead to wrong annotations as well as to a loss of small plasmid genomes. Although long-read approaches are beneficial for building the scaffold of a genome, they do not fulfill all requirements for a thorough assessment, as information can be missed or errors can be incorporated. Despite short-read sequencing being currently the most popular way to investigate the genetic background, it is insufficient for certain purposes. In particular, when detecting and characterizing extrachromosomal plasmids, short reads alone did not allow the linkage to a plasmid marker as well as closing of the respective contigs. This sequencing even missed duplications of certain resistance genes. This makes a correct plasmid profiling, to be included in the assessment of antibiotic resistance dissemination, rather difficult. While short-read sequencing and assembling is reliable to some extent in gene detection and resistance phenotype estimation, it remains insufficient for drawing complex conclusions. Short- and long-read approaches both have pros and cons, depending on the purpose of use. However, when the aim was to investigate extrachromosomal structures like plasmids, hybrid assembly led to the most comprehensive results, as it led to more appropriate resistance gene and phenotype detection. In addition, it combined the information of large contigs and the information of smaller reads missed in the long-read-only assembly. However, the source of the long-read sequences, whether from PacBio or ONT, did not result in an extensive difference for the detection and characterization of extrachromosomal DNA.

Overall, we consider a hybrid assembly as a necessary approach for a detailed strain characterization, since it benefits from a thorough overview of various sized extrachromosomal DNA and correct resistance gene estimation. Overall, it will be worth extending the routine sequence diagnostic from short-read sequencing to additional long-read sequencing for a hybrid assembly approach, when a reference-grade complete bacterial genome is desired, or extrachromosomal structures need to be fully understood.

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Author Contributions: K.J., J.A.H., B.M. conceived the study and designed the experiments. K.J. and M.B. performed long-read whole-genome sequencing. S.O. supported the development of the DNA extraction method for long-read sequencing. K.J., C.D. and S.H.T. performed bioinformatics analyses. K.J., C.D., S.H.T., S.S. and J.A.H. interpreted the data. J.A.H. and C.D. supervised the project. K.J. provided the draft manuscript. J.A.H., B.M., C.D., S.H.T., M.B., S.O., D.M., A.K. and S.S. decisively contributed in editing the manuscript. All authors were involved in project discussion and approved the final manuscript.

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2.2 Publication II

Juraschek K, Deneke C, Schmoger S, Grobbel M, Malorny B, Käsbohrer A, Schwarz S, Meemken D, Hammerl JA.

Phenotypic and Genotypic Properties of Fluoroquinolone-Resistant, *qnr*-Carrying *Escherichia coli* Isolated from the German Food Chain in 2017

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1.	Design of the project including design of individual experiments	80%
2.	Performing the experimental part of the study	75%
3.	Analysis of the experiments	90%
4.	Presentation and discussion of the study in article form	85%

microorganisms

MDPI

Article

Phenotypic and Genotypic Properties of Fluoroquinolone-Resistant, *qnr*-Carrying *Escherichia coli* Isolated from the German Food Chain in 2017

Katharina Juraschek [1,*], Carlus Deneke [1], Silvia Schmoger [1], Mirjam Grobbel [1], Burkhard Malorny [1], Annemarie Käsbohrer [1,2], Stefan Schwarz [3], Diana Meemken [4] and Jens Andre Hammerl [1,*]

1 German Federal Institute for Risk Assessment (BfR), Max-Dohrn Str. 8-10, 10589 Berlin, Germany; Carlus.Deneke@bfr.bund.de (C.D.); silvia.schmoger@bfr.bund.de (S.S.); mirjam.grobbel@bfr.bund.de (M.G.); Burkhard.Malorny@bfr.bund.de (B.M.); annemarie.kaesbohrer@bfr.bund.de (A.K.)
2 Unit for Veterinary Public Health and Epidemiology, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria
3 Department of Veterinary Medicine, Institute of Microbiology and Epizootics, Freie Universität Berlin, 14163 Berlin, Germany; Stefan.Schwarz@fu-berlin.de
4 Department of Veterinary Medicine, Institute of Food Safety and Food Hygiene, Freie Universität Berlin, 14163 Berlin, Germany; diana.meemken@fu-berlin.de
* Correspondence: Katharina.Juraschek@bfr.bund.de (K.J.); Jens-Andre.Hammerl@bfr.bund.de (J.A.H.)

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Abstract: Fluoroquinolones are the highest priority, critically important antimicrobial agents. Resistance development can occur via different mechanisms, with plasmid-mediated quinolone resistance (PMQR) being prevalent in the livestock and food area. Especially, *qnr* genes, commonly located on mobile genetic elements, are major drivers for the spread of resistance determinants against fluoroquinolones. We investigated the prevalence and characteristics of *qnr*-positive *Escherichia (E.) coli* obtained from different monitoring programs in Germany in 2017. Furthermore, we aimed to evaluate commonalities of *qnr*-carrying plasmids in *E. coli*. We found *qnr* to be broadly spread over different livestock and food matrices, and to be present in various sequence types. The *qnr*-positive isolates were predominantly detected within selectively isolated ESBL (extended spectrum beta-lactamase)-producing *E. coli*, leading to a frequent association with other resistance genes, especially cephalosporin determinants. Furthermore, we found that *qnr* correlates with the presence of genes involved in resistance development against quaternary ammonium compounds (*qac*). The detection of additional point mutations in many isolates within the chromosomal QRDR region led to even higher MIC values against fluoroquinolones for the investigated *E. coli*. All of these attributes should be carefully taken into account in the risk assessment of *qnr*-carrying *E. coli* from livestock and food.

Keywords: *E. coli*; typing; genomes; plasmid; livestock; food; fluoroquinolones

1. Introduction

Antimicrobial resistance (AMR), especially against the highest priority, critically important substances (e.g., quinolones and fluoroquinolones) is a global threat for humans and animals. Food-producing animals are considered an important reservoir of AMR-carrying bacteria [1,2]. Therefore, annual monitoring programs in the EU are conducted to observe trends in the development and dynamics of resistances in specific target animals. Commensal *Escherichia* (*E.*) *coli* serves as an indicator bacterium among *Enterobacteriaceae* for estimating changes in the prevalence of resistance genes in food and livestock in European countries.

Quinolones and fluoroquinolones, further named (fluoro)quinolones, are antimicrobial agents, considered as clinically highly important substances [3], and are used for the treatment of animal infections and human diseases in Europe. In the last years, EFSA notified a steadily increasing trend in (fluoro)quinolone-resistant bacteria, isolated from

food-producing animals. Therewith, a high proportion of *Salmonella enterica* and *E. coli*, mainly isolated from poultry, were classified as not susceptible against ciprofloxacin. Next to the livestock sector, the human sector also registered an increase in ciprofloxacin resistance from 1.7% (in 2016) to 4.6% (in 2018) in certain *Enterobacteriaceae* [4]. The prevailing trend in European countries indicates a spread of (fluoro)quinolone-resistant bacteria, which poses a threat to animal and human health.

Resistance development against (fluoro)quinolones can occur via various mechanisms ranging from alterations of chromosomal genes to the acquisition of specific transferable genes. Mutations in the chromosomal elements encoding the target enzyme DNA gyrase (*gyrA*, *gyrB*) and topoisomerase IV (*parC*, *parE*) can alter the susceptibility of the isolates considerably. Other resistance mechanisms are involved in an overexpression of quinolone efflux pumps, alteration of the membrane permeability, or enzymatic inactivation of specific (fluoro)quinolones. These mechanisms can be induced by plasmid-mediated quinolone resistances (PMQR) including pentapeptide-encoding *qnr* genes, efflux pump-encoding genes (e.g., *qepA*), and the aminoglycoside acetyltransferase-coding *aac-(6′)-Ib-cr* gene [5]. PMQRs represent an inevitable threat, as they play an important role in the dissemination of (fluoro)quinolone resistance genes through horizontal gene transfer and the development of new (fluoro)quinolone-resistant bacteria. Hence, they are assumed accountable for the increased resistance to (fluoro)quinolones [6,7]. Horizontal gene transfer (HGT) is an efficient mechanism for adaptation of bacteria to prevailing environmental conditions. The exchange of resistance genes is a common response of bacteria to overcome antimicrobial selection pressures. Among them, *qnr* represents a highly prevalent PMQR gene in livestock with a broad overall distribution [8]. It has frequently been reported that *qnr* genes in *Enterobacteriaceae* increase (fluoro)quinolone resistance by enhancing the degree of resistance at which they can be selected [9]. Furthermore, some reports have linked the presence of PMQRs with a successive development of chromosomal alterations in the genes *gyrA*, *gyrB*, or *parC*, known to be associated with increased (fluoro)quinolone resistance when mutated at specific positions [10,11]. In addition, *qnr* genes were often observed in combination with other mobile determinants involved in resistance development against other critically important antimicrobials (i.e., extended spectrum β-lactamases (ESBLs), carbapenemases, and colistin) [12–14]. The co-occurrence of genes associated with resistances against antimicrobial agents routinely used in human medicine is of great concern, as it limits the therapeutic options for treatment of infections [15]. To estimate the specific impact of *qnr*-carrying isolates for the emergence and dissemination of (fluoro)quinolone-resistant *E. coli* in livestock and food, a deeper understanding of the occurrence, genetic variability, and elements involved in their spread is needed.

This study was conducted to assess the prevalence and diversity of *qnr*-carrying isolates among (fluoro)quinolone-resistant, commensal *E. coli* gained during the annual German AMR monitoring in livestock and food in 2017. These isolates were characterised in detail for their resistance phenotype and genetic characteristics. Furthermore, the commonality of plasmids carrying *qnr* genes along with their diversity and transmissibility were determined. Finally, a potential association of *qnr*-genes with (fluoro)quinolone resistance enhancing point mutations among German livestock *E. coli* was evaluated.

2. Materials and Methods

2.1. Bacterial Isolates and Culture Conditions

In total, 2799 *E. coli* from different food and livestock sources [16], especially from faecal samples of deer and fattening pigs, from cecum contents of fattening pigs and veal calves, as well as samples from pork, veal, and game meat where analysed. Samples from faecal and cecum sources are analysed as the same source in this study. All isolates were recovered during the German AMR monitoring of commensal (ZoMo, unselective cultivation conditions) and ESBL and/or AmpC-producing *E. coli* from food and livestock (ESBL-monitoring, selective cultivation conditions using cefotaxime) in 2017. The isolates were investigated according to the European Commission Implementing Decision

2013/652/EU in the National Reference Laboratory for Antimicrobial Resistance (NRL-AR). The isolates constitute the positive findings of a representative collection of samples taken in all 16 German federal states. If not stated otherwise, all isolates were cultivated in lysogeny-broth-based media for 16–18 h at 37 °C for further characterisation.

2.2. Antimicrobial Susceptibility Testing

Minimum inhibitory concentrations (MIC) were determined by using broth microdilution according to EUCAST recommendations on a standardised European antimicrobial test panel (EUVSEC/EUVSEC2; Sensititre™, TREK Diagnostic Systems, Altrincham, Cheshire, UK). The tested antimicrobials covered the substances and ranges fixed in the European Commission Implementing Decision No. 2013/652/EU [17]. The following antimicrobial agents were used in ranges as specified: ampicillin (1 to 64 mg/L), azithromycin (2 to 64 mg/L), cefepime (0.06 to 32 mg/L), ciprofloxacin (0.015 to 8 mg/L), colistin (1 to 16 mg/L), ertapenem (0.015 to 2 mg/L), cefoxitin (0.5 to 64 mg/L), gentamicin (0.5 to 32 mg/L), imipenem (0.12 to 16 mg/L), meropenem (0.03 to 16 mg/L), nalidixic acid (4 to 128 mg/L), cefotaxime (0.25 to 64 mg/L), ceftazidime (0.25 to 128 mg/L), temocillin (2 to 128 mg/L), tetracycline (2 to 64 mg/L), tigecycline (0.25 to 8 mg/L), trimethoprim (0.25 to 32 mg/L), chloramphenicol (8 to 128 mg/L), sulfamethoxazole (8 to 1024 mg/L), cefotaxime/clavulanic acid (0.06/4 to 64/4 mg/L), and ceftazidime/clavulanic acid (0.12/4 to 128/4 mg/L). For quality assessment, the *E. coli* strain ATCC 25,922 was included. MIC values were interpreted according to EUCAST epidemiological cut-off values (ECOFFs) [18].

2.3. Molecular Screening on qnr Genes

Genomic DNA from isolates exhibiting a non-wild-type phenotype for nalidixic acid (NAL $\geq$ 16 mg/L) and/or ciprofloxacin (CIP $\geq$ 0.06 mg/L) were subjected to boiling DNA preparation [19]. The DNA extracts were used for molecular screening of *qnr* genes. PCR amplification for detecting *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, and *qnrVC* was conducted using primers and conditions as previously described [20,21] (Table S1). Product amplification was performed in a Bio-Rad CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Feldkirchen, Germany).

2.4. Determination of Isolate-Specific Macrorestriction Patterns and Plasmid Profiles

For determination of the genetic relationship between isolates, macrorestriction profiles using pulsed-field gel electrophoresis (PFGE) according to the PulseNet laboratory protocol [22] were performed. For digestion, the restriction endonuclease XbaI (10 U/µL, Thermo Fischer Scientific, Darmstadt, Germany) was used. For plasmid profiling, bacteria were treated with S1 nuclease (180 U/µL, Thermo Fischer Scientific) and S1-PFGE was conducted as previously described [22]. Separation of DNA was conducted on a CHEF-DR III system (Bio-Rad Laboratories, Madrid, Spain). The *Salmonella enterica* (H9812) serovar Braenderup was used as a molecular weight standard for size determination. Detection of *qnr* gene-carrying fragments was performed by Southern blotting and DNA-DNA hybridisation of S1-PFGE agarose gels. Hybridisation was conducted using digoxigenin-labelled (Roche Diagnostics, Mannheim-Penzberg, Germany) PCR probes of *qnr* genes and were prepared as previously described [23] (Table S1).

2.5. Whole-Genome Sequencing (WGS) and Bioinformatics Analysis

Genomic DNA of the isolates was prepared using the PureLink Genomic DNA Mini Kit (Invitrogen-Thermo Fisher, Schwerte, Germany) according to the manufacturer's recommendation. The sequencing library was generated with the Nextera DNA Flex Library Preparation Kit (Illumina®, San Diego, CA, USA) as previously described [24]. Short-read, paired-end, whole-genome sequencing was performed in 2 × 151 cycles with the Illumina® NextSeq™ 500/550 Mid Output Kit v2.5 (300 Cycles). After trimming of reads with aquamis (version 1.33) [25], unicycler (version 0.4.4) [26] was used for de novo assembly of raw reads. Quality assessment of genome assemblies was conducted using QUAST

5.0.2 [27]. Assembled contigs were analysed for virulence factors and resistance genes as well as for plasmid markers (i.e., replicon types) with AMRfinder (version 3.6.7) and its database [28] and abricate (version 0.9.8) [29] each, through bakcharak [30]. Cluster analyses and sequence alignments were conducted using PATRIC with the RASTtk-enabled Genome Annotation Service and default parameters [31] for the service "Codon Tree". Visualisation was conducted in R with the packages ggplot2 (version 3.3.0) [32], ggtree (version 1.4.11), and treeio (version 3.10) [33].

The PointFinder tool [34] and the deposited database (updated—2 July 2019; access date—25 June 2020) were used to identify alterations in chromosomal genes that were confirmed to be associated with (fluoro)quinolone resistances for *E. coli*. In-silico-based multilocus sequence typing (MLST, according to the Achtmann scheme) was conducted using bakcharak [30] and the pubMLST database [35]. The screening for respective Inc groups was based on the PlasmidFinder database [36]. The detection of resistance genes was conducted with ResFinder 4.1 [37].

To determine the diversity of *qnr*-carrying plasmids, a reference database comprising all accessible *qnr* plasmid genomes of the NCBI RefSeq database (access date—17 April 2020) was developed. All available plasmids were checked for completeness through the keywords "complete sequence" or "complete plasmid". Abricate was performed with the NCBI AMRfinder database to screen for *qnr* plasmids. The resulted database was used for the subsequent reference search with RefSNPer [38] as described elsewhere [39]. The trimmed reads of all individual isolates were mapped to each reference plasmid (using bowtie2 [40], version 2.3.5). Subsequently, the coverage breadth and depth of each reference plasmid as well as the number of single-nucleotide polymorphisms (SNPs) are computed with SAMtools version 1.10 and BEDTools version 2.29.0 [41], thus providing the *qnr* plasmids that most closely match each isolate.

2.6. Analysis and Statistics

Data analysis and visualisation was conducted using R (version 3.6.3). Choropleth figure was visualised with R (version 3.6.3) using the packages maptools (version 0.9-9), sp (version 1.4-0), rgeos (version 0.5-2), and rgdal (v1.4-8). The SpatialPolygonDataFrame of Germany was provided by the Bundesamt für Kartographie und Geodäsie database (https://gdz.bkg.bund.de/index.php/default/open-data/gebietseinheiten-1-2-500-000-ge2500.html, access date—24 March 2020). Dependencies between the presence of antimicrobial resistance genes were calculated in R (version 3.6.3). The occurrence of resistance genes was translated into binary data. Correlation between certain resistance determinants was predicted by Fisher's exact test. A ρ-value of <0.05 was considered as a statistically significant correlation.

3. Results and Discussion

3.1. qnrS Is the Most Prevalent qnr Gene in E. coli from Livestock and Food

Antimicrobial resistance-testing (AST) revealed that 391 out of 2799 investigated isolates (14%) exhibited a non-wild-type phenotype (phenotypical resistance) against ciprofloxacin (CIP: MIC $\geq$ 0.06 mg/L) and/or nalidixic acid (NAL: MIC $\geq$ 16 mg/L) (Table S1). Of those, 80 isolates were recovered from the ESBL-monitoring and 23 from ZoMo. PCR screening revealed seven different *qnr* genes within the 103 *qnr*-positive *E. coli* (Table 1). Among them, *qnrS* (n = 95) was the most prevalent gene, followed by *qnrB* (n = 6), while *qnrA* and *qnrVC* occurred only once each. No *qnrC*- or *qnrD*-carrying isolates were detected. The rather low prevalence of (fluoro)quinolone-resistant *E. coli* of 14% in the investigated nonpoultry matrices is in good agreement with the data summarised by EFSA [42]. While CIP and NAL resistance were reported by several European countries at high levels in broiler and turkey, the EU medians in pigs and calves were rather low (6.2% and 4.2% for NAL and 7.4% and 8.4% for CIP per matrix, respectively) [43]. Regarding the detection of specific *qnr* genes, our result is in good agreement with previous reports in which *qnrS* and *qnrB* were the most frequently detected

qnr genes in livestock sources [44,45]. In contrast to livestock, *qnrA* genes are often present in isolates from hospitalised patients [46].

Table 1. Occurrence and frequencies of determined *qnr* genes within *qnr*-carrying, (fluoro)quinolone-resistant *E. coli* isolates.

Gene *1	Gene *2	Occurrence	Frequency #
qnrA	*qnrA1*	1	1.0%
qnrB	*qnrB1*	3	5.8%
	qnrB2	1	
	qnrB19	2	
qnrS	*qnrS1*	92	92.2%
	qnrS2	3	
qnrVC	*qnrVC4*	1	1.0%

*1: determined with PCR, *2: determined with WGS, # frequency of genes as determined by PCR.

We detected *qnr*-positive *E. coli* in veal faeces (n = 56, 8.2% of all veal faeces samples from the ZoMo- and ESBL-monitoring in 2017), fattening pig faeces (n = 38, 3.6%), minced meat (n = 3, 4.7%), beef (n = 2, 5.1%), and pork (n = 2, 5.9%), as well as in deer faeces (n = 1, 0.2%) and deer meat (n = 1, 0.5%) (detailed data for each isolate are presented in Table S2). Overall, the highest relative proportion of (fluoro)quinolone-resistant and *qnr*-positive isolates during the monitoring program in 2017 was determined in Lower Saxony (9.3%), followed by North Rhine-Westphalia (7.9%). However, these two federal states have the highest livestock population and contributed most to the overall sample size. The prevalence for other federal states was below 5%. An overview on the regional prevalence of (fluoro)quinolone-resistant and *qnr*-positive isolates is given in Figure 1.

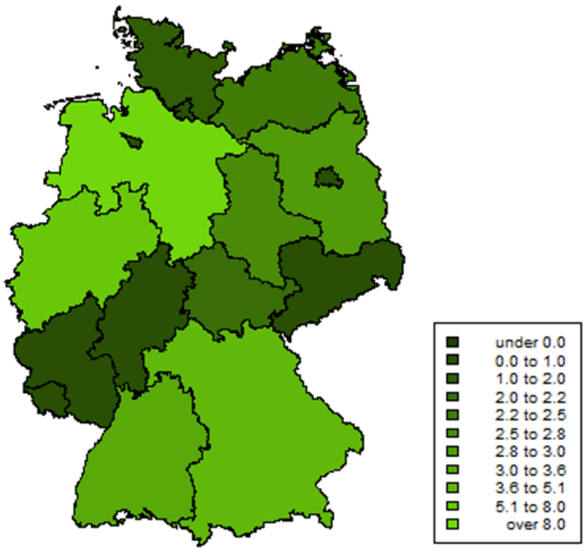

Figure 1. Choropleth of the proportion of (fluoro)quinolone-resistant and *qnr*-positive *E. coli* recovered during the ZoMo- and ESBL-monitoring in Germany in 2017. Prevalence was calculated as the proportion of (fluoro)quinolone-resistant and *qnr*-positive *E. coli* isolates divided by all investigated samples per federal state.

The analysis of the XbaI-macrorestriction patterns revealed a high phylogenetic heterogeneity of *E. coli* carrying *qnr* genes. For further typing purposes, the isolates were subjected to WGS and bioinformatics analysis. Here, 45 different sequence types (STs) were determined by in silico analysis. ST10 (21%), ST2325 (6%), and ST58 (5%) represented the predominant types. Overall, a broad distribution of *qnr*-carrying isolates in different ST-types was observed. Of ST10, 12 isolates were gained from veal faeces, nine from pork faeces, and one from veal meat. Four isolates from veal faeces, one from fattening pig

faeces, and one from veal meat were assigned to ST2325. ST58 was evenly distributed between isolates from faecal samples from veal and fattening pigs. Two isolates could not be assigned to previously described STs. The observed results respond well to prevailing reports, in which *qnr* genes were found to be prevalent in ST10 isolates of livestock and of human origin and support the hypothesis on their impact as possible distributors of plasmid-associated *qnr* genes between livestock and human [47]. To the best of our knowledge, ST2325 isolates have yet not been described to be associated with *qnr* genes.

In Figure 2, the phylogenetic relationship of the isolates, based on WGS data of the individual isolates, is shown. As expected, the clusters correspond with the prevailing ST, but seemed not to be associated with a certain food/livestock matrix. In addition, diverse resistance profiles were observed in different clusters and STs, as well as over different matrices for the tested strains (Figure 2). Overall, we found a high diversity of *E. coli* carrying *qnr*. This widespread occurrence of *qnr* has been reported before [48]. Especially, *E. coli* of the clonal group ST10 are often associated with AMR plasmids [49] and often reported to carry *qnr*-positive plasmids [50]. Further, ST10 is characteristic for *E. coli* defined as ESBL [51]. As we analysed 80 *E. coli* isolates from the ESBL-monitoring, the observation of ST10 being related to ESBL *E. coli* was confirmed. Our findings support the current knowledge that resistance genes such as *qnr* can spread over different sources and are not restricted to certain *E. coli* sequence types.

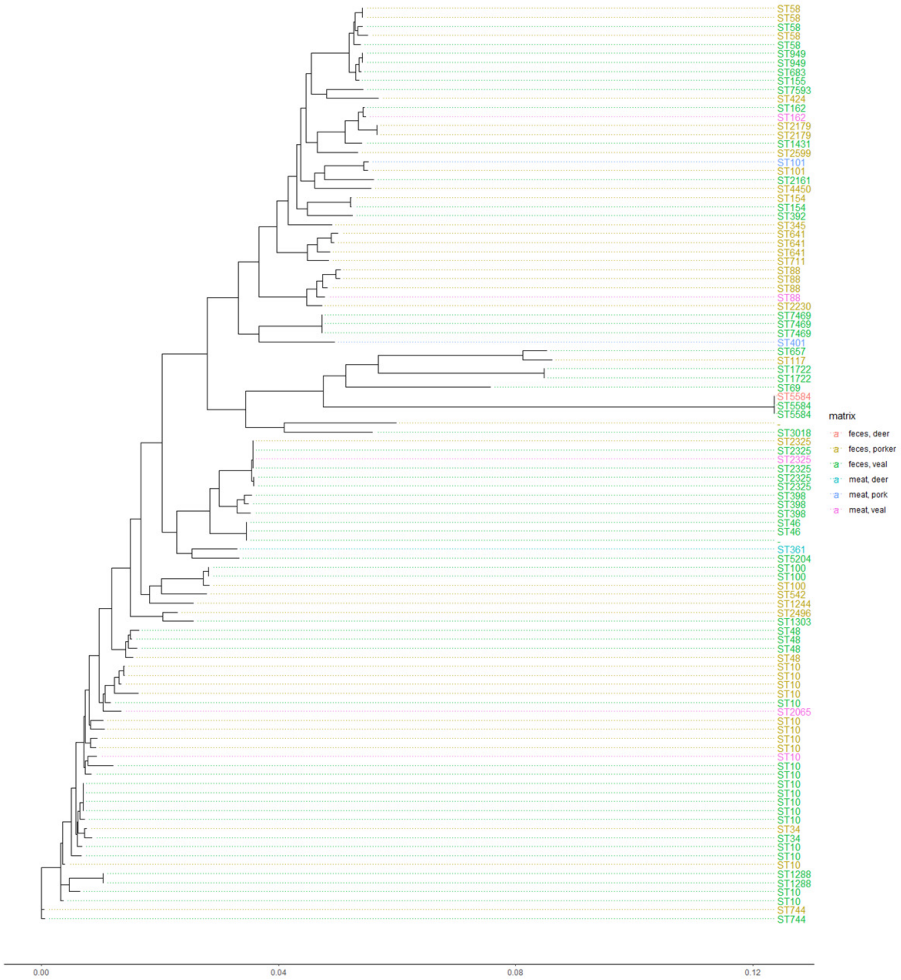

Figure 2. Phylogenetic relationship of (fluoro)quinolone-resistant and *qnr*-positive *E. coli* with metadata based on codon and protein differences, presented in a maximum-likelihood tree. The respective sequence type (ST) of the *E. coli* is shown as geom_tiplab and connected with dotted lines. The ST as well as the dots are coloured according to the matrix code of the recovered *E. coli*.

3.2. qnr-Carrying E. coli Isolates Exhibit Diverse Resistance Phenotypes Including Multidrug Resistances

In antimicrobial sensitivity testing, the *qnr*-carrying *E. coli* showed highly diverse resistance profiles. Most of them exhibited a phenotypic resistance for antimicrobial classes (Table S2) other than (fluoro)quinolones, which is linked to the fact that most isolates originated from ESBL-monitoring. Considering only the 23 ZoMo-isolates, the resistant phenotype ranged from resistance to only one to five different classes. When only ESBL-monitoring isolates were analysed, the numbers ranged from three to eight different classes.

Thus, for the ESBL-monitoring isolates, 90% exhibited resistance phenotype against more than three antimicrobial classes, 79% showed resistance phenotypes against five antimicrobial agents, and 34% exhibited increased MIC values against six to eight antimicrobial agents.

In general, besides ciprofloxacin (100% from ESBL-monitoring, 91% from ZoMo), resistant phenotypes for ampicillin (99% and 91%), cephalosporine (100% and 20%), and tetracycline (80% and 52%) were most common among the investigated *E. coli*. Table 2 shows the distribution of resistant phenotypes relative to the respective matrix. This distribution is presented graphically in the Supplementary material FS1. Overall, we only detected two isolates from the ZoMo, which were only resistant to (fluoro)quinolones. Thus, our data, in which (fluoro)quinolone resistance is frequently associated with multidrug resistance in *E. coli*, coincides well with the results of other studies [52]. As a result of a direct selection pressure, the treatment of livestock with a specific antimicrobial agent supports the maintenance of resistance genes directed against this antimicrobial agent [53]. As resistance genes can occur within a multidrug-resistant isolate or on a multiresistance plasmid, disseminating through selective forces, this could enhance the prevalence of other resistance genes on the same plasmid or within the same isolate. Our result supports that there is a potential risk of coselection, maintenance, transmission, and propagation of multidrug-resistant *E. coli* and their plasmids [15]. From our findings, one could assume that multidrug-resistant clones with (fluoro)quinolone resistance exist, especially in combination with ESBL genes, which might be of special concern.

Table 2. Absolute number of the phenotypic resistance of *qnr*-carrying isolates and absolute number of isolates gained from the respective matrix. In brackets the absolute number of isolates from the ZoMo-/ESBL-Monitoring is indicated.

Matrix	Matrix Occurrence	AMP	AZI	CHL	CIP	COL	FOT	GEN	MERO	NAL	SMX	TAZ	TET	TMP
faeces, veal calves	56 (52/4)	54 (51/3)	4 (4/0)	19 (17/2)	55 (52/3)	1 (1/0)	53 (52/1)	6 (6/0)	1 (1/0)	8 (7/1)	37 (36/1)	52 (52/0)	48 (44/4)	40 (37/3)
faeces, deer	1 (1/0)	1	0	0	1	0	1	0	0	0	1	1	1	1
faeces, pigs	38 (24/14)	38 (24/14)	6 (5/1)	11 (9/2)	37 (24/13)	0	27 (24/3)	4 (4/0)	0	10 (7/3)	22 (18/4)	27 (24/3)	22 (17/5)	20 (17/3)
meat, veal	2 (2/0)	2	0	2	2	0	2	0	0	1	1	1	1	2
meat, deer	1 (1/0)	1	1	0	1	0	1	1	0	0	1	1	1	1
meat, pork	2 (0/2)	1	0	2	2	0	0	0	0	0	1	0	1	2
minced meat	3 (0/3)	3	0	0	3	0	1	0	0	0	0	1	2	0

AMP—Ampicillin, AZI—Azithromycin, CHL—Chloramphenicol, CIP—Ciprofloxacin, COL—Colistin, FOT—Cefotaxime, GEN—Gentamicin, MERO—Meropenem, NAL—Nalidixic acid, SMX—Sulfamethoxazole, TAZ—Ceftazidime, TET—Tetracycline, TMP—Trimethoprim.

3.3. qnr-Carrying E. coli Isolates Are Associated with Highly Diverse Resistomes

Overall, the most abundant resistance genes of *qnr*-carrying isolates were bla_{EC} (accession number: A0A244BQ89) (100% of all *qnr*-carrying strains), as well as different variants of the *tet* (96%) and $bla_{CTX\text{-}M}$ (74%) genes. In general, we found *qnrS1* to be in frequent connection with *tet*(34) and *tet*(A). By analysing the nucleotide-sequence of the *tet*(34) gene

of *E. coli*, a sequence coverage of only 76% to the reference (accession number: A7J11_00001) was detected. Besides this defective gene, tetracycline-resistant isolates usually carried other determinants like *tet*(A) or *tet*(B). Interestingly, none of the *qnr*-harbouring *E. coli* carried a PMQR (*aac(6′)-Ib-cr*, *qepA*, or *oqxAB*) other than *qnr*.

We further split the isolates by the ZoMo- and ESBL-monitoring source. For the ZoMo isolates, we found a significant correlation for the co-occurrence of *qnrVC* with bla_{OXA10}, *cmlA5*, and the pesticide-resistance encoding gene *qacF*. For the other *qnr* gene variants, no significant correlation for co-occurrence was detected (Table S3). No statistically significant correlation was observed for *qnrS1* and $bla_{\text{TEM-1}}$ (p-value 0.059). However, the low number of ZoMo isolates hinders a thorough analysis for this potential correlation. When we analysed the ESBL-monitoring isolates, some co-occurrence of *qnr* and other resistance genes was detected (Table 3). Due to the selective isolation procedure, a high correlation of *qnr* and *bla* genes was observed. However, *qnrA* was rather associated with $bla_{\text{ACC-1}}$ and $bla_{\text{VIM-1}}$ as well as *qnrB* with $bla_{\text{OXA-1}}$, while *qnrS* correlated with $bla_{\text{CTX-M-65}}$ and $bla_{\text{OXA-1}}$. Other co-occurring resistance genes with *qnr* are presented in Table 3. Especially, *qnrS1* was often detected in combination with multiple resistance genes as well as with the pesticide resistance encoding gene *qacEΔ1*.

Table 3. p-value for co-occurrence of selected resistance genes and *qnr* genes in ESBL-monitoring isolates; p-values below 0.05 are highlighted in red and represent statistical significance. The value 1 reflects that the two genes were not detected.

	qnrA1	***qnrB1***	***qnrB2***	***qnrB19***	***qnrS1***	***qnrS2***
aadA1	0.1625	0.00348101	1	1	0.00462503	0.41693038
aph(3′)-lia	1	1	1	1	0.1125	0.0375
aph(3′)-XV	0.0125	1	1	1	0.1125	1
arr-3	1	1	1	1	0.011392405	0.00094937
$bla_{\text{ACC-1}}$	0.0125	1	1	1	0.1125	1
$bla_{\text{CTX-M-65}}$	1	1	1	1	0.060414269	0.00559883
$bla_{\text{OXA-1}}$	1	0.00012171	1	1	0.000000524	0.00925024
$bla_{\text{VIM-1}}$	0.0125	1	1	1	0.1125	1
catA1	0.0625	0.00012171	1	1	0.000377371	1
catB2	0.0125	1	1	1	0.1125	1
catB3	1	1	1	0.0375	0.001022395	0.00282376
dfrA25	1	1	0.0125	1	0.1125	1
floR	0.25	1	1	1	0.038946034	0.01387537
mef(C)	1	0.10966407	1	1	0.032132425	0.10966407
mph(A)	0.2125	1	1	1	0.090321713	0.00827653
mph(G)	1	0.10966407	1	1	0.032132425	0.10966407
qacEΔ1	0.2	0.49289192	0.2	0.2	0.001541798	0.10029211
sul1	0.175	0.44303798	0.175	0.175	0.006847169	0.44303798

This coexistence of multiple resistance genes can pose a higher risk, as their presence may contribute to a better adaption to different environmental conditions and enhance the persistence of the plasmid. It has been reported in previous publications that *E. coli* isolates from different livestock matrices carried both ESBL and PMQR genes, as they often coexist on the same plasmid. Mainly, a coexistence of *qnr* and $bla_{\text{CTX-M-15}}$ as well as bla_{SHV} was previously described [12]. In general, ESBL-producing *E. coli* are an emerging public-health threat and their rise will further reduce the available treatment options in human medicine. The co-occurrence of *qnr* and ESBL genes represent another risk as the bacteria exhibit resistances against antimicrobials of two important classes. Especially, the spread of plasmids bearing resistance determinants of both antimicrobial classes will further force the development of multidrug-resistant isolates. A correlation of *qnr*-positive ESBL *E. coli* was previously reported for human sources. The presence of genes conferring resistances against two critically important antimicrobial agents on the same plasmid or within one isolate can constitute an important issue for treatment failures when using the respective antimicrobial agents for therapeutic application in hospitalised patients. As

Salah et al. mentioned, these plasmid-mediated resistances highly facilitate the spread and increase their frequency [54]. They also found that every *qnr*-positive strain investigated in their study was ESBL-producing. However, as we mainly screened ESBL-preselected *E. coli*, the observation of *qnr* in these ESBL-producers presumably relates on a conditional probability. Although the coexistence of multiple PMQR genes has been described as a frequent event [52], we did not detect PMQRs other than *qnr*.

As mentioned, a significant co-occurrence of *qnrS1* and *qacE*Δ1 as well as of *qnrVC* and *qacF* was observed. These determinants confer resistance to quaternary ammonium compound disinfectants [55]. The awareness for this plasmid-associated antiseptic resistance gene is broadly present, as it enhances the tolerance to several disinfectants that might increase the ability of AMR-carrying isolates to persist in the environment [56]. Quaternary ammonium compounds are widely used as disinfectant in farm environments. It has been observed that *qac* genes are often associated with multidrug-resistant isolates [57]. Thus, they might support the evolution (i.e., adaptation to specific environmental conditions) of bacterial resistance to multiple antimicrobial agents. Here, the presence of the biocide resistance genes reveals another risk harboured by the *qnr*-carrying isolates, as it represents an additional determinant for resistances against biocides.

3.4. Virulence Genes Associated with qnr-Carrying E. coli

As this study was based on commensal *E. coli*, the number of virulence-associated genes in the isolates was expected to be low. In our samples, between 34 and 108 potential virulence factors (according to the Virulence Factor Database) per isolate were identified. Ninety-two isolates were found to carry *fimH*, a D-mannose-specific adhesin, type 1 fimbriae-encoding gene. Frequent detection of the potential surface virulence factors *fimH* is quite common among *E. coli*. However, FimH mediates adherence to cells and, therewith, helps the formation of bacterial biofilms [58]. It confers the possibility of colonisation and, when certain mutations occur, can represent a virulence factor [59,60]. Additionally, other surface virulence factor encoding genes such as *afa* (n = 2), *focG* (n = 1), *paa* (n = 1), *pap* (n = 1), and *saf* (n = 1) were found. Moreover, we detected one isolate with *eae*, an intimin-encoding gene. The protein, encoded by *eae*, plays a critical role on the intestinal colonisation and, therefore, STEC (shiga toxin-producing *E. coli*) or another aggregative *E. coli* pathogenesis [61]. Some isolates harboured toxin genes or toxin subunits such as *astA* (n = 16), *cdtA* (n = 3), *cnf1* (n = 3), *eltA* (n = 1), and *faeC* (n = 1). The presence of *astA* has been detected in subgroups of enteroaggregative *E. coli* [62]. Further, two isolates possessed the *hlyA* gene, an important secretory virulence factor. Therewith, the presence of the virulence genes was unrelated to the different monitoring programs from where the *E. coli* was isolated. Thus, we showed that important virulence factors could sporadically occur in *qnr*-positive *E. coli* isolates. As many virulence factors are also located on mobile genetic elements, like plasmids, their potential spread with resistance determinants should be taken into account. Thus, through horizontal gene transfer, not only the resistance genes but additional virulence factors are spread. In these cases, antibiotic treatment failure, due to resistance, may give rise to potential impacts of certain virulence factors; therewith, representing an evolutionary pathway to pathogenicity [63]. However, most of the virulence factors detected in this study represent individual components of different complex systems. As the different virulence factors of *E. coli* are quite complex in their interaction, they may not have a high impact on the isolate's pathogenicity on their own [64].

3.5. In-Silico-Based Prediction of Plasmids Types Carrying qnr

Plasmids play a major role in bacterial evolution and resistance gene transmission. Understanding factors influencing plasmid composition and evolution are essential for reliable assessments. Therefore, we detected the best-matching references to our *qnr*-carrying plasmids with the refSNPer tool. Therewith, reference plasmids that were covered by up to 100% and 90% were determined for 31 and 35 datasets, respectively, to their reference plasmids. Four WGS datasets showed no significant matches (best reference < 50%

coverage) to any *qnr*-plasmid genome of the reference database and could be considered as new plasmids. The most frequently detected references were plasmid tig00003056 of *E. coli* strain AR_0162 (NZ_CP021681, *n* = 15), plasmid C of *E. coli* strain D9 (NZ_CP010155, *n* = 8), an unnamed plasmid of *Shigella flexneri* 1a strain0670 (NZ_CP020088, *n* = 8), and pKpvST101_6 of the *Klebsiella pneumoniae* strain KpvST101_OXA-48 (NZ_CP031373, *n* = 6).

Plasmids exhibiting nucleotide similarities of >80% to the best matched plasmid tig00003056 (NZ_CP021681) have been described in hospitalised patients in the USA (CP026200.1, CP044008.1), the UK (LT906492.1, LT882487.1), in Taiwan (CP046430.1), and in Pakistan (CP040574.1). Plasmid C (NZ_CP010155)-like genomes were found in China and Japan, isolated from wastewater (CP035315.1, CP045998.1, AP019678.1, MT219825.1, CP051432.1, CP046002.1) or dog faeces (NZ_CP010155). Both plasmids were mainly isolated from *E. coli* but also found in other *Enterobacteriaceae*. This geographical spread, as well as the different reservoirs, provide no further evidence on a common source/origin for *qnr*-carrying plasmids. In addition, NZ_CP020088-like plasmids seemed broadly distributed. They have been detected in Brazil (MK965545.1) and Norway (MH507589.1) in chicken and turkey meat, and in rook faeces in the Czech Republic (KF362122.2, MH121702.1). However, they also have been isolated from hospitalised patients in China (CP020088.1, KJ201886.1, CP012734.1, and CP020341.1). With NZ_CP020088-like plasmids being reported mainly in poultry origin, it supports our findings of this plasmid in the livestock reservoir. NZ_CP031373 was detected in the Netherlands (KX618696.1), Czech Republic (MH594478.1), and the UK (CP031373.2). Overall, the best matched plasmid-references to our identified *qnr*-carrying plasmids mainly originate from *E. coli* (*n* = 50) and *Shigella flexneri* (*n* = 20). However, similar plasmid-types were also identified in a broad range of other *Enterobacteriaceae* like *Enterobacter*, *Salmonella*, and *Serratia*. The broad distribution of these plasmids, closely related to our identified *qnr*-carrying plasmids, demonstrates the high ability of spread among *Enterobacteriaceae*. The diverse host adaption is clear evidence for a broad host spectrum of *qnr*-plasmids. Further, the distant locations of plasmid isolation demonstrate the putative exchange of global resistance transfer over plasmids, especially for *qnr* here.

As shown in Table 4 (and Table S4), the most abundant plasmid replicon types among our identified *qnr* reference plasmids were IncN (*n* = 12), IncY (*n* = 19), as well as a combination of IncX1 and IncX3 (*n* = 29). However, a total of 21 different plasmid-type combinations were identified. Therewith, IncN plasmids are reported as broad host range types, with the ability for conjugative transfer and the carriage of drug resistance genes. Close phylogenetic relationships from environmental and clinical samples are described for IncN plasmids [65]. Further, IncN plasmids are known for carrying a great variety of resistance genes against extended-spectrum β-lactams, sulphonamides, quinolones, aminoglycosides, tetracyclines, and streptomycin [66]. Here, we found the IncN reference plasmid to carry the $bla_{\text{TEM-1}}$ resistance gene, encoding β-lactamase. IncN is widely found in *Enterobacteriaceae* and recognised as conjugative. Plasmids from the IncY type are known for frequently harbouring $bla_{\text{CTX-M-5}}$ or $bla_{\text{SHV-2}}$ resistance genes, associated with ESBL development [66]. Further, plasmids of these group have been shown to carry *mcr*-1 [67]. Here, we found the reference plasmid from the IncY group to harbour multiple resistance genes, including $bla_{\text{CTX-M-5}}$ and $bla_{\text{TEM-1}}$. Although plasmids belonging to the IncX group are known to be narrow host range plasmids, commonly found in *Enterobacteriaceae*, they often carry a wide spectrum of multidrug resistance enabling genes and were often found in the guts of animals [68]. Genes encoding carbapenemases as well as the colistin resistance genes *mcr*-1 and *mcr*-2 are frequently reported on IncX plasmids [69,70]. Dobiasova and Dolejska [71] found that IncX plasmids are widely distributed in *E. coli* in European animals and predominantly associated with (fluoro)quinolone resistance genes, particularly with *qnrS*. All of the prevalent plasmid incompatibility (Inc)-groups were often associated with multiple resistance genes besides *qnr*, which increases the risk of the dissemination of those plasmids. As shown in Table 4, the plasmids from the IncX group did carry multiple resistance genes next to *qnrS*. Thus, we detected $bla_{\text{TEM-1}}$ and bla_{SHV} located on the

same plasmids. However, while we identified certain clusters of the mentioned plasmid Inc-groups, the *qnr* gene was rather broadly distributed over different plasmid types.

Table 4. Inc group and resistance genes of the best matching reference plasmid to the most prevalent plasmids carrying *qnr*-genes detected in this study. Identified with RefSNPer.

Plasmid Type and Resistance Genes on Matching Reference	Frequency
IncN	∑ 12
aac(3)-IId, *qnrS1*	1
$bla_{\text{TEM-1}}$, *qnrS1*	5
qnrB19	1
qnrS1	5
IncR, IncX1	∑ 1
aadA2, *blaTEM-1*, *dfrA12*, *floR*, *qnrS1*, *sul2*, *tet(A)*, *tet(M)*	1
IncX1	∑ 9
aph(3′)-Ia, *floR*, *qnrS2*	8
$bla_{\text{TEM-1}}$, *qnrS1*, *tet(M)*	1
IncX1, IncX3	∑ 14
$bla_{\text{TEM-1}}$, *qnrS1*	14
IncX3	∑ 6
bla_{SHV}, *qnrS1*	6
IncY	∑ 19
aph(3″)-Ib, *aph(6)-Id*, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, *qnrS1*, *sul2*, *tet(A)*	15
aph(3″)-Ib, *aph(6)-Id*, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, *dfrA14*, *qnrS1*, *sul2*, *tet(A)*	2
aph(3″)-Ib, *aph(6)-Id*, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, *qnrS1*, *sul2*	2

Further, we examined the size distribution of extrachromosomal DNA elements through S1-PFGE analysis and successive DNA–DNA hybridisation against the different *qnr* genes. Overall, we found a broad size diversity of plasmids carrying *qnr* (Figure 3).

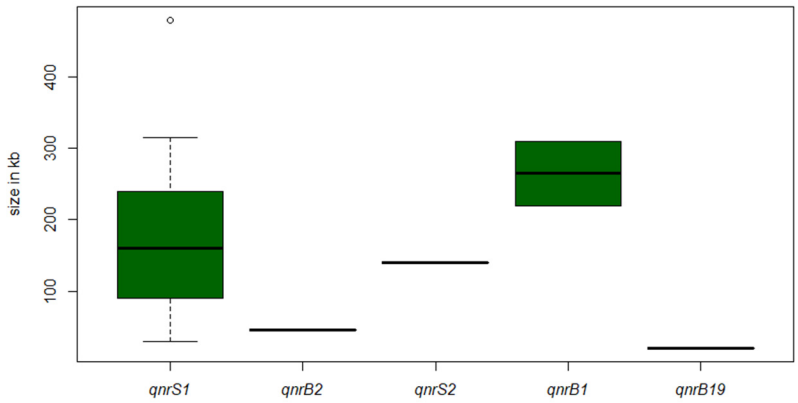

Figure 3. Boxplots of the size distribution of analysed plasmids harboring *qnr*, estimated through S1-PFGE. The white circle represents an outlier.

The *qnrS1* gene was detected on plasmids ranging between 30 kb and 480 kb. Once again, this emphasises the ability of *qnr* to combine with different plasmid types of various sizes and, therewith, the adaptability to different niches.

Apart from *qnr*, the most frequently found resistance genes on the same reference plasmid were *aph(3″)-Ib*, *aph(6)-Id*, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1}}$, and *sul*2, independent of the monitoring program. The occurrence of this resistance gene combination was not exclusively for a certain Inc group, but rather broadly distributed. Hence, *qnr* genes are detectable on plasmids of various sizes and are seldom the only resistance gene located on the plasmid, but instead related to different resistance genes. The transfer of plasmids carrying *qnr* has been described as associated with the transfer of genes leading to multidrug resistance [72]. This suggests that provoking *qnr* resistance by overuse of an antimicrobial agent can support the expansion of multidrug-resistant isolates in livestock and food sources.

3.6. Frequent Detection of Point Mutations in the gyrA, parC and parE Genes of qnrS-Carrying E. coli

In our investigations, we detected isolates with phenotypes resistant against ciproflo xacin but lacking phenotype resistance against nalidixic acid (Table S2). Resistance to fluoroquinolones without resistance to quinolones is mainly associated with mutations in the chromosome within the *gyrA* and *parC* genes [58,59]. Further, the EFSA assumed that the resistance to ciprofloxacin without resistance against nalidixic acid indicates an increasing occurrence of plasmid-mediated quinolone resistance [2]. In general, besides PMQR genes, mutations in genes encoding DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) are highly associated with increased (fluoro)quinolone resistance. Previous studies showed that a high level of resistance to (fluoro)quinolones is mainly associated with mutations in the *gyrA* and an additional mutation in the *parC* gene in *E. coli* [73,74]. In this study, 16 out of 103 *qnr*-carrying isolates were identified to exhibit a point mutation in the QRDR regions of *gyrA* (n = 9, two mutations; n = 5, one mutation) or *parC* (n = 2, two mutations; n = 10, one mutation) and *parE* (n = 4, one mutation) genes. Subsequently, these 16 isolates had high MIC values for nalidixic acid (mostly > 128 mg/L) and ciprofloxacin (mostly > 8 mg/L). Interestingly, we also detected three other isolates with a MIC for nalidixic acid > 128 mg/L, but no alteration in the chromosomal genes associated with (fluoro)quinolone resistance. However, all isolates but one had a point mutation in the *parC* region leading to the amino acid substation E62K. This strongly suggests an influence of this mutation for the (fluoro)quinolone resistance in *E. coli*. Vingopoulou et al. [75] described this ParC E62K substitution previously. They detected this new amino acid exchange in enrofloxacin-resistant *E. coli* isolated from dog otitis and faecal samples. As enrofloxacin is classified to the group of the (fluoro)quinolones, this would support the hypothesis of the E62KL exchange enhancing the (fluoro)quinolone resistance. In total, 87 of the analysed isolates were (fluoro)quinolone-resistant but carried only a *qnr* gene without any other PMQR genes associated with the development of the resistance. Possibly, the presence of *qnr* genes is sufficient to increase the MIC for NAL to a degree of resistance. One could also consider the development of yet unknown genes or point mutations in *E. coli* involved in this (fluoro)quinolone resistance. This clearly demonstrates the urgency of monitoring for PMQR to estimate resistance against (fluoro)quinolones and the possible capability of *qnr* to enhance (fluoro)quinolone resistance in *E. coli*.

4. Conclusions

In this study, we determined the prevalence of *qnr*-genes among (fluoro)quinolone-resistant *E. coli* from livestock and food in Germany and analysed the potential risks associated with the dissemination of the respective plasmids. While the prevalence of 3.7% of *qnr*-carrying isolates within (fluoro)quinolone-resistant *E. coli* was rather low, the risk linked with *qnr*-positive isolates is due to other reasons. Most of the *qnr*-positive *E. coli* also carried other resistance genes leading to a multidrug resistance phenotype of the isolate, especially when *E. coli* isolates from the ESBL-monitoring were analysed. Next to the occurrence of resistance genes, we detected genes leading to pesticide resistance or virulence genes within the same *qnr*-positive isolate. Further, we found that *qnr*-plasmids were widely distributed. Hence, the spread of the *qnr*-plasmid is not restricted to specific matrices or certain Inc groups. Further, we could confirm findings reporting that reported that the sole presence of *qnr* can lead to phenotypic (fluoro)quinolone resistance. We found isolates being resistant to nalidixic acid and ciprofloxacin that only carried a *qnr* gene without further PMQR or point mutations in the respective area of the chromosome. Since *qnr* is mostly identified on mobile genetic elements, this finding stresses the possible spread of this resistance determinant. The outgoing risk from *qnr* genes needs to be taken seriously, especially when evaluating (fluoro)quinolone resistance in *E. coli* isolated from livestock and food.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/microorganisms9061308/s1. Supplemental Figure S1: Grouped bar chart of total number of resistant isolates per antimicrobial agent and matrix. Table S1: Sequences, annealing temperatures, and references of used primers for *qnr* detection. Table S2: Matrix and MIC values of investigated (fluoro)quinolone-resistant and *qnr*-positive *E. coli* isolates, as well as their resistance profile. Table S3: *p*-value of respective Fisher's exact test; *p*-values < 0.05 represent statistical significance and are highlighted in red. The value 1 reflects that the two genes were not detected; columns with only 1 as value are not presented. Table (A) shows the correlation for genes detected in the ESBL-monitoring isolates. Table (B) shows the correlation for genes detected in the ZoMo isolates. Table S4: Inc-group and resistance genes of all the best-matching reference plasmids to the plasmids carrying *qnr* genes detected in this study. Identified with RefSNPer.

Author Contributions: K.J. and J.A.H. conceived the study and designed and conducted the experiments. J.A.H. supervised the project. J.A.H., M.G., A.K. and S.S. (Silvia Schmoger) performed and managed investigations regarding the monitoring programs. K.J. and C.D. performed the bioinformatics analyses. K.J., C.D. and J.A.H. analysed these bioinformatics data. K.J. provided the draft manuscript. J.A.H., B.M., D.M., A.K. and S.S. (Stefan Schwarz) decisively contributed to editing the manuscript. All authors have read and agreed to the published version of the manuscript.

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2.3 Publication III

Juraschek K, Käsbohrer A, Malorny B, Schwarz S, Meemken D, Hammerl JA.

Dissection of Highly Prevalent *qnrS1*-Carrying IncX Plasmid Types in Commensal *Escherichia coli* from German Food and Livestock

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		contribution
1.	Design of the project including design of individual experiments	80%
2.	Performing the experimental part of the study	75%
3.	Analysis of the experiments	90%
4.	Presentation and discussion of the study in article form	80%

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Article

Dissection of Highly Prevalent *qnrS1*-Carrying IncX Plasmid Types in Commensal *Escherichia coli* from German Food and Livestock

Katharina Juraschek [1,*], Annemarie Käsbohrer [1,2], Burkhard Malorny [1], Stefan Schwarz [3], Diana Meemken [4] and Jens André Hammerl [1,*]

1 Department Biological Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn Str. 8-10, 10589 Berlin, Germany; annemarie.kaesbohrer@bfr.bund.de (A.K.); Burkhard.Malorny@bfr.bund.de (B.M.)
2 Unit for Veterinary Public Health and Epidemiology, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria
3 Department of Veterinary Medicine, Institute of Microbiology and Epizootics, Freie Universität Berlin, 14163 Berlin, Germany; stefan.schwarz@fu-berlin.de
4 Department of Veterinary Medicine, Institute of Food Safety and Food Hygiene, Freie Universität Berlin, 14163 Berlin, Germany; Diana.Meemken@fu-berlin.de
* Correspondence: Katharina.Juraschek@bfr.bund.de (K.J.); Jens-Andre.Hammerl@bfr.bund.de (J.A.H.)

Abstract: Plasmids are mobile genetic elements, contributing to the spread of resistance determinants by horizontal gene transfer. Plasmid-mediated quinolone resistances (PMQRs) are important determinants able to decrease the antimicrobial susceptibility of bacteria against fluoroquinolones and quinolones. The PMQR gene *qnrS1*, especially, is broadly present in the livestock and food sector. Thus, it is of interest to understand the characteristics of plasmids able to carry and disseminate this determinant and therewith contribute to the resistance development against this class of high-priority, critically important antimicrobials. Therefore, we investigated all commensal *Escherichia* (*E.*) *coli* isolates, with reduced susceptibility to quinolones, recovered during the annual zoonosis monitoring 2017 in the pork and beef production chain in Germany (n = 2799). Through short-read whole-genome sequencing and bioinformatics analysis, the composition of the plasmids and factors involved in their occurrence were determined. We analysed the presence and structures of predominant plasmids carrying the PMQR *qnrS1*. This gene was most frequently located on IncX plasmids. Although the *E. coli* harbouring these IncX plasmids were highly diverse in their sequence types as well as their phenotypic resistance profiles, the IncX plasmids-carrying the *qnrS1* gene were rather conserved. Thus, we only detected three distinct IncX plasmids carrying *qnrS1* in the investigated isolates. The IncX plasmids were assigned either to IncX1 or to IncX3. All *qnrS1*-carrying IncX plasmids further harboured a β-lactamase gene (*bla*). In addition, all investigated IncX plasmids were transmissible. Overall, we found highly heterogenic *E. coli* harbouring conserved IncX plasmids as vehicles for the most prevalent *qnr* gene *qnrS1*. These IncX plasmids may play an important role in the dissemination of those two resistance determinants and their presence, transfer and co-selection properties require a deeper understanding for a thorough risk assessment.

Keywords: *E. coli*; *qnrS1*; IncX; fluoroquinolones; plasmids; transferability

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1. Introduction

The World Health Organization (WHO) recognizes fluoroquinolones (FQ) as critically important antimicrobials (CIA) for the treatment of human infections [1,2]. Further resistance development against antimicrobials of this class should thus be avoided. The food production chain, starting from livestock and ending at the food product, plays an important role in the transmission of antimicrobial-resistant microorganisms, as well as for their evolution and dissemination [1,3,4]. *Escherichia* (*E.*) *coli*, a commensal species of the

gastrointestinal tract of animals and humans, is a suitable indicator organism for monitoring the emergence of genes, leading to antimicrobial resistance (AMR) in gram-negative bacteria [5,6]. *E. coli* is also a common reservoir for mobile genetic elements (MGEs), such as plasmids, involved in the dissemination of genetic information to other commensal or pathogenic enteric microorganisms [7]. The screening of antimicrobial-resistant *E. coli* from livestock and food is widely established to estimate the prevailing AMR situations and dynamics over time.

Plasmids and other MGEs are major contributors to the spread of genetic information by horizontal gene transfer [8–10]. In general, they support the evolution and diversification of bacteria for e.g., developing resistances or novel pathotypes. In bacteria, horizontal gene transfer is mainly attributed to the spread of MGEs as gene cassettes, transposons, integrative conjugative elements (ICEs), and plasmids [11–14]. For the spread of FQ resistances, plasmid-mediated quinolone resistance (PMQR) genes play a major role. PMQR genes are notified to be substantially involved in the spread of FQ resistance in livestock [7,8]. Furthermore, several PMQR genes (*qnr*, *aac*(6′)-*lb-cr*, *qepA* and *oqxAB*) are known to be associated with a decrease in susceptibility against FQ. *qnrS1* especially was frequently reported as transmissible FQ-resistance gene in *E. coli* from food and livestock [15–21]. *qnrS*1 is of special concern, as this gene is often reported to be co-localized with resistance genes against extended spectrum cephalosporins (ESC) or resistance determinants to other antimicrobial classes. This gene has been shown to occur on plasmids carrying $bla_{\text{CTX-M}}$ genes [6,8,16,18,22]. The co-occurrence of antimicrobial resistance genes on plasmids can lead to a long-term persistence of these elements by antimicrobial co-selection, which provides not only a selective advantage but also promotes their spread [10,23]. Qnr proteins are known to be associated with low-level resistances against FQ [24]. However, the genes are recognized for facilitating the selection of high-level FQ resistance in gram-negative bacteria [1,25,26]. Moreover, it has been shown that isolates carrying PMQR genes support the alteration of chromosomal sequences also involved in FQ resistance development [1,27–30]. Thus, further information on the occurrence of *qnrS1* in livestock and food will support a deeper understanding of potential sources of this determinant, mechanisms involved in its dissemination and the diversity of associated plasmids. Plasmids are mainly subdivided on the basis of specific incompatibility sequences (*inc* groups). This classification takes into account their stable co-residence in the same bacterial cell without any selection pressure [9,31]. The determination of predominant plasmid types will provide further information on the impact of specific MGEs in the spread of *qnrS1* and will help to evaluate the risk of FQ resistance development in other compartments, such as the human sector.

This study aims to identify prevalent *inc* plasmid types carrying *qnrS1* originating from the German livestock and food sector for the beef and pork production chain. We aimed to determine the genetic basis of elements involved in FQ resistance development, and to derive the core plasmid backbones of predominant *qnrS1*-carrying plasmids by whole-genome sequencing (WGS) and bioinformatics analysis. Furthermore, the commonalities and dissimilarities of the most prevalent *qnrS1*-carrying plasmids were depicted. Such studies will help to evaluate potential evolutionary processes associated with the occurrence and spread of *qnrS1*-carrying plasmids. Further, the conjugation ability of those plasmids was investigated to better assess the likelihood of *qnrS1* transmission.

2. Results and Discussion

2.1. qnrS1 Is Highly Prevalent on IncX Plasmids in Commensal E. coli

Out of the investigated *E. coli* from livestock and food, *qnrS1* was the most prevalent PMQR gene. Of 2799 *E. coli* isolates obtained during the German monitoring programs in 2017, we identified 391 isolates representing a non-wildtype against ciprofloxacin (MIC $\geq$ 0.06 µg/mL) and/or nalidixic acid (MIC $\geq$ 16 µg/mL). PCR amplification revealed that 97 isolates carried *qnrS1*, while all other *qnr* determinants were detected only sporadically. S1-PFGE of *qnrS1*-positive *E. coli* coupled with Southern-blotting and DNA-DNA hybridization indicated that 85 isolates carried the *qnrS1* gene on a plasmid. The

12 chromosomally encoded *qnrS1* genes were detected in isolates representing eight distinct multi-locus sequence types (STs). This observation suggested a high heterogeneity of the *E. coli* carrying this PMQR gene within the chromosome. We found *qnrS1* to be the most frequent PMQR gene in isolates from the here investigated veal and pork source, as investigated in the monitoring program in 2017 (Table 1). To assign the plasmids to specific *inc* groups, we mapped the WGS data to all available *qnr* plasmid genomes published on NCBI as references, using the plasmidID tool. By this analysis, a high heterogeneity of *qnr* plasmids was detected. Out of our investigated WGS data of our isolates, two main clusters represented by IncY (n = 19) and IncX (n = 29) plasmids were determined. However, the *E. coli* comprising *qnrS1*-carrying IncX-plasmids were found to be highly diverse. The corresponding isolates exhibiting diverse STs were from different origins and exhibited distinct resistance profiles. Based on the XbaI-macrorestriction profiles, the high diversity of *E. coli* could be confirmed (data not shown), indicating that the occurrence of *qnrS1*-positive isolates is mainly triggered by the transmission of *qnrS1*-carrying plasmids. However, 23 of the 29 investigated *E. coli* with a *qnrS1* on an IncX plasmid were phenotypically resistant against ampicillin, demonstrating the potential link of *qnrS1* and *bla* genes.

Table 1. Characteristics of *E. coli* carrying *qnrS*1 on an IncX plasmid.

Isolate	ST	Resistance Genes *	Source	Phenotypic Resistance Profile
17-AB00542	1288	*aph*(3′′)-*Ib*, *aph*(6)-*Id*, bla_{EC}, bla_{TEM-1}, *qnrS1*, *tet*(B)	calf, faeces	TET
17-AB00544	155	*aph*(3′)-*Ia*, bla_{EC-18}, $bla_{TEM-176}$, *dfrA14*, *floR*$^{+}$, *qnrS*1, *tet*(A)	calf, faeces	AMP, CHL, CIP, TET, TMP
17-AB00639	10	*aac*(3)-*IVa*, *aph*(3′′)-*Ib*, *aph*(4)-*Ia*, *aph*(6)-*Id*, $bla_{CTX-M-1}$, bla_{EC}, bla_{TEM-1}, *dfrA5*, *mph(A)*, *qnrS1*, *sul2*	pig, faeces	AMP, CIP, FOT, GEN, SMX, TAZ, TMP
17-AB00742	10	*aadA1*, bla_{EC-15}, bla_{TEM-1}, *qnrS*1	pig, faeces	AMP, CIP
17-AB00995	392	*aph*(3′′)-*Ib*, *aph*(6)-*Id*, bla_{EC-18}, bla_{TEM-1}, *qnrS1*, *sul2*, *tet*(B)	calf, faeces	AMP, CIP, SMX, TET
17-AB01005	1244	*aadA1*, *aph*(3′′)-*Ib*, *aph*(6)-*Id*, bla_{EC}, bla_{SHV-12}, *qnrS1*, *tet*(B)	calf, faeces	FEP, FOT, TAZ
17-AB01006	10	bla_{EC-15}, bla_{SHV-12}, *qnrS1*	calf, faeces	FEP, FOT, TAZ
17-AB01018	88	*aph*(3′′)-*Ib*, *aph*(3′)-*Ia*, *aph*(6)-*Id*, bla_{EC-13}, bla_{SHV-12}, bla_{TEM-1}, *dfrA5*, *floR*, *qnrS1*, *sul2*, *tet*(A)	pig, faeces	AMP, CHL, CIP, FOT, SMX, TAZ, TET, TMP
17-AB01105	58	*aadA5*, $bla_{CTX-M-1}$, bla_{EC-18}, bla_{TEM-1}, *dfrA*17, *dfrA5*, *qnrS1*, *sul2*, *tet*(A)	pig, faeces	FEP, FOT, TAZ
17-AB01352	88	bla_{EC-13}, bla_{TEM-1}, *qnrS1*, *tet*(A)	calf, meat	AMP, CIP, TET
17-AB01531	34	bla_{EC}, bla_{TEM-1}, *qnrS1*, *sul2*,	pig, faeces	AMP, CIP, SMX
17-AB01539	10	*aadA1*, bla_{EC}, bla_{TEM-1}, *qnrS1*	pig, faeces	AMP, CIP
17-AB01619	10	*aac*(3)-*IIa*, *aadA5*, *aph*(3′)-*Ia*, $bla_{CTX-M-15}$, bla_{EC}, $bla_{TEM-176}$, *dfrA14*, *dfrA17*, *floR* $^{+}$, *mph*(A), *qacE*Δ*1*, *qnrS1*, *sul1*, *sul2*, *tet*(A), *tet*(B)	calf, faeces	AMP, AZI, CHL, CIP, FOT, GEN, NAL, SMX, TAZ, TET, TMP
17-AB01686	1288	*aph*(3′′)-*Ib*, *aph*(3′)-*Ia*, *aph*(6)-*Id*, $bla_{CTX-M-15}$, bla_{EC}, $bla_{TEM-176}$, *dfrA14*, *floR*$^{+}$, *qnrS1*, *tet*(A), *tet*(B)	calf, faeces	AMP, CHL, CIP, FOT, SMX, TAZ, TET, TMP
17-AB01707	10	*aph*(3′)-*Ia*, bla_{EC}, $bla_{TEM-176}$, *dfrA14*, *floR* $^{+}$, *qnrS1*, *tet*(A)	calf, faeces	AMP, CHL, CIP, TET, TMP
17-AB01752	641	*aph*(3′′)-*Ib*, *aph*(6)-*Id*, bla_{EC-13}, bla_{TEM-1}, *qnrS1*, *tet*(B)	pig, faeces	AMP, CIP, TET
17-AB01792	101	*aadA5*, $bla_{CTX-M-1}$, bla_{EC-18}, bla_{TEM-1}, *dfrA17*, *qnrS1*, *sul2*	pig, faeces	FEP, FOT, TAZ
17-AB01795	10	bla_{EC-15}, bla_{TEM-1}, *qnrS1*	pig, faeces	AMP, CIP
17-AB01798	641	*aadA1*, *aadA2*, *aph*(3′′)-*Ib*, *aph*(6)-*Id*, bla_{EC-13}, bla_{SHV-12}, bla_{TEM-1}, *cmlA1*, *dfrA32*, *ere*(A), *mef*(B), *qacE*Δ*1*, *qacL*, *qnrS1*, *sul1*, *sul3*, *tet*(A)	pig, faeces	FEP, FOT, TAZ

Table 1. *Cont.*

Isolate	ST	Resistance Genes *	Source	Phenotypic Resistance Profile
17-AB01875	10	*aph(3′)-Ia*, $bla_{\text{CTX-M-1}}$, bla_{EC}, $bla_{\text{TEM-176}}$, *dfrA14*, *floR*$^{+}$, *mph*(A), *qnrS1*, *tet*(A)	calf, faeces	AMP, CHL, CIP, FOT, TAZ, TET, TMP
17-AB01969	711	$bla_{\text{EC-18}}$, $bla_{\text{TEM-1}}$, *mph*(A), *mph*(E), *msr*(E), *qnrS1*	calf, faeces	AMP, AZI, CIP
17-AB02071	58	$bla_{\text{EC-18}}$, $bla_{\text{SHV-12}}$, *qnrS1*	calf, faeces	AMP, CIP, FOT, TAZ
17-AB02090	48	$bla_{\text{CTX-M-1}}$, $bla_{\text{EC-15}}$, $bla_{\text{TEM-1}}$, *qnrS1*	calf, faeces	AMP, CIP, FOT, TAZ
17-AB02355	2230	*aadA1*, *aadA2*, *aph(6)-Id*, $bla_{\text{EC-13}}$, $bla_{\text{SHV-12}}$, $bla_{\text{TEM-1}}$, *cmlA1*, *dfrA14*, *qacL*, *qnrS1*, *sul2*, *sul3*, *tet*(A)	pig, faeces	AMP, CIP, FOT, SMX, TAZ, TET, TMP
17-AB02707	58	*aac*(3)-*IVa*, *aph*(3′′)-*Ib*, *aph(6)-Id*, $bla_{\text{CTX-M-1}}$, $bla_{\text{EC-18}}$, $bla_{\text{TEM-1}}$, *qnrS1*	calf, faeces	AMP, CIP, FOT, TAZ
17-AB02711	7469	*aadA2*, *aph*(3′)-*Ia*, $bla_{\text{CTX-M-1}}$, $bla_{\text{EC-8}}$, $bla_{\text{TEM-176}}$, *dfrA14*, *floR*$^{+}$, *lnu*(F), *mph*(A), *qnrS1*, *tet*(A)	calf, faeces	AMP, CIP, FOT, SMX, TAZ, TET, TMP
17-AB02721	7469	*aadA2*, *aph*(3′)-*Ia*, $bla_{\text{CTX-M-1}}$, $bla_{\text{EC-8}}$, $bla_{\text{TEM-176}}$, *dfrA14*, *floR* $^{+}$, *lnu*(F), *mph*(A), *qnrS1*, *tet*(A)	calf, faeces	FEP, FOT, TAZ
17-AB02726	7469	*aadA2*, *aph*(3′)-*Ia*, $bla_{\text{CTX-M-1}}$, $bla_{\text{EC-8}}$, $bla_{\text{TEM-147}}$, *dfrA14*, *floR* $^{+}$, *lnu*(F), *mph*(A), *qnrS1*, *tet*(A)	calf, faeces	FEP, FOT, TAZ
17-AB02951	2496	*aadA1*, *aadA5*, *aph*(3′′)-*Ib*, *aph(6)-Id*, $bla_{\text{CTX-M-1}}$, $bla_{\text{EC-15}}$, $bla_{\text{TEM-1}}$, *dfrA1*, *dfrA17*, *mef*(C), *mph*(B), *mph*(G), *qacΔ1*, *qnrS1*, *sul1*, *sul2*	pig, faeces	FEP, FOT, TAZ

$^{+}$ Identity below 100%; * If possible, the variant for bla_{EC}-like genes is given. However, for some genes the variant could not be determined. Abbreviations: AMP = ampicillin, CHL = chloramphenicol, CIP = ciprofloxacin, FOT = cefotaxime, GEN = gentamicin, NAL = nalidixic acid, SMX = sulfamethoxazole, TAZ = ceftazidime, TET = tetracycline, TMP = trimethoprim.

The data of our analysis are in good agreement with previously published results. Similar to our observation of calf and pig isolates, *qnrS1* was also identified as the most prevalent PMQR gene in *E. coli* from investigated turkeys, broilers and layers worldwide [15,20,21]. Based on the combination of our results and the prevailing literature, *qnrS1* seems to be the most frequent PMQR gene in farm animals. It also seems that there is a strong association of *qnrS1* to IncX plasmids. Several plasmids of this incompatibility group have been described as efficient carriers of this gene in *E. coli* [1,8,16,19,32–34]. Dolejska et al. [6] further detected *qnrS*-carrying IncX plasmids in other sources i.e., horses, environmental samples and flies at an equine clinic.

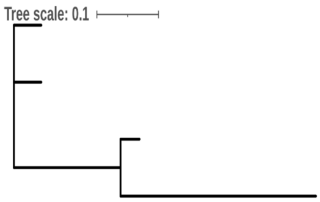
In this study, *qnrS1* was found to be the most prevalent PMQR gene in livestock and food, frequently associated with plasmids of the IncX group. The *qnrS1*-carrying IncX plasmids were found to be disseminated among different *E. coli* STs recovered from various sources. As such, plasmids are often found in various genera or species of the Enterobacteriaceae, the main routes of transmission and spread need to be determined. To assess this further, in silico analysis of the genomes was performed to achieve deeper knowledge on the evolution of the plasmids, their stability and its dissemination.

2.2. Three Prevalent IncX Plasmids, Carrying qnrS1 in German Livestock Were Detected

The investigation of the *qnrS*1 IncX genomes resulted in the detection of three distinct reference plasmids representing the most frequent plasmid types present in German livestock in 2017. Table 2 includes the phylogenetic relationship of the plasmids. Therewith, the short-read sequences of only one isolate resemble the unnamed reference plasmid of strain R1701 (NZ_CP039972.1, *Klebsiella pneumoniae*). All other reference plasmids are represented by 14 (NZ_CP020088.1, unnamed plasmid identified in *Shigella flexneri*), eight (NZ_CP037995, psg_ww281 plasmid identified in *Salmonella enterica* subsp. *enterica* serovar Brancaster) and six (NZ_CP031373.1, pKpvST101_6 plasmid identified in *Klebsiella pneumoniae*) WGS datasets from our study. In general, the plasmid sequences are highly conserved (94% to 100% mapped) in comparison to their reference plasmids, indicating that only

a minor evolutionary adaption prevails. All of the most frequently detected reference plasmids were larger in size and carried a *bla* gene, as presented in Table 2.

Table 2. Characteristics of best-matching IncX plasmids carrying *qnrS*1 with aligned neighbour-joining tree without distance corrections of all four best matching reference plasmids based on Clustal Omega multiple sequence alignment.

	Plasmid Name	Reference Plasmid	AMR Genes	Inc	bp
	unnamed plasmid of strain R17071	NZ_CP039972.1	bla_{TEM} *	IncR, IncX1	16,795
	pKpvST101_6	NZ_CP031373.1	bla_{SHV}, *qnrS1*	IncX3	43,670
	unnamed plasmid of strain 0670	NZ_CP020088.1	bla_{TEM-1}, *qnrS1*	IncX1, IncX3	47,674
	psg_ww281 plasmid	NZ_CP037995.1	*aph(3′)-Ia*, $bla_{TEM-176}$, *dfrA14*, *floR*, *qnrS1*, *tet*(A)	IncX1	48,223

* a bla_{TEM} derivative was detected but is only covered by 52.45%.

Dolejska et al. [35] emphasized the correlation between these IncX plasmids comprising bla_{TEM} and $bla_{CTX-M-15}$ genes in association with *qnrS*, resulting in ESBL-producing *E. coli*. The frequent observation of *qnrS1*-carrying plasmids comprising ESBL-enhancing resistance genes stresses the necessity of thorough screening and a better characterization of *qnr*-positive *E. coli* for risk assessment. Furthermore, Guo et al. [36] described an IncX plasmid carrying a mobile colistin resistance gene (*mcr*). Thus, IncX plasmids seemed to be a potential reservoir for diverse combinations of resistances, decreasing the susceptibility against clinically important antimicrobials and antimicrobials of the last resort. IncX plasmids have regularly been described as a group harbouring *qnrS*1. Therefore, we decided to dissect this group of plasmids even further.

2.2.1. The Genomes of Prevalent *qnrS1*-Carrying IncX Plasmids

In general, IncX plasmids can be assigned to six distinct subgroups, namely IncX1 to IncX6 [37,38]. Here, we only detected IncX1, IncX3 or a combination of both as carriers for *qnrS1*. Overall, the unnamed reference plasmid of the strain 0670 (NZ_CP020088) was the most prevalent IncX plasmid type (WGS data of 14 isolates) detected to carry a *qnrS1* gene.

2.2.2. Characteristics of Plasmids Assigned to the Unnamed Reference Plasmid of the Strain 0670

Twelve out of 14 WGS datasets resemble the reference plasmid under the number NZ_CP020088 (Figure 1). Due to the frequent occurrence of these plasmids, we can conclude that its genome structure represents the most prevalent *qnrS1* plasmid of *E. coli* from German livestock in 2017. The plasmid is 47,674 bp in size and harbours an IncX1 (100%) and an IncX3 (80.59% identical to NZ_CP020088) replicon sequence. Further, the resistance determinants *qnrS1* and bla_{TEM-1} are present on the reference plasmids, as well as on our detected plasmids.

The unnamed plasmid (NZ_CP020088) originates from a *Shigella flexneri* isolate recovered in Hangzhou, China from human origin. Comparable plasmids were shown to be spread worldwide, as close relatives were detected i.e., in *E. coli* from turkey meat (LR882060) or chicken meat (MK965545) in Norway and Brazil, respectively. Resistance determinants and associated IS elements or transposases of these plasmids are located in a single DNA region of approx. 15 kb. Downstream of *qnrS1*, the *hin* DNA-invertase was detected, which was in vicinity to a IS*Kra4* and a Tn*3* transposase. Upstream of the Tn*3* transposase, the bla_{TEM-1} gene is located. Outside of the resistance-IS region, different components of the type IV secretion systems (*virB4*, *virD4*, *ptlE*, *virB9*) were detected. The plasmid of 17-AB00639 lacks a 1325 bp DNA region, which encoded an additional Tn*3* family transposase present on the reference plasmid. Transmissibility evaluation using the

mob-suite for these plasmids yielded an assignment of self-transmissibility (conjugative). All plasmids carried the MOB_P relaxase and the MPF_T mating pair formation (*mpf*) region. In vitro filter mating experiments demonstrated that all *qnrS*1 IncX-like plasmids were self-transmissible among *E. coli* at 37 °C. Verification of the plasmid structure within the *E. coli* J53 recipient showed no obvious differences between the plasmids of the donor strains and the transconjugants by PFGE and DNA-DNA hybridization. We thus conclude that the plasmids seemed to be genetically stable.

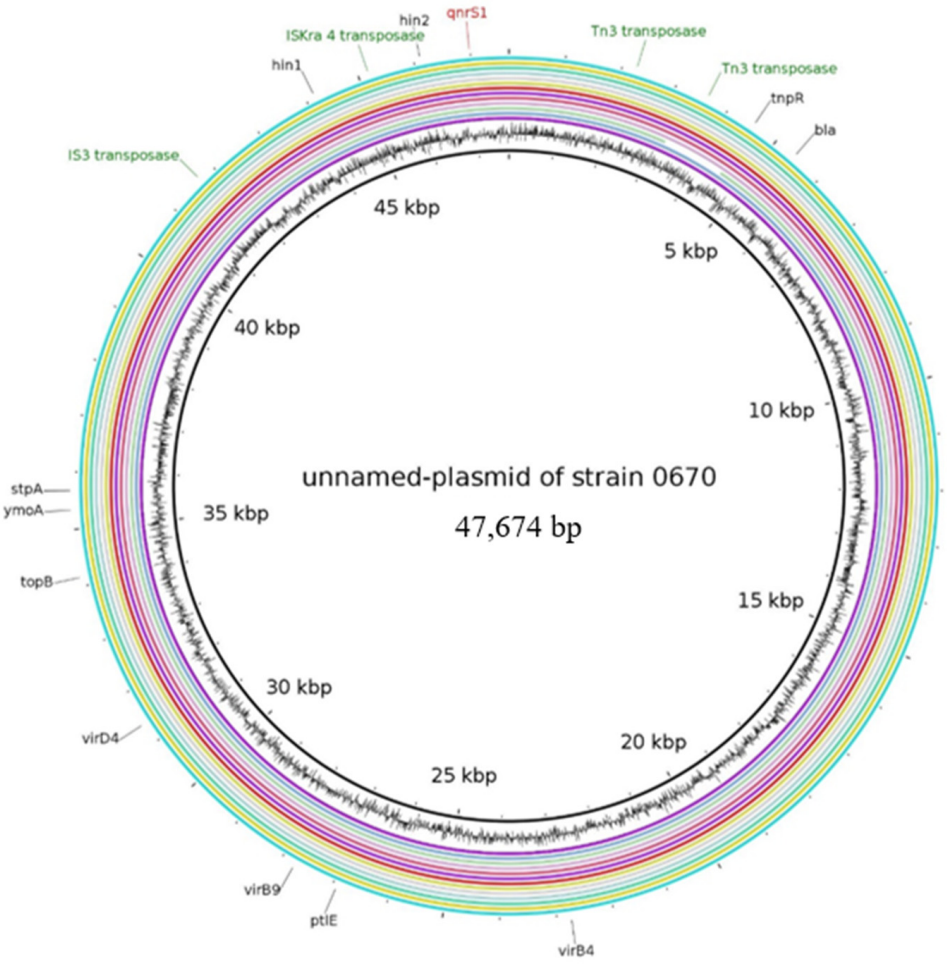


Figure 1. BRIG image of sequence contigs of individual isolates assigned to the unnamed reference plasmid (NZ_CP0200800). Contigs belonging to the isolates as indicated (from inner to outer ring): unnamed-plasmid of strain 0670, 17-AB00542, 17-AB00639, 17-AB00742, 17-AB00995, 17-AB01105, 17-AB01352, 17-AB01539, 17-AB01752, 17-AB01792, 17-AB01795, 17-AB01969, 17-AB02090, 17-AB02707 and 17-AB02951.

A frequent occurrence of *qnrS1* and bla_{TEM-1}-carrying InX1 plasmids was previously described by Dobiasova and colleagues [39]. They found the presence of these plasmids in Enterobacteriaceae from food-producing animals and wildlife in Europe. Therewith, the combined existence of *qnrS1* and bla_{TEM-1} was mentioned as common. Furthermore, the highly conserved backbone consisting of *taxC* (relaxase encoding gene), *qnrS1* and bla_{TEM} of these plasmids was discussed. Due to the detection of 12 closed plasmid structures out of 14 matching plasmids, this study confirms the frequent occurrence of this conserved plasmid structure. The co-occurrence of IncX1 and IncX3 replicons represents a multi-replicon type that might be beneficial for the plasmid as it is useful for stable replication in isolates carrying either IncX1 or IncX3 plasmids. This plasmid structure again represents a possible evolvement for the dynamics of the *qnrS1* plasmid dissemination. All 14 plasmids

carried a *pir* gene (encoding for replication initiation) and the type IV secretion system, necessary for conjugational transfer. A similar high conservation of the plasmid backbone was also described before [40]. The DNA-invertase gene *hin* as well as both resistance determinants were present in all detected plasmids matching to the reference plasmid. However, two plasmids comprised slightly altered structures indicating a possible hot spot for further evolutionary adaptions or acquisition of further resistance determinants.

2.2.3. Characteristics of Plasmids Assigned to the Reference Plasmid pKpvST101_6

Plasmid pKpvST101_6-like (NZ_CP031373) structures were detected in six WGS datasets of the investigated isolates. pKpvST101_6 is 43,670 bp in size and carries an IncX3 replicon. The DNA region encoding the IS elements (IS*6*, IS*Kra4*), transposases and resistance determinants is 8.5 kb in size (Figure 2). This region includes an IS*Kra4* transposase gene, followed by the DNA-invertase genes *hin1* and *hin2*, and *qnrS1* encoding the pentapeptide repeat protein. Right after this structure the bla_{SHV} genes are present, flanked by the IS*6* transposase gene on each site. Outside of the resistance determinant carrying DNA region, the plasmid harbours the type IV secretion system genes *ptlH*, *virB4* and the conjugational transfer gene *traG*.

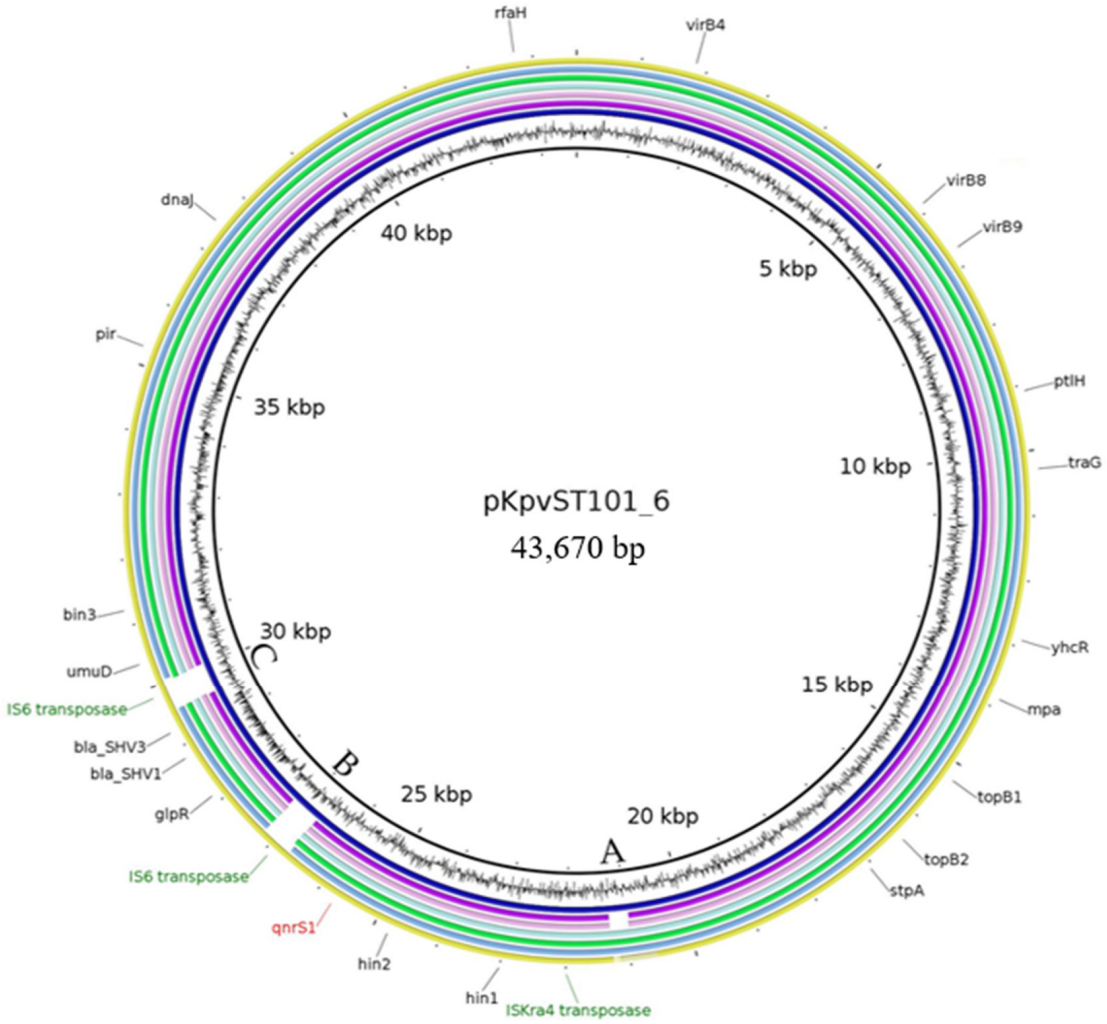


Figure 2. BRIG image of sequence contigs of individual isolates assigned to the reference plasmid pKpvST101_6 (NZ_CP031373). Contigs belonging to the isolates as indicated (from inner to outer ring): pKpvST101_6, 17-AB01005, 17-AB01006, 17-AB01018, 17-AB01798, 17-AB02071 and 17-AB02673. Regions lacking within the investigated are indicated by the capital letters A, B and C.

This plasmid was first detected in a *Klebsiella pneumoniae* strain from a hospital in the United Kingdom (CP031373.2). Similar plasmids were reported from an *E. coli* of poultry origin from the Netherlands (KX618696.1) and from *Citrobacter freundii* of a healthcare environment in Spain (MT720906.1). The plasmids detected in our study lacked certain regions compared to the reference plasmid, except for the plasmid occurring in isolate 17-AB02673. All other datasets were lacking two regions (B and C in Figure 2). Furthermore, the pKpvST101_6-like plasmids of 17-AB01005 and 17-AB01006 lacked an additional region. This included two IS*6* transposase genes, which are located downstream and upstream of the bla_{SHV}. The area A is missing in the two plasmids detected in 17-AB01005 and 17-AB01006 (Figure 2). However, it did not cover a CDS. This missing sequence was located next to the IS*Kra4* transposase gene. Besides the reference plasmid pKpvST101_6, also the reconstructed plasmids out of the livestock isolates were assigned to be self-transmissible using the mob-suite. Furthermore, this prediction could be experimentally confirmed by in vitro filter mating studies leading to an efficient self-transfer between donor and recipient *E. coli*.

IncX3 plasmids, as carriers for *qnrS1* and bla_{SHV} genes, have been described as common in central Europe [39] and China [41]. Especially the presence of IS*26* (IS*6* family transposase) in the vicinity to the bla_{SHV} gene was notified before [42] and described as mobilizing-factor for the β-lactam resistance gene. Moreover, IncX3 plasmids have been described as carriers of carbapenem resistance genes, such as bla_{NDM}, in clinical environments [43,44]. Thus, such plasmids play an important role in the dissemination of resistances against last resort antimicrobials. Furthermore, several IncX3 plasmids have been reported to carry *qnrB*, *qnrS* and *bla* genes [41,42,44,45], highlighting the importance of this plasmid for the dissemination of antimicrobial resistance genes in Enterobacteriaceae [37]. In contrast to the published results, the majority of our IncX3 plasmids of this study lacked the IS*6* transposase gene. In addition, a non-coding area next to the IS*Kra4* transposase gene was not detected in two plasmids, while present in the reference plasmid. This could suggest the alteration of the plasmid in its resistance determinant area. Therefore, as conjugative plasmid, carrying two important resistance determinants, the complete structure of this IncX3 plasmid should be investigated further.

2.2.4. Characteristics of Plasmids Assigned to the Reference Plasmid psg_ww281

Another frequently detected plasmid type matched to the reference plasmid psg_ww281 (NZ_CP037995). It was recognized as an IncX1 plasmid of 48,223 bp. This plasmid carried multiple resistance genes, including *aph(3′)-Ia*, $bla_{TEM-176}$, *dfrA14*, *floR*, *qnrS1*, and *tet*(A). Thus, it confers phenotypic resistance against antimicrobials of different classes. In total, eight WGS datasets of the livestock isolates resemble this reference plasmid. As shown in Figure 3, the best-matching plasmids lacked certain regions in comparison to the reference plasmid psg_ww281.

For the first time, this reference plasmid was reported in a *Salmonella enterica* from a wet market in Singapore. A close relative of psg_ww281 was also found in Singapore, but occurred in an *E. coli* (plasmid pSGMCR103 (MK731977.1)). Later on, a similar plasmid was described from an *E. coli* of the Czech Republic (plasmid pCE1594 (MT859327.1)). On the reference plasmids, the resistance determinants are scattered within a DNA region ranging between 12 to 30 kb (Figure 3). The core genome of this plasmid type carries the resistance gene *bla* followed by an IS*6* transposase gene, downstream followed the DNA-invertase gene *hin*, the PMQR gene *qnrS*1 followed by another IS*6* transposase gene. Further upstream, the resistance genes *floR* and *aph(3′)-Ia* were located. However, the IS*6* transposase gene in vicinity to $bla_{TEM-176}$ was lacking in our plasmids investigated here. The gene for a hypothetical protein downstream of *qnrS1,* as well as the IS*6* transposase gene downstream of *qnrS1,* were also not detectable. In addition, the IS*6* transposase genes flanking *dfrA* were missing, compared to the reference. The plasmid psg_ww281, as well as the reconstructed plasmids from our in silico analysis, were determined to be self-transmissible, using the mob-suite as the MOB_P relaxase and the MPF_T mating pair

formation type was detected in all psg_ww281-like genomes. The conjugative behaviour of the IncX1 plasmids could be confirmed by laboratory investigations and was determined to be efficient among *E. coli*.

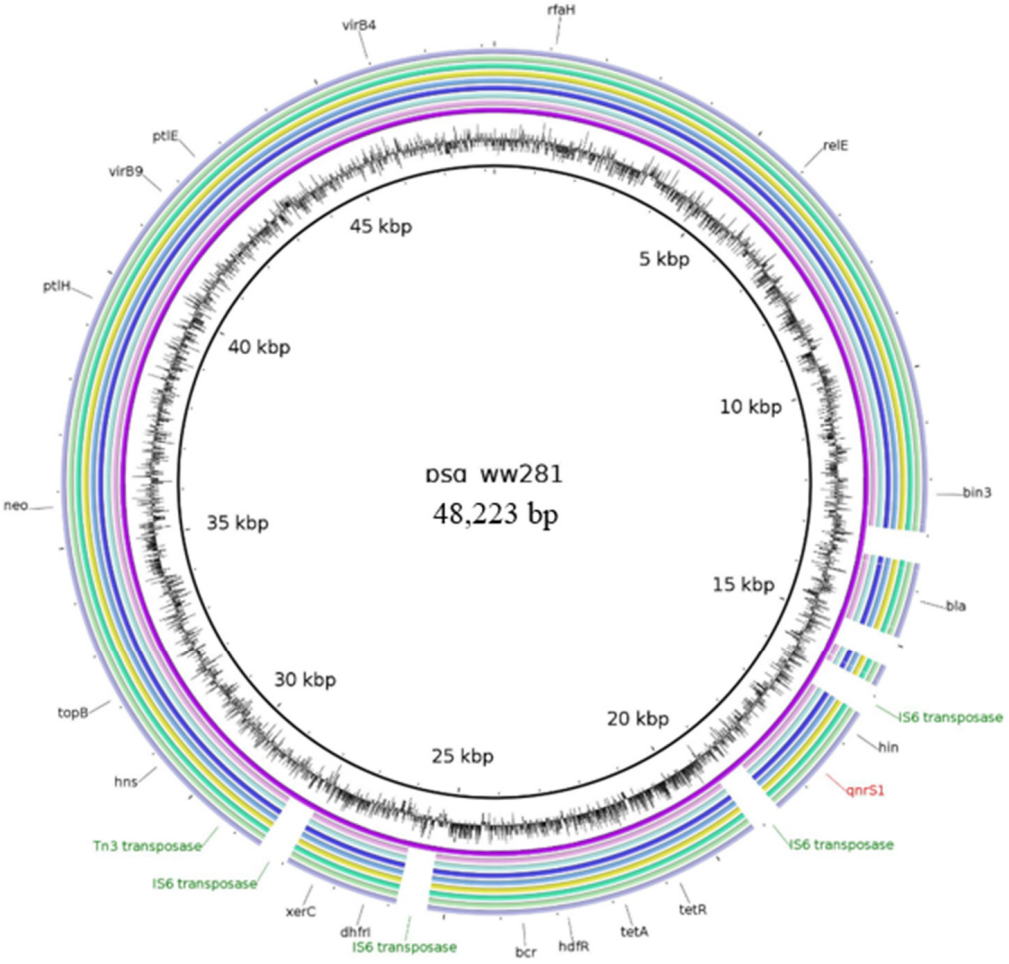


Figure 3. BRIG image of sequence contigs of the individual isolates assigned to the reference plasmid psg_ww281 (NZ_CP031373). Contigs belonging to the isolates as follows (from inner to outer ring): psg_ww281, 17-AB00544, 17-AB01619, 17-AB01686, 17-AB01707, 17-AB01875, 17-AB02711, 17-AB02721 and 17-AB02726.

The predominant IncX1 psg_ww281-plasmid is comparable to the aforementioned unnamed IncX1 reference plasmid (NZ_CP020088). The conserved sequence of this plasmid carried the DNA invertase (*hin*) and different components of the type IV secretion system (*ptl*, *vir*). However, in addition to the *qnrS1* and bla_{TEM} genes, this plasmid type acquired further resistance determinants, thus, presumably demonstrating the evolution of the plasmids regarding resistance development. Interestingly, the pattern of missing IS*6* elements, compared to the reference, was observed. This can be a result of assembly difficulties in the repeat-rich area of IS elements. It can also present a German counterpart plasmid, compared to the psg_ww281-plasmid. Thus, it would present a plasmid, lacking those IS6 elements and therewith the mobility of the respective resistance genes. As this plasmid type was frequently detected in Europe, it probably represents an important vehicle for resistance progression and should therefore be further monitored.

2.2.5. Characteristics of the Plasmid Assigned to the Unnamed Reference Plasmid of the Strain R1701

For one isolate, the best-matching reference plasmid was the unnamed plasmid of the strain R1701 (NZ_CP039972). This plasmid exhibited a size of 16,795 bp and did not carry any resistance determinants. The plasmid type was described first in a *Klebsiella pneumoniae* from human blood samples in the USA and seems to be rare, as no further relatives could be detected by blast searches. However, plasmids of larger size ranges with notable similarity to the reference genome were detected by nucleotide comparisons. Furthermore, the unnamed reference plasmid was assigned to the IncR group. When we investigated the contigs of our isolate matching the reference, we found that this plasmid seemed to be evolved into a *qnrS1*- and bla_{TEM}-carrying plasmid (Figure 4).

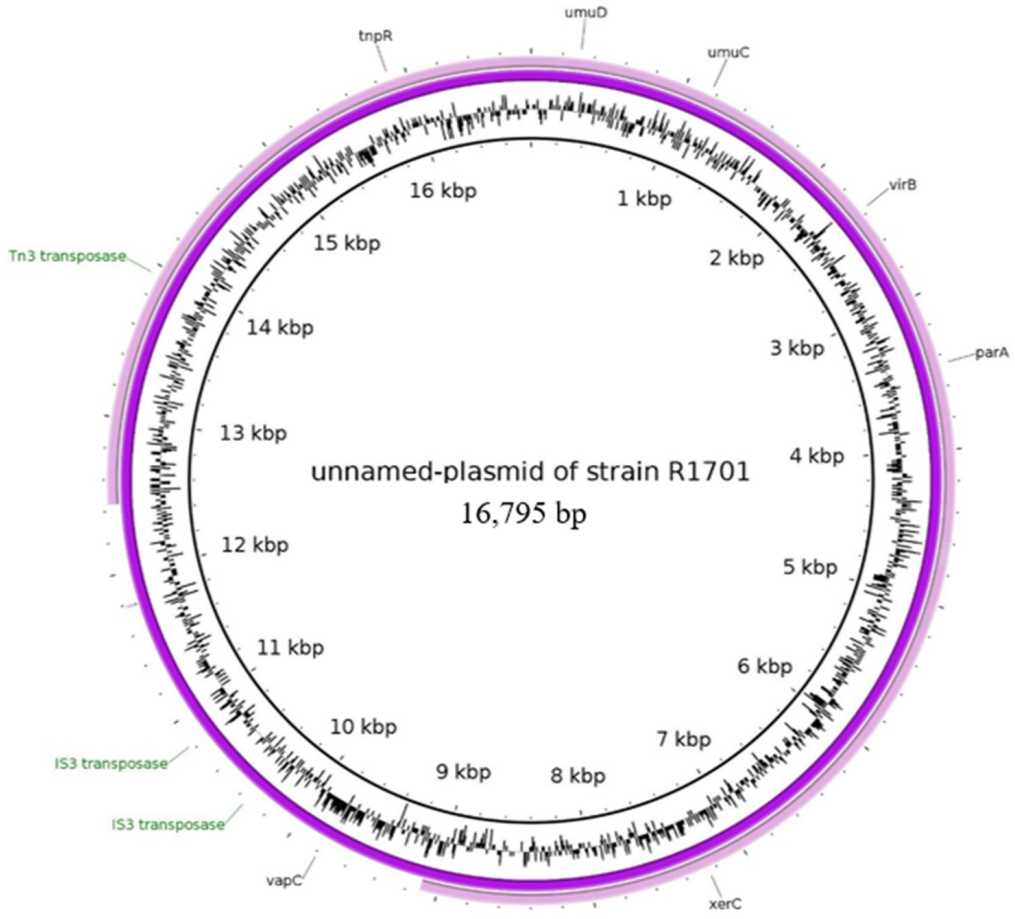


Figure 4. BRIG image of sequence contigs of individual isolates assigned to the unnamed reference plasmid (NZ_CP039972). Contigs belonging to the isolates as follows (from inner to outer ring): unnamed reference plasmid of the strain R1701 and 17-AB01531.

The plasmid (NZ_CP039972) carries multiple IS elements. In particular two IS3 transposase genes were detected. The in-silico generated organization of our plasmid is shown in Figure 5.

The structure presented in Figure 5 carries *qnrS*1 and bla_{TEM}. It represents the contig not present on the reference plasmid but assigned to it for our plasmid of the strain 17-AB01531. Similarly, to all other plasmid types described in this study, we found the DNA-invertase encoding genes *hin1* and *hin2*. The assignment of the contig to the plasmid led to the co-occurrence of the replicons IncX and IncR. Further in-silico analysis revealed the presence of remnant sequences of a bla_{TEM} gene on the reference plasmid. This remnant *bla* sequence was located from 16,346 to 16,795 bp and covers only 52.45% of the bla_{TEM} reference gene (NZ_CP039972). Using mob-suite, the reference plasmid was determined to

be non-conjugative. In addition, by experimental investigation no conjugative transfer of the plasmid was detected in *E. coli*.

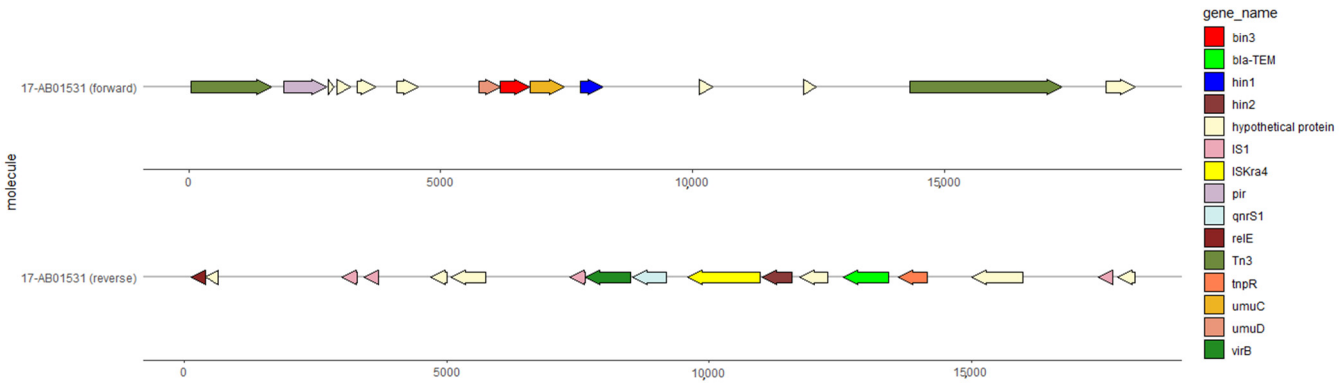


Figure 5. Structural organization of the *qnrS1*-carrying contig of isolate 17-AB01531 derived from mapping against the unnamed reference plasmid (NZ_CP039972). Upper line represents the annotated forward strand. Lower line represents the annotated reverse strand.

It is likely that the used reference plasmid does not represent the complete sequence plasmid correctly. As we detected a remnant sequence of the bla_{TEM} gene on the genome, it is possible, that the assigned contig sequence (Figure 5) could actually be present on the reference plasmid but not assembled correctly. This suggestion might be supported by the fact, that we could not detect any further plasmid of similar size but found rather larger genomes exhibiting larger DNA regions of high similarity to the reference plasmid. Thus, this type of plasmid might also present a platform for development of resistance gene accumulation. Moreover, the presence of two *inc* groups represents a potential hybrid of two distinct plasmids. This co-occurrence of different *inc* groups has been mentioned before, especially for IncX plasmids. Thus, IncX plasmid sequences were shown to co-integrate within different plasmid genomes, resulting in a broadening of the host range [46]. A project of Slettemeas et al. [1] confirmed this conjugation potential of IncX plasmids and states that these plasmids are successful and widely disseminated. Extending the narrow host range of IncX plasmids to a broader spectrum of potential host bacteria [33,40]. In general, it has been shown that this plasmid type is able to be spread to different species of Enterobacteriaceae. Although *qnrS1*-IncR plasmids had been described before [45], the combined presence of IncX and IncR seems to be rare, as we could not find any description in the current literature.

3. Materials and Methods

3.1. Isolate Characterization

All *E. coli* recovered during the annual zoonosis monitoring 2017 in Germany, covering the pork and beef production chain, were investigated regarding their minimum inhibitory concentration (MIC) on commercial test plates (EUVSEC/EUVSEC2; Sensititre™, TREK Diagnostic Systems, East Grinstead, UK). MIC values were interpreted according to EUCAST epidemiological cut-off values (ECOFFs) [47]. All isolates determined as being non-wildtype against nalidixic acid (MIC $\geq$16 µg/mL) and/or ciprofloxacin (MIC $\geq$ 0.06 µg/mL) were further subjected by PCR for *qnr* gene detection as described according to Cattoir et al. [48]. An extrachromosomal localization of the *qnrS* gene and a size prediction of the plasmid was performed by S1-nuclease pulsed-field gel electrophoresis (S1-PFGE) combined with Southern blotting and DNA-DNA hybridization against a digoxygenin-labelled *qnrS* probe [49]. The phylogenetic relationship of the *qnrS*-carrying *E. coli* was determined by XbaI-macrorestriction PFGE (XbaI-PFGE) in a CHEF-DR III system (Bio-Rad Laboratories, Madrid, Spain) according to the PulseNet standardized

laboratory protocol [49]. All *E. coli*, with a confirmed plasmidic localization of *qnrS* were subjected to whole-genome sequencing (WGS).

3.2. DNA Extraction and Sequencing

Genomic DNA of *E. coli* was prepared using the PureLink Genomic DNA Mini Kit (Invitrogen-Thermo Fisher, Schwerte, Germany), according to the manufacturer's recommendation. Sequencing DNA libraries were generated with the Nextera DNA Flex Library Preparation Kit (Illumina®, San Diego, CA, USA), as previously described [50]. Short-read, paired end whole-genome sequencing was performed in 2 × 151 cycles using the Illumina® NextSeq™ 500/550 Mid Output Kit v2.5 (300 Cycles). The Unicycler pipeline (version 0.4.4; Wick et al., 2017) recommended for bacterial genomes was used for de novo assembly. Evaluation and quality assessment of genome assemblies were conducted using QUAST 5.0.2 [51]. Assembled contigs were analysed for resistance genes and plasmid markers (i.e., replicon types) with bakcharak [52]. *E. coli* isolates determined to harbor a *qnrS1* gene on a plasmid with the most prevalent replicon type (IncX) were further investigated.

3.3. Bioinformatics Analysis, Characterization and Visualization of the WGS Data

To determine the most prevalent *qnrS1* plasmid type, a reference database comprising all available closed *qnr*-plasmid genomes of the Genbank database was developed. Raw reads of all individual isolates were aligned to the genomes of the *qnr*-plasmid database using plasmidID v1.6.5 (https://github.com/BU-ISCIII/plasmidID, accessed on 17 April 2021) to identify the matching reference based on the closest relationship. Further analysis and SNP difference prediction between the estimated reference and the actual investigated plasmid was performed using snippysnake (https://gitlab.com/bfr_bioinformatics/snippySnake, accessed on 17 April 2021).

Visualisation of DNA alignments was done with BRIG [53]. Investigation of similar plasmids was conducted through blast searches [54]. Determination of the multi-locus sequence types (MLST) and the identification of genes involved in antimicrobial resistance development was conducted using the bakcharak pipeline [52]. Annotation of genomes was operated with the annotation tool prokka (v1.14.5) [55]. Phylogenetic relationship of the plasmids was determined with Clustal Omega alignment (v1.2.4) [56] and visualised with iTOL (v6) [57]. Mapping of the corresponding sequences was conducted through visualisation and analysation of the bed-file in geneious (v2020.2.2) [58]. To determine the conjugational transfer of the respective plasmids, we further screened for MOB and MPF components with the mob-suite-tool [59].

3.4. Conjugational Test

The transferability of plasmids carrying *qnrS*1 was tested by in vitro filter mating studies. The filter mating experiments were conducted using the plasmid-free, sodium azide-resistant *E. coli* strain J53 as the recipient [60,61]. The conjugative transfer of plasmids was confirmed with S1-PFGE, and PCR as described above. The colonies were stored at −80 °C in a glycerol suspension.

4. Conclusions

Here, we determined the predominant *qnrS*1-carrying IncX plasmid types present in commensal and ESBL-producing *E. coli* of the German pork and beef production chain in 2017. Although the *E. coli* harbouring the respective IncX plasmids were highly heterogenic in their characteristics, the prevalent plasmids resemble a predominant genetic basis. In this study, we detected *qnrS1*-carrying IncX1 and IncX3 plasmids that also carried genes for resistance to other antimicrobials, such as *bla*. IncX plasmids seem to represent important carriers for the dissemination of resistance against clinically important antimicrobial agents. A deeper understanding and investigation of the persistence, evolutionary adaption and fitness of the plasmids is highly recommended.

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2.4 Publication IV

Juraschek K, Malekzadah J, Malorny B, Käsbohrer A, Schwarz S, Meemken D, Hammerl JA.

Characterization of *qnrB*-carrying plasmids from ESBL- and non-ESBL-producing *Escherichia coli*

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2.	Performing the experimental part of the study	75%
3.	Analysis of the experiments	95%
4.	Presentation and discussion of the study in article form	75%

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Characterization of *qnrB*-carrying plasmids from ESBL- and non-ESBL-producing *Escherichia coli*

Katharina Juraschek[1*], Janina Malekzadah[1], Burkhard Malorny[1], Annemarie Käsbohrer[1,2], Stefan Schwarz[3,4], Diana Meemken[4,5] and Jens Andre Hammerl[1*]

Abstract

Background: *Escherichia coli* carrying clinically important antimicrobial resistances [i.e., against extended-spectrum-beta-lactamases (ESBL)] are of high concern for human health and are increasingly detected worldwide. Worryingly, they are often identified as multidrug-resistant (MDR) isolates, frequently including resistances against quinolones/fluoroquinolones.

Results: Here, the occurrence and genetic basis of the fluoroquinolone resistance enhancing determinant *qnrB* in ESBL-/non-ESBL-producing *E. coli* was investigated. Overall, 33 *qnrB*-carrying isolates out of the annual German antimicrobial resistance (AMR) monitoring on commensal *E. coli* (incl. ESBL-/AmpC-producing *E. coli*) recovered from food and livestock between 2013 and 2018 were analysed in detail. Whole-genome sequencing, bioinformatics analyses and transferability evaluation was conducted to characterise the prevailing *qnrB*-associated plasmids. Furthermore, predominant *qnrB*-carrying plasmid-types were subjected to *in silico* genome reconstruction analysis. In general, the *qnrB*-carrying *E. coli* were found to be highly heterogenic in their multilocus sequence types (STs) and their phenotypic resistance profiles. Most of them appeared to be MDR and exhibited resistances against up to ten antimicrobials of different classes. With respect to *qnrB*-carrying plasmids, we found *qnrB*19 located on small Col440I plasmids to be most widespread among ESBL-producing *E. coli* from German livestock and food. This Col440I plasmid-type was found to be highly conserved by exhibiting *qnrB*19, a *pspF* operon and different genes of unassigned function. Furthermore, we detected plasmids of the incompatibility groups IncN and IncH as carriers of *qnrB*. All *qnrB*-carrying plasmids also exhibited virulence factors and various insertion sequences (IS). The majority of the *qnrB*-carrying plasmids were determined to be self-transmissible, indicating their possible contribution to the spread of resistances against (fluoro) quinolones and other antimicrobials.

Conclusion: In this study, a diversity of different plasmid types carrying *qnrB* alone or in combination with other resistance determinants (i.e., beta-lactamase genes) were found. The spread of these plasmids, especially those carrying antimicrobial resistance genes against highest priority critically important antimicrobial agents, is highly unfavourable and can pose a threat for public health. Therefore, the dissemination pathways and evolution of these plasmids need to be further monitored.

*Correspondence: kajuraschek@hotmail.de; Jens-Andre.Hammerl@bfr.bund.de

[1] Department Biological Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn Str. 8-10, 10589 Berlin, Germany
Full list of author information is available at the end of the article

Keywords: *E. coli*, *qnrB*, Fluoroquinolone, Plasmids, *Inc*-group

Background

The spread of antimicrobial-resistant bacteria is a global concern. Commensal *E. coli* can acquire and spread various antimicrobial resistance genes sometimes leading to an emergence of multidrug-resistant (MDR) isolates in the livestock, food, and human sector, worldwide. Among antimicrobial-resistant bacteria, extended-spectrum beta-lactamases (ESBL)-producing *E. coli* (ESBL-EC) are of particular concern. While ESBL-EC had become common in hospitalised and healthy people [1], they are also increasingly detected in livestock, food and the community [2]. Their occurrence in livestock and food poses a threat to public health due to a possible transmission to humans via direct contact to colonised animals or the consumption of contaminated food products [1]. Frequently, ESBL-EC carry additional AMR genes, which result in non-wildtype phenotypes against substances of other antimicrobial classes, which can affect an efficient treatment of infections during hospitalization. Prevailing reports highlighted that ESBL-EC isolated from food samples are often associated with increased minimum inhibitory concentrations (MICs) and resistances against avilamycin, colistin, quinolones and fluoroquinolones [3, 4]. Especially, the co-occurrence of genes conferring resistances against quinolones or fluoroquinolones [further designated as (fluoro)quinolones] are of great concern. Fluoroquinolones are classified as highest priority critically important antimicrobials for the treatment of human infections [5], but are also used in the veterinary sector. (Fluoro)quinolone resistance is mainly caused by alterations in the quinolone resistance-determining regions (QRDR) within the *E. coli* chromosome. However, plasmid-mediated quinolone resistance (PMQR) genes are also responsible for a decreased susceptibility or resistance of isolates [6]. As PMQR genes are usually present on mobile genetic elements (MGE) their spread to different bacteria and environments is feasible. Co-localization of PMQR genes and other resistance determinants on MGEs can contribute to MDR development and persistence by co-selection [7]. Interestingly, characterised ESBL-EC were increasingly reported as carriers of *qnr* genes (especially *qnrB*) [8–15] in Europe, the United States, Asia and Africa [16], with different genes and variants of both determinants (*bla* and *qnr*, predominantly *qnrB1* and $bla_{\text{CTX-M-9}}$, $bla_{\text{CTX-M-3}}$ or $bla_{\text{SHV-12}}$) co-localised on the same plasmids. As many different plasmid-types were shown to be responsible for the spread of *qnr* genes, a deeper understanding of their occurrence, diversity and transmission pathways is necessary.

In this study, we investigated the composition of plasmids carrying *qnrB* variants from ESBL-EC and non-ESBL-EC originating from the annual resistance monitoring from livestock and food in Germany. *In-depth* characterization of the plasmids was conducted by phenotypic analyses in combination with whole-genome sequencing (WGS) and bioinformatics analysis of the isolates. The isolates were studied to gain insight into the properties of *qnrB*-carrying MGEs as well as into their transmission potential. Thus, the risk for the spread of MDR *E. coli* outgoing by these *qnrB*-carrying ESBL-EC is discussed.

Results

Characteristics of ESBL-/non-ESBL-EC carrying qnrB

Based on available *E. coli* sequences from annual resistance monitoring programs conducted by the National Reference Laboratory for Antimicrobial Resistances (NRL-AR) hosted at the German Federal Institute for Risk Assessment, 33 ESBL ($n=29$)/non-ESBL-EC ($n=4$) carrying *qnrB* were identified and subjected to further characterization. The isolates originate from samples of livestock and food taken between 2013 and 2020 and were provided from different German federal state laboratories. In Table 1, essential information on the main characteristics of these isolates is given.

Overall, the 33 analysed *E. coli* were assigned to 26 different 7-locus ST (Supplemental Table 1). We also detected a broad spectrum of different serotypes ($n=20$), with O89 ($n=3$), O9 ($n=3$), O166 ($n=2$) and O25 ($n=2$) as the most prominent O-types. However, no MLST-O:H combination occurred twice, underlining the high heterogeneity of the investigated isolates.

Besides the general typing features, the *E. coli* genomes exhibited between 30 up to 79 genes potentially involved in pathogenicity or virulence. The most frequently detected determinants were *chu* gene variants ($n=12$), *iroN* ($n=13$), *kpsM* ($n=6$) and *irp* variants ($n=6$), *iuc* variants ($n=12$), *pap* variants ($n=4$), *pic* ($n=1$) and *vat* ($n=1$) genes and *csg*, *fim* variants (including *fimA, B, C, D, E, F, G, H,* and *I*), while *ompA* was present in all isolates.

Antimicrobial susceptibility testing (AST) confirmed non-wildtype phenotypes for the majority of the investigated isolates against the beta-lactam antimicrobials ampicillin, cefotaxime and ceftazidime as well as against the (fluoro)quinolones nalidixic acid and ciprofloxacin for the ESBL-EC. Thus, these isolates exhibited at least resistances against critically important antimicrobials of

Table 1 Main characteristics of ESBL-EC (A, $n=29$) and non-ESBL-EC (B, $n=4$) associated with *qnrB* from the WGS collection of the NRL-AR. Besides basic metadata (year of sampling and source), information on phenotypic resistance profiles, *in silico*-based prediction of acquired resistance determinants and chromosomal sequence alterations associated with (fluoro) quinolone resistance development as well as the MLST type is given

Isolate	Year of sampling	Matrix (source)	Resistance profile[a]	Acquired resistance determinants[b]	Chromosomal alterations associated with (fluoro)quinolone resistance	multilocus seuqence type (MLST)
13-AB00983	2013	Poultry, meat	AMP, CIP, COL, FOT, STR, TAZ	*acrF, ant(3'')-Ia, bla*$_{EC}$, *bla*$_{SHV-12}$, *emrD, lnu(F), mcr*-1.1, *mdtM, qnrB*19	n.d	753
14-AB00641	2014	Poultry, meat	AMP, CIP, COL, FOT, TAZ	*acrF, ant(3'')-Ia, bla*$_{EC}$, *bla*$_{SHV-12}$, *emrD, lnu(F), mcr*-1.1, *mdtM, qnrB*19	n.d	48
16-AB00831	2016	Poultry, meat	AMP, CIP, COL, NAL, TET	*acrF, bla*$_{EC}$, *bla*$_{TEM-1}$, *emrD, mcr*-1.1, *mdtM, qnrB*19, *tet(A)*	GyrA (S83L), ParC (S80I), ParE (S458A)	1196
16-AB01284	2016	Poultry, feces	AMP, CHL, CIP, COL, GEN, SMX, TET	*aac(3)-IId, aadA*1, *aadA*2, *acrF, bla*$_{EC}$, *bla*$_{TEM-1}$, *cmlA*1, *emrD, mcr*-1.1, *mdtM, qnrB*19, *sul*3, *tet(A)*	n.d	58
16-AB01309	2016	Poultry, feces	AMP, CHL, CIP, COL, FOT, NAL, SMX, TAZ, TET, TMP	*aac(6')-Ib-cr5, aadA*1, *acrF, arr*-3, *bla*$_{CTX-M-65}$, *bla*$_{EC}$, *bla*$_{OXA-1}$, *bla*$_{TEM-135}$, *catB*3, *cmlA*1, *dfrA*15, *emrD, floR, mcr*-1.1, *mdtM, qnrB*19, *qnrS*1, *qnrS2, sul*3, *tet(A)*	GyrA (S83L), ParC(S80I)	2179
16-AB02042	2016	Poultry, meat	AMP, CHL, CIP, COL, GEN, SMX, TET	*aac(3)-IIe, aadA*1, *aadA*2, *acrF, aph(3'')-Ib, aph(3')-Ia, aph(6)-Id, bla*$_{EC}$, *bla*$_{TEM-135}$, *catA*1, *cmlA*1, *emrD, mcr*-1.1, *mdtM, qnrB*19, *sul*3, *tet(A)*	n.d	155
16-AB03538	2016	Poultry, feces	AMP, CHL, CIP, COL, SMX, TET	*aadA*1, *aadA*2, *acrF, aph(3')-Ia, bla*$_{EC}$, *bla*$_{TEM-135}$, *cmlA*1, *emrD, mcr*-1.1, *mdtM, qnrB*19, *sul*3, *tet(A)*	n.d	48
17-AB00706	2017	Pig, feces	AMP, CIP, FOT, NAL, SMX, TAZ, TET, TMP	*aadA5, acrF, aph(3'')-Ib, aph(6)-Id, bla*$_{EC}$, *bla*$_{TEM-1}$, *catB*3, *dfrA*1, *emrD, mdtM, qnrB*19, *sul*1, *sul*2, *tet(A)*	GyrA (S83L, D87N)	88
17-AB01697	2017	Cattle, feces	AMP, CIP, FOT, SMX, TAZ, TMP	*acrF, bla*$_{CTX-M-1}$, *bla*$_{EC}$, *dfrA*25, *emrD, mdtM, qnrB2, sul*1	n.d	657
17-AB02713	2017	Cattle, feces	AMP, CIP, FOT, SMX, TAZ, TET, TMP	*aac(6')-Ib-cr5, aadA*1, *acrF, aph(3'')-Ib, aph(6)-Id, bla*$_{CTX-M-15}$, *bla*$_{EC}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$, *catA*1, *catB*3, *dfrA*1, *dfrA*14, *emrD, mdtM, mef(C), mph(G), qnrB*1, *sul*1, *sul2, tet(A)*	n.d	398
17-AB02827	2017	Pig, feces	AMP, CHL, CIP, FOT, GEN, NAL, SMX, TAZ, TET, TMP	*aac(3)-IIe, aac(6')-Ib-cr5, aadA*1, *acrF, aph(3'')-Ib, aph(6)-Id, bla*$_{CTX-M-15}$, *bla*$_{EC}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$, *catA*1, *catB*3, *dfrA*14, *emrD, mdtM, qnrB*1, *sul2, tet(A)*	ParC (A56T, S80I)	744
18-AB00078	2017	Cattle, feced	AMP, CHL, CIP, FOT, SMX, TAZ, TET, TMP	*aac(6')-Ib-cr5, aadA*1, *acrF, aph(3'')-Ib, aph(6)-Id, bla*$_{CTX-M-15}$, *bla*$_{EC}$, *bla*$_{OXA-1}$, *bla*$_{TEM-1}$, *catA*1, *catB*3, *dfrA*14, *emrD, floR, mdtM, qnrB*1, *sul2, tet(A)*	n.d	154

Table 1 (continued)

Isolate	Year of sampling	Matrix (source)	Resistance profile[a]	Acquired resistance determinants[b]	Chromosomal alterations associated with (fluoro)quinolone resistance	multilocus seuqence type (MLST)
20-AB00274	2020	Poultry, meat	AMP, CIP, FOT, TAZ, TET	*acrF, aph(3')-Ia, bla*$_{CTX-M-55}$*, bla*$_{EC}$*, emrD, mdtM, qnrB*19*, tet(A)*	n.d	1011
20-AB00375	2020	Poultry, feces	AMP, CIP, FOT, NAL, TAZ, TET	*acrF, bla*$_{EC}$*, bla*$_{SHV-12}$*, bla*$_{TEM-1}$*, emrD, mdtM, qnrB*19*, qnrS*1*, tet(A)*	n.d	1626
20-AB00564	2020	Poultry, feces	AMP, CIP, FOT, TAZ	*acrF, bla*$_{EC}$*, bla*$_{SHV-12}$*, emrD, mdtM, qnrB*19	n.d	155
20-AB00569	2020	Poultry, feces	AMP, CIP, FOT, TAZ	*aadA*2*, acrF, bla*$_{EC}$*, bla*$_{TEM-52}$*, emrD, lnu(F), mdtM, qnrB*19	n.d	192
20-AB00611	2020	Poultry, feces	AMP, CIP, FOT, NAL, TAZ	*acrF, blaCMY-2, bla*$_{EC}$*, bla*$_{TEM-1}$*, emrD, qnrB*19	GyrA (S83L), ParC (E84K)	131
20-AB00922	2020	Poultry, feces	AMP, CIP, FOT, NAL, TAZ	*acrF, bla*$_{CMY-2}$*, bla*$_{EC}$*, emrD, qnrB*19	GyrA (S83L)	131
20-AB01255	2020	Poultry, feces	AMP, CIP, FOT, TAZ	*acrF, bla*$_{EC}$*, bla*$_{SHV-12}$*, emrD, mdtM, qnrB*19	n.d	1406
20-AB01339	2020	Poultry, feces	AMP, CIP, FOT, TAZ	*aadA*22*, acrF, bla*$_{EC}$*, bla*$_{SHV-12}$*, emrD, lnu(F), mdtM, qnrB*19*, qnrS*1	n.d	162
20-AB01569	2020	Poultry,feces	AMP, CHL, CIP, FOT, NAL, SMX, TAZ, TET, TMP	*aadA*1*, acrF, aph(3")-Ib, aph(6)-Id, bla*$_{CTX-M-27}$*, bla*$_{EC}$*, dfrA*1*, emrD, floR, mdtM, qnrB*19*, sul*1*, sul*2*, tet(A), tet(B)*	GyrA (D87N, S83L), ParC (S80I)	533
20-AB01574	2020	Poultry, feces	AMP, CHL, CIP, FOT, SMX, TAZ, TET, TMP	*aac(3)-IIe, aac(6')-Ib-cr5, aadA*1*, acrF, aph(3")-Ib, aph(6)-Id, bla*$_{CTX-M-15}$*, bla*$_{EC}$*, bla*$_{OXA-1}$*, bla*$_{TEM-1}$*, catA*1*, catB*3*, dfrA*14*, emrD, mdtM, qnrB*1*, sul*2*, tet(A)*	n.d	3058
20-AB01775	2020	Poultry, feces	AMP, CIP, COL, FOT, TAZ	*acrF, bla*$_{EC}$*, bla*$_{TEM-52}$*, emrD, mdtM, qnrB*19	n.d	226
20-MO00017	2018	Pig, feces	AMP, CIP, FOT, GEN, SMX, TAZ, TET	*aac(3)-IIe, aadA*1*, acrF, aph(3')-Ia, bla*$_{CTX-M-55}$*, bla*$_{EC}$*, emrD, mdtM, qnrB*19*, sul*3*, tet(A)*	n.d	10
20-MO00019	2018	Pig, feces	AMP, CIP, FOT, GEN, SMX, TAZ, TET	*aadA*1*, acrF, bla*$_{CTX-M-1}$*, bla*$_{EC}$*, bla*$_{TEM}$*, emrD, mdtM, qnrB*19*, tet(A)*	n.d	10
20-MO00028	2017	Poultry, feces	AMP, CIP, FOT, SMX, TAZ, TET	*acrF, bla*$_{CTX-M-1}$*, bla*$_{EC}$*, emrD, mdtM, qnrB*19*, sul*2*, tet(A)*	n.d	3995
20-MO00045	2017	Poultry, feces	AMP, CIP, FOT, SMX, TAZ, TET	*aadA*2*, acrF, bla*$_{CTX-M-1}$*, bla*$_{EC}$*, emrD, lnu(F), mdtM, qnrB*19*, sul*2*, tet(A)*	n.d	3995
20-MO00078	2018	Poultry, feces	AMP, CIP, FOT, NAL, SMX, TAZ, TET, TMP	*aadA*1*, aadA*5*, acrF, aph(3")-Ib, aph(6)-Id, bla*$_{CTX-M-1}$*, bla*$_{EC}$*, bla*$_{TEM-1}$*, dfrA*1*, dfrA*17*, emrD, lnu(F), mdtM, mph(B), qnrB*19*, sul*1*, sul*2*, tet(A)*	GyrA (S83L), ParE (I355T)	4994
20-MO00080	2018	Poultry, feces	AMP, CIP, FOT, GEN, NAL, SMX, TAZ, TET, TMP	*aac(3)-IIe, aadA*1*, acrF, bla*$_{EC}$*, bla*$_{SHV-2}$*, bla*$_{TEM}$*, dfrA*1*, emrD, lnu(F), mdtM, qnrB*19*, sul*1*, tet(A)*	GyrA (S83L, D97N), ParC (S80I)	533

Table 1 (continued)

Isolate	Year of sampling	Matrix (source)	Resistance profile[a]	Acquired resistance determinants[b]	Chromosomal alterations associated with (fluoro)quinolone resistance	multilocus seuqence type (MLST)
14-AB01030	2016	Poultry, feces	AMP, CHL, CIP, COL, SMX, TMP	*aadA*1, *aadA*2, *acrF*, bla_{EC}, $bla_{TEM-135}$, *catA*1, *cmlA*1, *dfrA*1, *emrD*, *mcr*-1.1, *qnrB*19, *sul*1, *sul*3, *tet(M)*	ParE (I529L)	131
17-AB00065	2016	Poultry, feces	AMP, CIP, GEN, TET	*aac(3)*-VIa, *aadA*1, *acrF*, bla_{EC}, bla_{TEM-1}, *emrD*, *mdtM*, *qnrB*19, *tet(A)*	n.d	349
17-AB00089	2016	Poultry, feces	CIP	*acrF*, bla_{EC}, *emrD*, *mdtM*, *qnrB*19	n.d	69
17-AB00375	2017	Pig, feces	CIP	*acrF*, *aph(3")-Ib*, *aph(6)-Id*, bla_{EC}, *emrD*, *mdtM*, *qnrB*19	n.d	117

[a] *AMP* Ampicillin, *CHL* Chloramphenicol, *CIP* Ciprofloxacin, *COL* Colistin, *FOT* cefotaxime, *GEN* Gentamicin, *NAL* Nalidixic Acid, *SMX* Sulfamethoxazole, *STR* Streptomycin, *TAZ* ceftazidime, *TET* Tetracycline, *TMP* Trimethoprim, *n.d.* not detected, *GyrA* Gyrase subunit A, *ParC* DNA topisomerase IV subunit, *ParE* DNA topoisomerase IV subunit.

[b] If no gene variant is given, the bioinformatics analysis was unable to specify the gene variant

two different classes. However, a few isolates also exhibited non-wildtype phenotypes against other important antimicrobials like colistin ($n=8$). The phenotypic data was in good agreement with the AMR genes detected by bioinformatics analysis. In silico-typing revealed the presence of multiple AMR genes per isolate (Supplemental Table 1 and Table 1). Most frequently, the beta-lactamase gene $bla_{\text{TEM-1}}$ and $bla_{\text{TEM-135}}$ were detected, while *qnrB*19 represented the dominant (fluoro)quinolone resistance determinant. Analysing the point mutations within the chromosomal sequence of *gyrA*, *gyrB*, *parC*, *parE*, *pmrA*, *pmrB*, *folP*, 23S/16S rRNA as well as the *ampC* and *rpoB* regions, of which some of the genes are known to be associated with (fluoro)quinolone resistance, we found yet uncharacterised alterations in the above-mentioned sequences within every isolate. For ten isolates, previously characterised point mutations were detected, known to be involved in a decrease of the susceptibility against (fluoro)quinolones. The most frequently appearing point mutations within the *E. coli* are alterations of *gyrA* leading to a decreased susceptibility against nalidixic acid and ciprofloxacin (Table 1). Besides, we detected mutations leading to changes of the amino acid sequence of ParC and ParE also affecting the (fluoro) quinolone susceptibility of the isolates.

To assess a potential transferability of the AMR genes and the association of *qnrB* genes and mobile elements, WGS data of the isolates were used. Potential plasmid sequences of all isolates could be assigned to 24 distinct incompatibility groups (Supplemental Table 2).

Characterization of qnrB-carrying plasmids

Among the 33 *qnrB*-carrying *E. coli* isolates, four harboured *qnrB*1, one *qnrB*2 and 28 *qnrB*19. The *in silico* analysis further revealed an association of *qnrB* to contigs of plasmidic origin. Twenty-four isolates harboured *qnrB* on a Col440I replicon carrying plasmid contig. Furthermore, individual plasmid types represented by replicon sequences of the incompatibility groups IncN and ColE10 were detected ($n=2$). For the remaining isolates ($n=7$), the *qnrB*-carrying contig could not be associated with a specific replicon type backed in the PlasmidFinder database. Overall, we were able to close the *qnrB*-carrying plasmid genomes of 12 isolates, solely through assembling with unicycler. Interestingly, the majority of those closed plasmids belonged to the Col440I type, while one was assigned to ColE10 (Table 2). We were able to close the remaining plasmids carrying the *qnrB* gene on a Col440I plasmid through primer walking.

For the determination of the *qnrB*-plasmid diversity, the genetic basis of the most prominent types was analysed, including Col440I-, IncN- and IncH2/IncH2A-plasmids.

qnrB19 genes on Col440I plasmids

The *qnrB*-associated Col440I plasmids of this study exhibited genome sizes ranging between 2,700 and 3,500 bp with GC-contents of 47.7% to 50.9%. Therewith, we found five reference plasmids representing the different Col440I plasmid-types (Table 2). According to a phylogenetic comparison as shown in Fig. 1, the *qnrB*19 Col440I plasmids clustered into three distinct clades. While clade (A) was only represented by the reference plasmid pEC14-9 (NC_013782), the remaining clades exhibited a slight diversity in their assigned reference genomes. However, Clade (B) was best assigned to the reference plasmid p14-7355.2 (NZ_CP039609), while the most distant clade (C) matched the best to the reference plasmid p3_12888 (NZ_CP045448).

All Col440I plasmids were equipped with a *pspF* operon transcriptional activator, upstream of the coding sequence (CDS) of the pentapeptide-encoding gene *qnrB*19. Close to the replication initiation protein, the gene for the transcription factor *Sp1* was identified, as well as a putative CDS of a yet uncharacterised function (Fig. 2). The main differences of the phylogenetic clusters are caused by the occurrence of these sequences encoding proteins of unknown function. On the Col440I genomes, no further AMR, virulence, or biocide resistance genes were detected. Interestingly, these plasmids also lack insertion sequences potentially associated with a further dissemination of the *qnrB* gene. In Fig. 2, the genetic diversity of the Col440I-plasmid is shown. Furthermore, the organization and function of the CDS predicted on the Col440I-genomes are given. Based on the prevailing data a core genome of this plasmid type can be assigned to the presence of *qnrB*19 and *pspF* gene.

By analysing the Col440I genomes with the in silico mob-suite tool, the plasmids were assigned to be mobilizable. In general, the origin of transfer (*oriT*) of the plasmids was subjected to the conjugative transfer system of the MOB_{P} type, but no type IV secretion system (T4SS) was detected. Thus, the plasmid is transmissible by a helper plasmid but not self-transmissible. However, in vitro studies using the *E. coli* isolates for filter mating with the sodium acid-resistant *E. coli* strain J53, yield negative results under double selective conditions (NAL/SAC), suggesting that the plasmid could not be transmitted due to a lack of a suitable helper plasmid or based on missing determinants or altered sequences involved in mobilization.

Characterization of IncN plasmids carrying qnrB2

Out of the prevailing *qnrB* collection, only one ESBL-producing *E. coli*, from cattle, represented a *qnrB*2 determinant. This one was located on an IncN plasmid. *qnrB*2 was identified on a 39,563 bp contig, which could not be

Table 2 Incompatibility group (*inc*) prediction based on *inc*-typing of the *qnrB*-carrying contigs and assignment to the best-matched reference plasmid. The frequency of occurrence of *inc* types is provided as absolute numbers in brackets

***inc* prediction based on *qnrB*-carrying contig**	**Number of isolates**	**No. of closed plasmids**	***inc* group of best-matching reference plasmid**	**Accession no. of the best-matching reference plasmids**
Col440I	24	22	Col440I (*n* = 22)	NC_013782.1 (*n* = 4), NZ_CP039508.1 (*n* = 3), NZ_CP039609.1 (*n* = 11), NZ_CP045445.1 (*n* = 1), NZ_CP045448.1 (*n* = 3)
			ColRNAI (*n* = 2)	NZ_LT985269.1
ColE10	1	1	n.d. (*n* = 1)	n.d
IncN	1	0	IncN (*n* = 1)	NC_019098.1
n.d	7	0	Col440I (*n* = 1)	NC_013782.1 (*n* = 1)
			InH2, IncH2A (*n* = 4)	NZ_CP048350.1 (*n* = 3), NZ_CP024813.1 (*n* = 1)
			- (*n* = 2)	NZ_CP039985.1

n.d. not detected

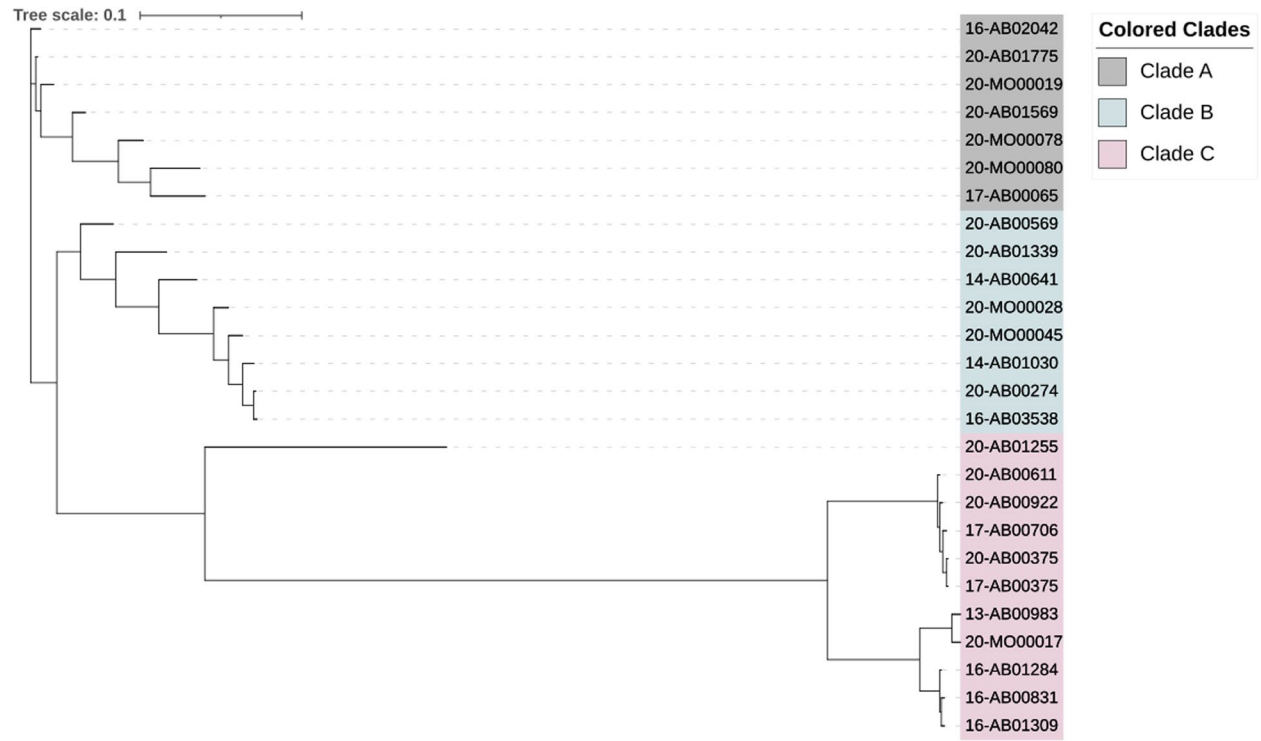

Fig. 1 Phylogenetic tree of the Col-plasmids carrying *qnrB*19. The phylogenetic tree was visualised with iTOL v6.3 after creating a nexus file with a multiple Clustal Omega alignment. All tools were used with default parameters

circularised to a complete plasmid genome using unicycler alone. However, reference-based mapping with plasmidID against a complete plasmid database revealed a close relationship (92.33% reference coverage) to the *E. coli* plasmid pHHA45 (NC_019098). This plasmid represents an IncN plasmid of 39,510 bp with a GC content of 50.5%. A sequence comparison of both plasmid sequences revealed the presence of repetitive sequences on pHHA45, which might be responsible for failing circularisation by using the unicycler program.

However, the derived contigs of the isolate comprised all essential information for the evaluation of the impact of this IncN plasmid type. In proximity to *qnrB*2, the plasmid carried a $bla_{\text{CTX-M-1}}$ gene, which is also present on the reference plasmid pHHA45 (NC_019098) (Fig. 3). The $bla_{\text{CTX-M-1}}$ gene is flanked by

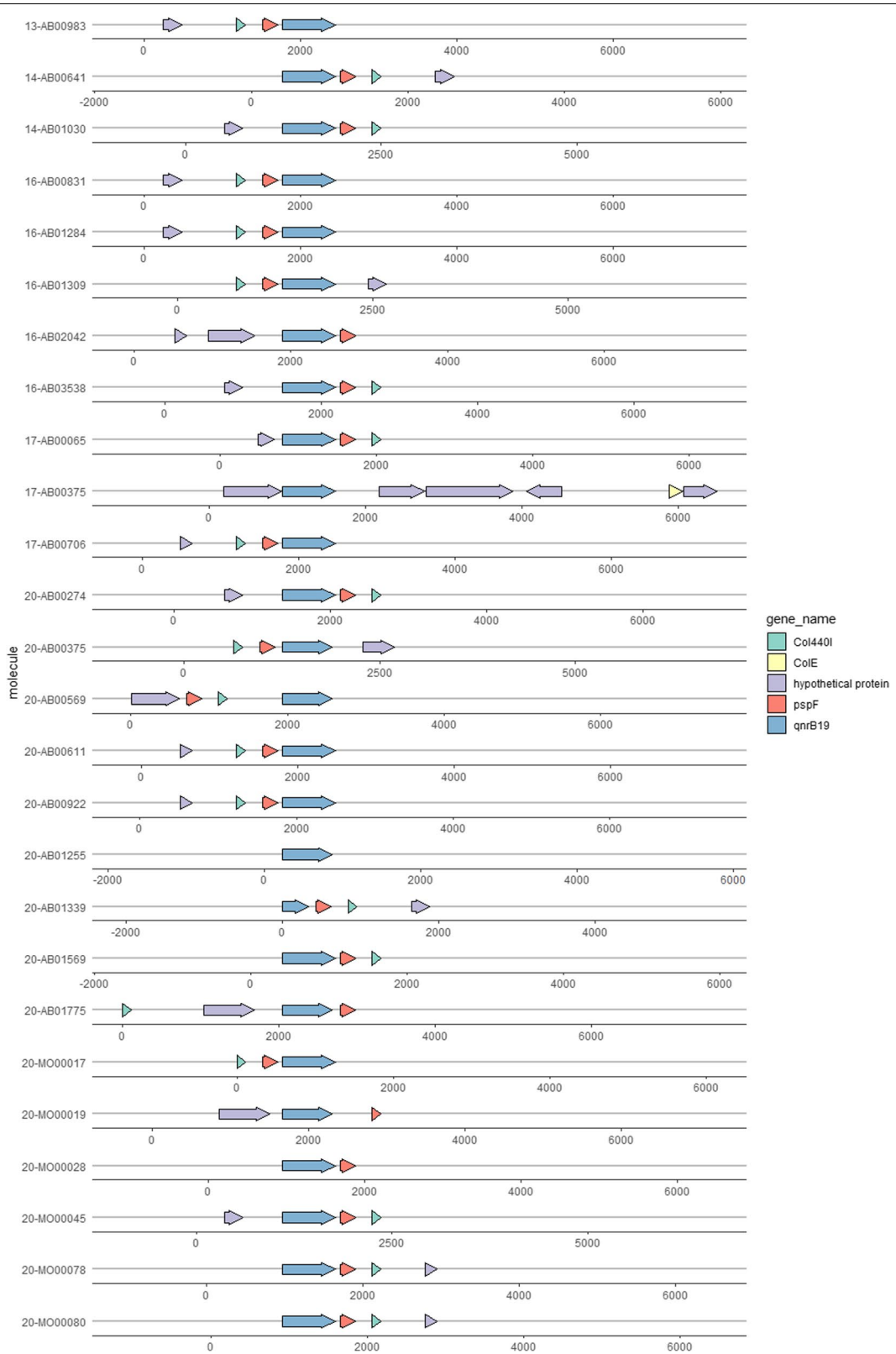

Fig. 2 Arrow graph of annotated plasmids assigned to the Col plasmid group, visualised with the ggplot2 package gggenes in RStudio. Genes were aligned according to the position of *qnrB*19. The schematic illustration provides no information on the actual plasmid size but the size and position of the annotated genes. The ruler given below the gene map represents an artificial indicator for the size of the region

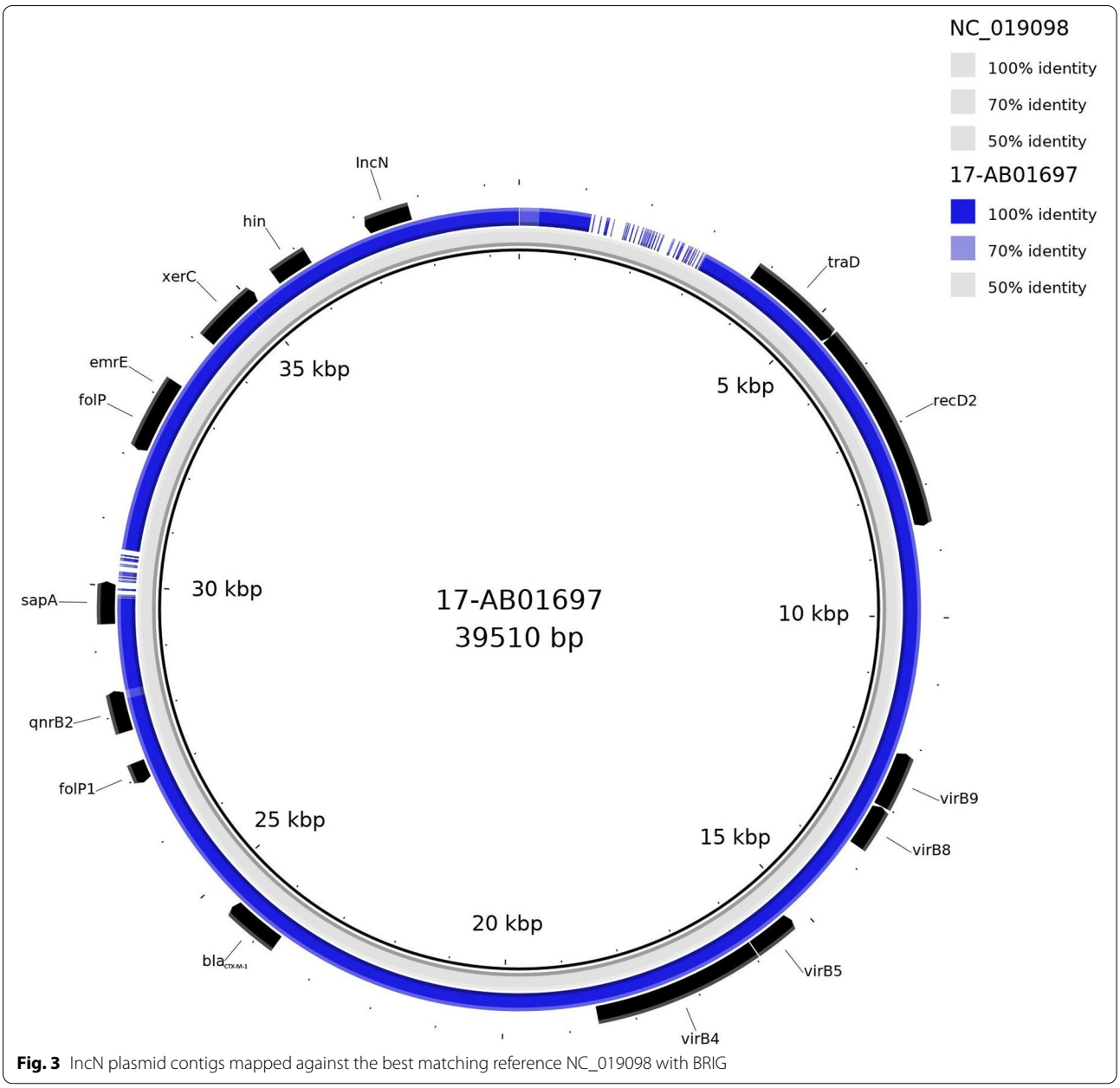

Fig. 3 IncN plasmid contigs mapped against the best matching reference NC_019098 with BRIG

IS*26* elements. Upstream to *qnrB*2, the dihydropteroate synthase (encoded by *folP*) and $bla_{\text{CTX-M-1}}$ flanked by IS*26* elements are located. Downstream of *qnrB*2, *sapA* encoding a peptide transport periplasmic protein and a further dihydropteroate synthase was identified. Moreover, the multidrug transporter gene *emrE*, associated to the transposase IS*Ssu9*, was located on this plasmid. The mob-suite analysis predicted that the IncN plasmid is self-transmissible (conjugative). In silico analysis revealed the presence of MOB_F relaxase and the MpfT mating pair formation (*mpf*) system. The predicted self-transmissibility of the plasmid could be confirmed by *in vitro* filter mating studies. We observed a transmission rate of 10^5 to 10^6 per donor cell.

Characterization of IncH2/IncH2A plasmids carrying qnrB1

Four of the investigated *E. coli* revealed plasmid sequences associated with *qnrB*1 and IncHI2-IncHI2A replicon sequences. Here, we were able to assign contigs, bioinformatically clustering together, from our isolates to a reference plasmids, carrying the *qnrB1* as well as the IncH replicon sequence on the same plasmid. Therewith, these contigs of our isolate fully covered the *qnrB1* and IncH replicon sequence region.

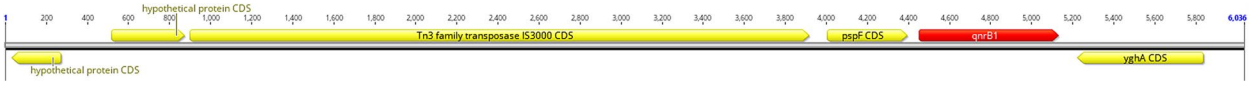

Fig. 4 Organization of the *qnrB*1 coding region on the IncH plasmids. The region represents the contig as detected for the four investigated *qnrB1* carrying *E. coli* of our study. This contig, bioinformatically mapped to the reference plasmids containing the IncH replicon sequence, did always consist of the same structure as presented here

Two of the isolates were recovered from cattle, one from pig and one from poultry. De novo assemblies yielded *qnrB*1-carrying contigs of 6 and 12 kb for three and one isolate, respectively. Using the plasmidID tool we were able to identify p23_A-OXA140 (NZ_CP048350; 99% coverage) and pCRENT-193_1 (NZ_CP024813; 99% coverage) as the closest relatives. The respective reference plasmids ranged between 279 and 298 kb in size and exhibited GC contents of 48%. Both reference plasmids are 92% identical at nucleotide level to each other. Here, *qnrB*1 was located downstream to the *pspF* and a Tn*3* family transposase gene (Fig. 4).

Here, the IS*6* family transposons (IS*26*) flanked an area, containing the AMR genes *qnrB*1, *aac*(3)-lle, *aac*(6')-Ib-cr5 and $bla_{\text{OXA-1}}$. All AMR genes detected on these plasmids are located in a region of 40 kb. in silico analysis revealed a self-transmissibility of the plasmid due to the detection of the Mpf_{T} system and the MOB_{H} relaxase type. The plasmid pCRENT-193_1(NZ_CP024813) carries multiple AMR genes like *aac*(3)-IIe, *aac*(6')-Ib-cr5, *aadA*1, *aph*(3")-Ib, *aph*(6)-Id, $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$, *catA*1, *catB*3, *dfrA*14, *qnrB*1, *sul*2 and *tet*(A). The same genes were detected in the WGS data of 18-AB00078 analysed in this study. A similar resistance gene profile, only lacking *aadA*1 and *catA*1, was detected on the p23_A-OXA140 genome (NZ_CP048350). However, *aadA*1 and *catA*1 were present in the respective matching plasmids from our investigated isolates and resembled to the reconstructed *qnrB*1 plasmid. Furthermore, we detected the *hipA* gene, coding for a serine/threonine-protein kinase toxin, which is the toxic component of a type II toxin-antitoxin (TA) system. The binding partner HipB encoding gene was not detected in proximity of *hipA* and could not be identified in the complete WGS dataset representing the complete *E. coli* isolate. We also detected the tellurium ion resistance gene *terC*, often associated with pathogenic bacteria [17], in the investigated plasmids. Overall, all *qnrB*-carrying plasmids were shown to be self-transmissible under natural condition based on the observed transfer rates of 10^2 to 10^4 per donor cell.

Discussion

Characteristics of ESBL and non-ESBL E. coli carrying qnrB-plasmids

In this study, 33 ESBL-/non-ESBL-EC carrying *qnrB* on an extrachromosomal element were characterised in detail. This co-occurrence of ESBL and (fluoro)quinolone resistance genes in *E. coli* poses a threat to public health, as these antimicrobials are highest priority critically important substances in human medicine.

Among the investigated isolates, a broad variety of sources of *qnrB*-carrying *E. coli* was found. While poultry seems to be the predominant source for *qnr* genes [18], plasmids with PMQR were also found in other sources. The presence of *qnrB* in ESBL-EC from poultry is frequently reported. The latest summary report of the European Food Safety Authority (EFSA) on AMR in zoonotic and indicator bacteria from humans, animals and food [18] also addresses this trend. Although the incidence of ESBL-EC was generally low, it was most often detected in broiler isolates (Member State group level of up to 30%). However, EFSA further reported a high level of ciprofloxacin- and nalidixic acid-resistant *E. coli* especially from broilers (median 73.5% for ciprofloxacin and 64.1% for nalidixic acid) and turkeys. Thus, poultry seems to be a common reservoir for ESBL-EC and (fluoro)quinolone-resistant *E. coli*. General, the occurrence of MDR *E. coli*, as characterised in this study, along the food chain poses a risk for a transmission of these bacteria to human via food products.

Interestingly, *qnrB*-positive ESBL-EC were also detected among isolates of the international high-risk clone ST131 [19] and O89 serotype. ST131 isolates are known to represent a predominant sequence type among extraintestinal pathogenic *E. coli*, which comprise ESBL-positive as well as (fluoro)quinolone-resistant isolates [20]. *E. coli* of the serotype O89 are often associated with MDR [21]. Based on the results of this study, a similar association was observed for these *E. coli* types. The occurrence of *qnrB*-carrying plasmids in various STs of ESBL-EC further demonstrated that these plasmids exhibit a broad adaptability to *E. coli* of different ST.

It had been discussed before, that *qnrB* represents the dominant PMQR group in humans, while *qnrS* seemed to be more frequent in the environment [22], the veterinary and food sector [23–26]. This emphasises the need for a better understanding of the composition and impact of *qnrB*-carrying plasmids to estimate the transmission possibilities from animal to humans.

Here, the ESBL-EC were mostly phenotypically resistant against penicillins, cephalosporins and (fluoro)

quinolones. Further, every isolate carried a *bla* gene coding for different TEM- or less frequently CTX-enzyme variants. For *qnrB*, the variant *qnrB*19 seemed to be predominant. Interestingly, some isolates showed genes encoding penicillinases and ceftazidime resistance. This observation needs to be further verified and characterised in detail to determine, if also other mechanisms like mutations in PBP3 and efflux pumps can cause these effects. However, all isolates of this study further exhibited phenotypical resistance to antimicrobials of other classes and were shown to carry various AMR genes co-occurring in the same isolate.

The detected virulence factors (*ompA*, *csg*, *fim, chu*, *iroN*, *kpsM*, *irp*, *iuc*, *pap*, *pic* and *vat*) may contribute to an increase in the pathogenic potential of these *E. coli*. The outer membrane protein A (OmpA) contributes to pathogenesis. The capsular antigen (KpsM) represents a protection factor against phagocytosis. The siderophore aerobactin gene (*iuc*) as well as *irp, iroN* and *chu* are associated with iron uptake often present in uropathogenic *E. coli* (UPEC). The genes *pap* (coding for P fimbriae), *fim* (type 1 fimbriae) and *csg* (curli fibers) contribute to the adhesion properties of the *E. coli.* The serine protease autotransporter encoding gene *pic* and the vacuolating autotransporter encoding gene *vat* do represent toxins. All detected factors individually contribute to an increase in the pathogenic potential of *E. coli* [27]. The presence of these virulence factors, in *qnr*-carrying ESBL-EC demonstrates an aggregated risk. As virulence factors are also frequently present on plasmids [28], their potential spread can increase the clinical impact of the bacterium dramatically. Further subtyping results, i.e., the phylotype and detected *fim* variants are given in Supplemental Table 3.

While PMQR genes are the main contributors for horizontal (fluoro)quinolone resistance transmission, alterations in the sequences of the DNA gyrase and topoisomerase IV genes are the main reason for resistance against (fluoro)quinolones in *E. coli* [29]. We detected previously determined single nucleotide polymorphisms (SNPs) leading to high-level resistances within ten of 33 isolates. The SNPs where mainly identified in the genes *gyrA* and *parC*. Especially the mutations in the S83L (in GyrA) and D87N (in ParC), as found here, are common [30, 31]. These mutations, in combination with the carriage of a *qnr* gene, are responsible for the (fluoro) quinolone resistance phenotype [32]. However, we further detected yet uncharacterised SNPs in the QRDR for every analysed isolate (Supplement Table 4). As the study only focused on (fluoro)quinolone-resistant *E. coli*, it might be possible that these mutations also contribute to the observed (fluoro)quinolone resistance. Another appropriate interpretation might be a higher contribution of the *qnrB* genes to (fluoro)quinolone resistance, than commonly expected [33].

Prevalent qnrB-carrying plasmids in ESBL-/non-ESBL-EC

We identified a 39.5 kb IncN plasmid carrying *qnrB*2 in combination with $bla_{\text{CTX-M-1}}$ surrounded by IS*26* elements as previously described [34, 35]. The $bla_{\text{CTX-M-1}}$ in proximity to IS*26* elements was also detected on other plasmid types, suggesting that transmission of this specific region took place via IS*26*-mediated transfer [36]. The *folP* gene, identified upstream of the *qnrB*2 gene, is another characteristic of this plasmid. The *folP* gene encodes the dihydropteroate synthase (DHPS) enzyme, which is usually encoded on the chromosome and represents the target of sulfonamides [37, 38]. The presence of *folP* on the plasmid may represent a genetic advantage for *E. coli*, as it ensures the folate biosynthesis pathway. Furthermore, the MDR transporter, encoded by *ermD* was detected. It represents a small MDR transporter known to confer resistance to a broad spectrum of disinfectants and quaternary cation compounds [39]. The gene *ermD* was detected close to the IS*Ssu9*, also suggesting IS-mediated transfer. In this study, we isolated the plasmid from bovine *E. coli*. However, similar IncN plasmids were identified in isolates of various sources (food, livestock and humans) from the Czech Republic, Poland, Denmark and Italy [40]. Dolejkska et al. [41] described comparable plasmids also carrying a $bla_{\text{CTX-M-1}}$, in addition to *qnrS*1 or *qnrB*19, but to the best of our knowledge not together on the same IncN plasmid. As this plasmid was determined to be conjugative as well as to be a broad-host range plasmid [40], the risk resulting from this special IncN plasmid and the evolvement of AMR gene accumulation should be further monitored. Another detected plasmid type in this study were plasmids of the IncHI2-IncHI2A incompatibility group. We detected this plasmid type in association with a *qnrB*1 gene in isolates of different animal sources, which suggests a possible broad dissemination. We identified multiple AMR genes on our *qnrB*1-carrying plasmids. All IncH plasmids from this study exhibited the following AMR genes: *aac*(3)-IIe, *aac*(6')-Ib-cr5, *aadA*1, *aph*(3")-Ib, *aph*(6)-Id, $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$, $bla_{\text{TEM-1}}$, *catA*1, *catB*3, *dfrA*14, *qnrB*1, *sul*2 *and tet*(A), leading to resistance against aminoglycosides, beta-lactams, chloramphenicol, trimethoprim, (fluoro) quinolones, sulphonamides, and tetracyclines. IncH-like plasmids were previously reported as accumulators, carriers and spreaders of various resistances [42]. The carriage of multiple AMR genes, as present for this plasmid type, probably presents a risk when transmitted. The co-occurrence of genes conferring resistance against two broad antimicrobial classes is alarming. With the presence of $bla_{\text{CTX-M-15}}$, $bla_{\text{OXA-1}}$ and $bla_{\text{TEM-1}}$, three different

beta-lactamase genes were located on the same *qnrB*1-carrying plasmid. The IS*26* element in proximity to *qnrB*1 and $bla_{\text{OXA-1}}$ is known to be responsible for spreading multiple AMR genes [43]. Varani et al. described the importance of IS*26* in clinical settings. They mentioned an increased frequency of plasmids carrying IS*26* involved in aggregation of antimicrobial resistance genes [43]. Harmer and colleagues described IS*26* as key element for the dissemination of AMR genes in Gram-negative bacteria [44]. We further detected *hipA* on the IncH plasmid. We were not able to detect the antitoxin component *hipB*, neither in other plasmids nor in the chromosomal DNA of the respective isolates. Thus, it remains unclear how the *E. coli* copes with the burden of the toxin produced from *hipA*. We can assume that the presence of this component is a benefit for the plasmid stability within the isolate. The detected *terC* virulence factor on the plasmid is quite common for IncH plasmid types. It was described as responsible for the control of resistance to infections by some bacteriophages [45]. Thus, it may confer another advantage for the host to retain the respective plasmid. Further, IncH-type plasmids were often detected in animal and human isolates and seemed to be disseminated among different sources contributing to the spread of AMR genes from animals to humans or vice versa [45]. As we determined the self-transmissibility of the IncH-like plasmids characterised within this study, a possible spread of this plasmid carrying multiple AMR genes is indeed given.

The most frequently detected *qnrB* plasmid type in the ESBL-/non-ESBL-EC in this study belonged to the Col440I-like group. All Col440I plasmids carried a *qnrB*19 gene and a *pspF* operon, as well as the gene for the transcription factor *sp1*. The protein sequence of the hypothetical protein as well as the non-coding regions altered within the different clusters. With the detection of the mob_P relaxase gene, this plasmid was categorised as mobilizable, but not self-transmissible. The same plasmid (100% identity) has been described before with exactly the same genome structure but was assigned to a different plasmid type. Karczmarczyk and colleague identified this "ColE-like" plasmids from food samples in Colombia to carry *qnrB*19 [46]. Pallecchi et al. characterised the same structure as ColE-like plasmid. They found this small *qnrB*19-carrying plasmid in *E. coli* from humans around Latin America with a high frequency and suggested a major role of this small plasmid in *qnrB* dissemination. As the plasmid is small and contains only a few genes, the authors hypothesised that it could have undergone a subsequent excision [47]. This was supported by other studies, describing *qnrB*19 within a comparable genetic environment in larger plasmids, associated with IS*Ecp1C*-based transposons [48]. Moreno-Switt et al. [49] described that this small *qnrB*19-carrying plasmid was reported in Europe, the U.S.A. and South America in *Salmonella* obtained from food, animals and humans. They demonstrated how this *qnrB*19-carrying plasmid type was transmitted between different *Salmonella* serotypes through a P22-mediated transduction, probably explaining the frequent detection of this small plasmid. Although we only detected *qnrB*19 on the small Col440I plasmids, there are some studies available, presenting the *qnrB*19 on different plasmids also containing $bla_{\text{TEM-1}}$ or $bla_{\text{SHV-12}}$ [50]. Blasting the Col440I plasmid against the NCBI database, we detected an 11.3 kb plasmid (FDAARGOS_1249) containing the backbone of the *qnrB*19-carrying plasmid but also additional genes, like the plasmid mobilization gene encoding MOB_{C}.

Overall, we were able to thoroughly determine and characterize the structures of the plasmids carrying the *qnrB* gene. However, as we investigated the isolates with short-read sequencing the limitations for closing these plasmids has to be mentioned. Although, in silico estimation of the whole plasmid from short reads is getting more reliable, an optimised approach would include long-read sequencing of the plasmids of interest. As previously shown, especially for the determination of large plasmid genomes long read sequencing is necessary [51]. Due to the occurrence of mobile genetic elements or repetitive sequences, short read sequencing techniques represent limitations for addressing this issue.

Risk posed by qnrB-carrying plasmids in ESBL-/non-ESBL-EC

We detected different *qnrB* genes on plasmids within the *E. coli* isolates of this study. All investigated isolates were resistant to (fluoro)quinolones. Usually, plasmidic factors were accounted only with a decrease of the susceptibility of the isolate not necessarily resulting in a non-wildtype phenotype of the isolates. However, not all isolates carried a known mutation within the respective QRDRs. Thus, it might be possible that the presence of a *qnrB* gene without any other yet characterised chromosomal alteration in the PMQR or the presence of other plasmidic factors can lead to a resistance phenotype for (fluoro)quinolones. Different studies had already explained, how the *qnr* genes are able to alter the resistance against (fluoro)quinolones due to mutations in the chromosomal QRDR regions and how the presence does allow other antimicrobial resistance genes to enter and persist. Thus, Li and colleagues reported on how QnrB promotes DNA replication stress that leads to an increased bacterial mutation risk. In their investigations, they measured a two-fold increase in the mutation rate, when QnrB is expressed. Further, they found how QnrB is responsible for the accumulation of mutations, also

including quinolone resistance mutations. Overall, they suggested that QnrB could be in charge for promoting the persistence of plasmids leading to resistance against respective antimicrobial agents [52]. These results let us assume, that the presence of *qnrB* genes in our isolates are also responsible for an environment allowing some mutations to occur and therewith promote the presence of different resistance profiles.

When the presence of *qnrB* was investigated for the first time in-depth, the observation of its association with ESBL-producing bacteria was mentioned [53]. Jacoby et al. explained how the *qnrB*-carrying isolates, primarily detected in the U.S.A. and India, were always present with $bla_{\text{SHV-12}}$ or $bla_{\text{CTX-M-15}}$ genes on the same plasmid [53]. The combined presence of extended-spectrum beta-lactamases encoding *bla* and *qnr* genes together within the same isolate, and/or on the same plasmid, is highly unfavourable for medical treatment. Both genes are able to confer resistance against two important classes of antimicrobial agents. Their spread to different sources, as different bacterial species, different environments or to humans is a high risk. Kawamura et al. described how ESBL-EC have become common among healthy people worldwide. They explained how most ESBL-EC usually acquired co-resistance to (fluoro)quinolones and other clinically important antimicrobial agents [1]. As we detected the ESBL and non-ESBL-EC, carrying resistance determinants against (fluoro)quinolone within isolates recovered from livestock and food, one could assume, that the respective plasmids had spread over different areas, thus, demonstrating the necessity of a One-Health approach when estimating the risk especially arising from *qnr*-carrying ESBL-EC and from non-ESBL-EC.

Conclusion

In this study, we described and analysed the presence of different ESBL-/non-ESBL-EC carrying a *qnrB* resistance gene. We found *qnrB*1 *and qnrB*2 genes to be present on larger plasmids, carrying multiple antimicrobial resistance genes, including different *bla* genes (e.g. $bla_{CTX\text{-}M\text{-}15}$, bla_{TEM1}, $bla_{OXA\text{-}1}$). Further, we found *qnrB*19 in particular on small Col440I plasmids. The presence of these PMQR genes together with extended-spectrum beta-lactamases encoding different *bla* genes in the same isolate or even on the same plasmid harbors a risk for public health. Especially the small Col440I plasmids seem to play an important role in the dissemination of *qnrB*19 genes, as they frequently had been described in *E. coli* and other *Enterobacteriaceae* from various sources and areas. The spread of MDR conferring plasmids contributes to an impaired treatment possibility. Thus, their evolution should be studied further, especially with an One-Health approach.

Methods

Isolate selection

For this study, *E. coli* collected during the German AMR monitoring of commensal (ZoMo-monitoring) and ESBL-/AmpC-producing (ESBL-monitoring) from food and livestock were chosen, as directed by the Federal Office of Consumer Protection and Food Safety (BVL). In addition, isolates carrying *qnrB* genes from research projects (indicated in the designation of MO) were added. Characterization of the isolates was based on WGS data obtained from Illumina NextSeq sequencing. ESBL-/non-ESBL-EC carrying *qnrB* genes were further investigated here. In total 33 epidemiologically unrelated *E. coli*, carrying *qnrB* were included. All isolates were initially cultivated on lysogeny broth [Luria Broth Base (Miller's LB Broth Base), Invitrogen-Thermo Fisher, Darmstadt, Germany] for 16–18 h at 37 °C.

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing (AST), of *E. coli* was conducted by determining minimum inhibitory concentrations (MICs) (including sulfamethoxazole, trimethoprim, ciprofloxacin, tetracycline, meropenem, azithromycin, nalidixic acid, cefotaxime, chloramphenicol, tigecycline, colistin, ampicillin, gentamicin and ceftazidime). Broth microdilution was performed according to EUCAST recommendations on a standardised European antimicrobial test panel (EUVSEC/EUVSEC2; Sensititre™, TREK Diagnostic Systems, UK). Therewith, all antimicrobials were tested in ranges as given by the European Commission Implementing Decision No. 2013/652/EU [54]. The results were interpreted according to EUCAST epidemiological cut-off values (ECOFFs) [55]. For quality assessment during MIC evaluation, the *E. coli* isolate ATCC 25,922 was included in every measurement. An ESBL-phenotype was assigned when the following phenotypic patterns were detected: cefotaxime or ceftazidime > 1 mg/L and meropenem ≤ 0.12 mg/L and cefoxitin ≤ 8 mg/L and no combination of cefotaxime/clavulanic acid and/or ceftazidime/clavulanic acid. Strains were further determined as phenotypically resistant against (fluoro)quinolones when expressing a MIC of 32 mg/L for nalidixic acid and/or 0.12 mg/L for ciprofloxacin. For better monitoring, we chose all isolates representing these criteria or one MIC step lower.

Whole-genome sequencing

For conducting whole-genome-sequencing (WGS), genomic DNA of *E. coli* was extracted using the PureLink Genomic DNA Mini Kit (Invitrogen-Thermo Fisher) according to the manufacturer's recommendation. The library for sequencing purposes was generated with

the Nextera DNA Flex Library Preparation Kit (Illumina®, San Diego, CA, USA) as previously described by Borowiak et al. [56]. Short-read sequencing was performed in paired-end WGS mode in 2×151 cycles with the Illumina® NextSeq™ 500/550 Mid Output Kit v2.5 (300 Cycles). If appropriate for plasmid closing, a primer-walking approach was applied. Therefore, the gDNA was used as template with primers (Supplement Table 5) derived from the assembled plasmid genomes. The amplification was performed in a Bio-Rad Thermal Cycler (Bio-Rad, Feldkirchen, Germany). The purification of the PCR amplification products was performed using the illustra GFX PCR DNA and Gel Band Purification Kit (Cytiva Europe, Freiburg, Germany). Sanger sequencing was performed by Eurofins Genomics (Eurofins Genomics, Ebersberg, Germany).

Bioinformatics analysis

Raw reads were trimmed with Aquamis [57] and de novo assembled using unicycler [58]. Quality assessment of the assemblies was conducted with quast [59]. Analysis of virulence factors, antimicrobial resistance genes, serotype and plasmid markers was conducted with bakcharak [60]. The prediction of the 7-locus MLST for each *E. coli* was screened with the tool v0.2 (https://github.com/tseemann/mlst) [61]. The annotations of the generated fasta files were achieved with prokka v1.14.5 [62]. To estimate the regions of local similarity between sequences BLAST was utilised (https://blast.ncbi.nlm.nih.gov/Blast.cgi). We used the PointFinder tool v2.2 [63] for detecting alterations in the chromosome responsible for (fluoro)quinolone resistance in *E. coli*. The refSNPer tool v1.0.0 was used as described before [64] to find best matching reference plasmids. To confirm the estimation, we screened our contigs, whether they were assignable to the estimated reference plasmid. We conducted this screening with the gplas tool v0.6.0. The gplas tool bins the respective contigs and therefore allows accurate plasmid-prediction [65]. To visualize and confirm these findings we used the mapping-based and assembly-assisted plasmid identification tool plasmidID v1.6.5 (https://github.com/BU-ISCIII/plasmidID) with a plasmid database containing all available closed plasmids from NCBI. For detecting all present MGEs within one isolate, the MGEfinder v1.0.6 was used [66]. For estimating the conjugative properties of the isolates, through detection of the MOB and mpf determinants, mob-suite v3.0.1 was utilised [67]. To detect the presence of Toxin-Anti-Toxin systems, we used the sling tool v2.0 [68]. For estimation of the phylotype, the phylotyper superphy (https://github.com/superphy/insilico-subtyping) was used. Finally, BRIG was used for visualizing the circular comparison between genomes [69]. For generating and visualizing the phylogram, Clustal Omega was used to generate the newick file [70]. This format was then used in iTOL [71]. Further, the alignment was visualised in geneious [72]. An arrow-based alignment was created with the gggene v0.4.1 extension of ggplot2 in R (https://wilkox.org/gggenes/). If not mentioned otherwise, all tools were used with default parameters.

in vitro filter mating studies

The transferability of the *qnrB*-carrying plasmids was determined by filter mating studies on solid LB agar. Liquid cultures of donor and recipient bacteria were mixed in a ratio of 1:2 (500:1000 µl), centrifuged at $5{,}000 \times g$ for 5 min. Thereafter, the supernatant was discharged, and the pellet was resuspended in 150 µl LB broth, applied on a 0.2 µm pore-size filter on an LB agar plate and subjected to incubation at 37 °C. After 24 h, bacteria were resuspended from pore-size filters in 4 ml LB broth and 100 µl of the bacterial suspension was applied on double selective agar plates [nalidixic acid (NAL) 8 mg/L and sodium acid (SAC) 100 mg/L]. After 24 h of incubation at 37 °C, the plates were interpreted for presumptive transconjugants. To confirm successful plasmid transmission, the transconjugants were subcultivated on double selective LB agar (NAL/SAC) and further analysed for the presence of the *qnrB* gene by PCR.

Abbreviations

(fluoro)quinolones: Fluoroquinolones and quinolones; AMP: Ampicillin; AMR: Antimicrobial resistance; BLAST: Basic local alignment search tool; cg: Core genome; CHL: Chloramphenicol; CIP: Ciprofloxacin; COL: Colistin; DNA: Deoxyribonucleic acid; *E.*: *Escherichia*; ECOFF: Epidemiological cut-off values; EFSA: European Food Safety Authority; ESBL: Extended-spectrum β-lactamase; ESBL-EC: Extended-spectrum β-lactamase-producing *E. coli*; EU: European Union; FOT: Cefotaxime; GEN: Gentamicin; MGE: Mobile genetic element; MIC: Minimal inhibitory concentration; MLST: Multilocus sequence type; mpf: Mating pair formation; NAL: Nalidixic acid; NRL AR: National Reference Laboratory for Antimicrobial Resistances; OMQR: Plasmid-mediated quinolone resistance; ORF: Open reading frame; *oriT*: Origin of transfer; PCR: Polymerase chain reaction; PFGE: Pulsed-field gel-electrophoresis; QRDR: Quinolone resistance-determining regions; SMX: Sulfamethoxazole; SAC: Sodium acid; ST: Sequence type; TA: Toxin-antitoxin; TAZ: Ceftazidime; TET: Tetracycline; TMP: Trimethoprim; UK: United Kingdom; US: United States; ZoMo: Zoonosis monitoring.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-022-08564-y.

Additional file 1: Supplement Table 1. In silico detected ST, O:H, resistance genes and gene variants of every analysed isolate. **Supplement Table 2.** In silico detected plasmids marker for every analysed isolate. **Supplement Table 3.** Phylotype and fim variants of investigated isolates. **Supplement Table 4.** PointMutation within isolate, determined with PointFinder. **Supplement Table 5.** Nucleotide Sequence and Amplification Temperature of special designed Primers for Primer-Walking. **Supplement Table 6.** Accession Number of submitted plasmid fasta files.

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Authors' contributions
KJ, JAH conceived the study and designed the experiments. KJ and JM performed whole-genome sequencing. KJ performed bioinformatics analyses. KJ and JAH interpreted the data. JAH supervised the project. KJ provided the draft manuscript. JAH, BM, DM, AK and SS decisively contributed in editing the manuscript. All authors were involved in project discussion and approved the final manuscript.

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Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files. The datasets used and analysed during the current study are available from the corresponding author on reasonable request. Contigs representing the detected *qnr*-carrying plasmid are available under the project number PRJNA755260 in NCBI. Accession Numbers are presented in Supplemental Table 6.

Declarations

Ethics approval and consent to participate
Not applicable. No animals are directly involved in this study for the collection of isolates.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Author details
[1]Department Biological Safety, German Federal Institute for Risk Assessment (BfR), Max-Dohrn Str. 8-10, 10589 Berlin, Germany. [2]Unit for Veterinary Public Health and Epidemiology, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria. [3]Institute of Microbiology and Epizootics, Department of Veterinary Medicine, Veterinary Centre for Resistance Research (TZR), Freie Universität Berlin, 14163 Berlin, Germany. [4]Veterinary Centre for Resistance Research (TZR), Freie Universität Berlin, Robert-von-Ostertag-Str. 8, 14163 Berlin, Germany. [5]Institute of Food Safety and Food Hygiene, Department of Veterinary Medicine, Veterinary Centre for Resistance Research (TZR), Freie Universität Berlin, 14163 Berlin, Germany.

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Publisher's Note

3. Discussion

3.1. Resistance against quinolones and fluoroquinolones in livestock and food from a One-Health perspective

Antimicrobial resistance development and its spread come with an expensive consequence. It is estimated, that drug-resistance infections lead to the death of over 25,000 patients per year and can cost the EU over 1.5 billion Euro in healthcare [201]. Many action plans are trying to tackle this problem and strengthen the prevention to control the development and spread of AMR. The importance of a One-Health approach is emphasised, including the human, veterinary, food, and environmental sector. The WHO published some general evidence-based principles to guide a comprehensive and integrated antimicrobial stewardship. Next to the development of guidelines and the regulated access to antimicrobial agents, an improved awareness and education as well as a thorough surveillance and monitoring programme is recommended [202].

While surveillance and monitoring are a country-responsible task, improvement of awareness and education should be accomplished through internationally operating and all sectors including research projects. Thus, ARDIG examined the dynamics of AMR in a One-Health approach. Especially, knowledge on potential for transmission of resistance determinants was gathered. For this purpose, a broader overview of the situation within each country is necessary and needs comparison with the conditions of other countries. In particular, specific bacteria carrying critically important resistances need to be monitored to understand their occurrence and spread. Therefore, we investigated the present situation of *qnr*-carrying and quinolone- or fluoroquinolone-resistant *E. coli* isolates in Germany. The European Medicines Agency (EMA) summarizes annual trends in the sales of veterinary antimicrobial agents from 31 European countries. The overall sales of antimicrobial agents for veterinary use in 2017 was at 8.6 tonnes in Germany and an overall sale of 68.6 tonnes for the 31 selected countries. Of those, 1.1 tonnes of fluoroquinolones were sold in Germany and a total of 2.4 mg/PCU within 31 countries [203]. Those numbers increased in 2018 to 9.6 tonnes overall sales in Germany and 69.3 tonnes for all 31 countries, as well as fluoroquinolones at 2.5 mg/PCU for all 31 selected countries. However, the sale for fluoroquinolones decreased in 2018 in Germany to 0.9 tonnes [204]. As summarized in the German sales trends provided by the EMA, the sale of fluoroquinolones increased by 50% from 2011 to 2014 and decreased by 35% from 2014 to 2018. Data published by the BVL, specifies this decrease from 12.3 tonnes in 2014 down to 7.7 tonnes in 2018. The latest data published, shows a

further decrease down to 6.4 tonnes for the sale of fluoroquinolones in Germany. Thus, the overall trend for Germany is promising.

Regarding the JIACRA III Report published by the ECDC, EFSA and EMA the consumption of fluoroquinolones and quinolones in 2017 was higher in humans than in food-producing animals in the majority of the countries [205]. Further, a significant positive association for the consumption of fluoroquinolones and quinolones in humans and animals was observed between 2016 and 2018. In addition, a statistically significant association for this consumption and resistance in *E. coli* for fluoroquinolones and quinolones was reported. This was notified in humans as well as in food-producing animals. The correlation for food-producing animals was considered for *E. coli* isolates from broilers, turkeys, pigs and calves. The data is based on the susceptibility of the isolates against ciprofloxacin. This positive association was detected throughout the years 2014 to 2018. For the treatment of animals, fluoroquinolones should only be used if no other antimicrobial agents were effective. However, this statistically significant positive association was observed in a cross-sectional manner. Meaning, next to an association for the use and resistance development within bacteria from animals or humans, this positive association was also observed for the use in animals and the resistance development in humans and vice versa, during the years 2016 to 2018.

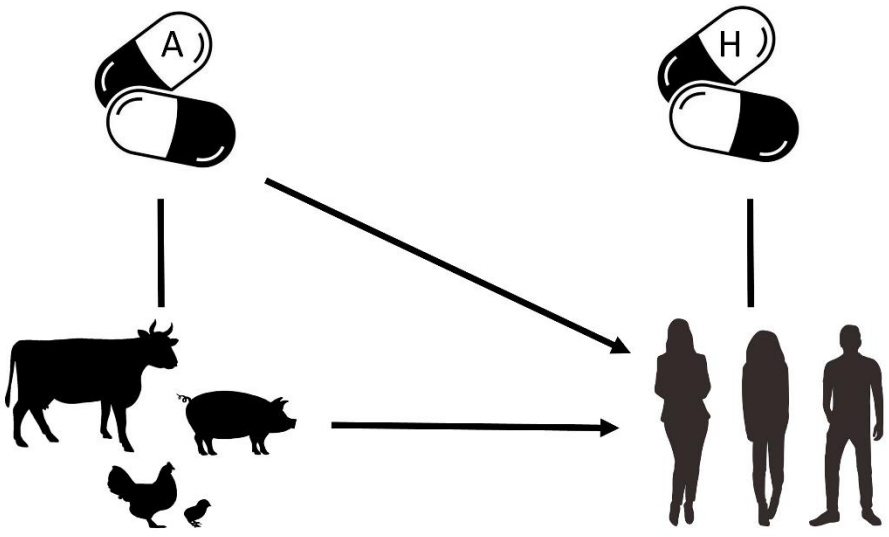

Figure 2. Schematic overview of the potential associations between antimicrobial consumption and antimicrobial resistance in humans and food-producing animals investigated for *E. coli* regarding fluoroquinolones and quinolones adapted from the JIACRA III [205]. Arrows symbolizing a statistically significance in univariate analysis, but not confirmed in multivariate analysis. Lines symbolising a statistically significance in a multivariate analysis. Symbols of pills with an A are antimicrobial consumption in food-producing animals. Symbols of pills with a H are demonstrating the antimicrobial use in humans. Vector icons were provided by: https://www.vecteezy.com/free-vector/sheep-silhouette, https://www.vecteezy.com/free-vector/human and https://www.vecteezy.com/free-vector/web

Although the consumption of fluoroquinolones and therewith presumably the resistance development was decreasing in the last years in Germany, AMR is a global problem. Therefore, it should always be discussed as such. A country can prohibit the use of certain antimicrobial agents and therewith decrease the selection pressure under which resistant bacteria can develop and spread [206]. It can monitor the application of certain substances in a healthcare environment. Germany, for example, demands the recording of an antibiogram, if fluoroquinolones are administered to animals or livestock. Thus, leading to a more targeted use of certain antimicrobial agents. These factors, as previously discussed, can help to tackle the problem of AMR development. However, in a time where people travel around the world, livestock is bought in different countries and food travels the world for processing, the influence of other countries antibiotic use policy is unavoidable. Complex travel networks allow a rapid spread of resistant organism between different countries [207]. There is a general intensive exchange of people and products around the world. Thus, policies of each country do have a significant impact on the whole world. The spread of AMR across borders is given. In some country, as India, no regulation for the use of antibiotic in livestock and food exists. Although forbidden in the EU since 2006, low doses of antimicrobial agents can be added to livestock's food to promote growth and therewith enhance the production efficiency [208]. Unfortunately, this is exactly the behaviour, favouring the development of resistances. The resistant microorganisms are then the source for spread into different compartments, including the human sector. Some studies demonstrate a strong effect that administration of low-doses of antimicrobial agents to foodstuff lead to resistance development, also in pathogenic organisms [209]. Thus, non-human antimicrobial usage results in human health threat. This is especially concerning, when antimicrobial agents are used in the livestock and food area, that are reserved for severe cases in the human health sector. The WHO considers fluoroquinolones as Highest Priority Critically Important Antimicrobials in human use. For the veterinary field, they are considered as Critically Important Antimicrobial Agents within the OIE (World Health Organisation for Animal Health) list. The broad spread of resistances against fluoroquinolones is also driven by PMQRs. Therewith, especially *qnr*, the pentapeptide repeat protein encoding PMQR, is of special interest. Qnr homologs have been described in many microorganisms, including Gram-negative and Gram-positive bacteria (e.g. *Bacillus*, *Enterococcus*, *Listeria*) [61, 96]. Furthermore, they are often described in livestock and food environment as well as within the human sector [210-212] worldwide [61]. They are described to only confer low-level resistances but can alter the susceptibility drastically in combination with certain point mutations within the chromosome. All these factors make *qnr* an important gene for the monitoring of the development of decreased susceptibility against quinolones or fluoroquinolones. A thorough monitoring and investigation of this gene is therefore an important step in understanding its broad spread.

Thus, *qnr* is broadly detectable in different species and in different matrices. However, in this work the focus was set on *E. coli.* We found that *qnr* is also present in a variety of different STs in *E. coli*. We found the characteristics of the *E. coli* carrying the *qnr* determinant to be highly heterogenic (publication II). The STs of the *E. coli* isolates as well as other resistance determinants or Inc groups present within the *E. coli* differed strongly. The plasmids carrying the *qnr* determinant were present within *E. coli* of rarely mentioned STs as well as in STs of high importance, such as ST10. ST10 is a predominant lineage among some pathogenic *E. coli* and was detected worldwide [150]. Furthermore, ST10 is frequently detected in human sources. This high heterogeneity of *E. coli* carrying the *qnr* determinant is an indicator for the wide adaptability of this PMQR gene. As the characteristics like the resistome, the carried virulence genes or the ability of pathogenicity, outgoing by the *E. coli* are highly diverse, the risk from *qnr*-carrying *E. coli* is also broad.

3.2. Impact of horizontal gene transfer in the dissemination of *qnr* resistance genes, evaluated in a One-Health perspective

Horizontal gene transfer (HGT) can lead to a broad dissemination of resistance genes and the spread to different species. While vertical gene transfer only results in a slow resistance gene inheritance within the same species, HGT is a driver of proliferation. For the PMQR resistance gene *qnr*, the drivers of its spread are transmissible plasmids. We detected those prevalent plasmid types, working as vehicles. The predominant *qnr* genes were *qnrS* and *qnrB*. They do predominantly exist on IncX or small Col-type plasmids, respectively. Thus, predominant plasmid types are responsible for the transfer of these PMQR genes. It is of utmost importance to understand the composition of these plasmids. This means, to understand their structure, their resistome, their mobile elements as well as the virulence factors harboured by these plasmids. Thus, understanding its success in proliferation and its ability to endure stably in their host.

It is known, that HGT is the reason for a rapid adaption to new environments for the microorganisms. However, only in rare cases, it is a fitness benefit for the strain to remain plasmids. Thus, only selective plasmids, necessary in certain conditions will sustain. In environments associated with fluoroquinolone and quinolone use, the adaption through resistance development is of high relevance. Studies were published, indicating this as the key role in evolutionary success for fluoroquinolone resistant clones [213-215]. Redgrave and his team also discussed a general benefit of fluoroquinolone resistance for *E. coli*. Thus, they report how long-term experiments resulted in mutations in genes responsible for supercoiling. This fitness benefit was reported as beneficial even under no antibiotic selective

pressure [215]. Thus, the HGT of *qnr* can be pervasive in its reach and stability. Next to the spread of resistance determinants through HGT, the mode of expansion of resistance determinants is another important factor. We were able to prove, that the PMQR *qnr* is frequently detectable on the same plasmid as other resistance genes. Thus, spreading the ability of resistance development against two or more important antimicrobial agents. This mutual spread has been described before. Lee at al. found that more than 20% of ESBL- and/or plasmidic AmpC-harbouring isolates carried a *qnr* gene. Most of these *qnr* genes were identified as *qnrS*1 or *qnrB*. The isolates were recovered from hospital-acquired infections from patients [216]. Koposta and his team reviewed all published carbapenem-resistant *Enterobacteriaceae* from 2013 and 2018 and found that *qnr* was most frequently associated with plasmid-borne carbapenemase genes [217]. In addition, bla_{CTX} was detected frequently on the same plasmid as *qnr* [174, 218]. Sanchez et al. further detected bla_{NDM} in correlation with *qnr*. Therewith these isolates, carrying plasmids with *bla* and *qnr* were isolated from different sources and multiple species. Isolation was performed from environmental water samples, from livestock and food samples as well as from hospitalized patients. This demonstrates the broad distribution of these plasmids. It shows the high impact of the co-selection of these two important resistance genes and the wide distribution, most probably through HGT. Overall, the high impact of HGT and the spread of co-selection should always be estimated in a One-Health approach. Studies have shown that foreign genes can enter the human genome over an evolutionary period. Usually, HGT can occur in different taxa that exist in the same environment [219]. The dynamics and dissemination pathways of resistance genes from environment, human and microorganisms intertwine. Overall, the One-Health approach of resistance gene dissemination considers multiple pathways. The irrigation of wastewater, the use of contaminated manure and a traditional livestock husbandry, living close to the animals, are all factors contributing to the dissemination of resistance genes within different matrices [221]. The basic definition of One-Health is “the collaborative effort of multiple health science professions, together with their related disciplines and institutions—working locally, nationally, and globally—to attain optimal health for people, domestic animals, wildlife, plants, and our environment” [222]. This mutual approach is inevitable, when realized that over 75 % of human infectious diseases are estimated to be of animal origin [223]. Especially as some antimicrobial agents are specifically reserved for the treatment of severe human infections, a dissemination of resistances against them should highly be avoided. However, the prophylactic treatment of crops or the growth-enhancing use of antimicrobial agents is still exploited [224]. The practice of low-dose long-term treatment is critical. Low-doses application of antimicrobial agents presents a perfect ground, for resistance development, as the dose is too low to result in a bactericidal effect but enhances the acquisition of resistance determinants leading

to a defence. This handling in the use of antimicrobial agents, leading to resistance, can ultimately cause a spread of resistant bacteria to the human population. For pathogenic bacteria, the combination of resistance and disease bringing microorganism can be fatal. The solely investigation of resistance genes presence in only one area is therefore not feasible anymore. Only a One-Health approach will lead to a proper understanding and appropriate arrangement for a plan of action against the burden of resistance-gene dissemination and the attempt to limit its harsh consequences.

3.3. Difficulties of correctly estimating the structure of the plasmid, carrying resistance determinants

As discussed in the chapters before, plasmids can harbour multiple resistance genes. It is of great importance, to understand whether a plasmid is prone to confer resistance to only one class of antimicrobial agents or whether it is responsible for conferring multiple resistances. In addition, the understanding of resistance gene cassettes, located on a plasmid or the ability of the plasmid to gain further resistance genes through IS elements or transposons helps for a proper risk assessment. As elucidated, the influence of HGT and the correct estimation of the location of the resistance gene is crucial. Thus, it is an important factor to consider if the respective gene is located on a plasmid or encoded chromosomally.

For many years, this information was only accessible through laborious work in the wet laboratory. Methods as plasmidic DNA extraction and corresponding PCR amplifications followed by gel electrophoresis were commonly used to detect resistance genes. Only with the time-consuming PFGE analysis and DNA-DNA hybridization experiments a conclusion could be drawn for the size of plasmids and a prediction of the gene location. However, those experiments only elucidated a tiny truth of the whole picture. It was not possible to conclude the mutual presence of resistance genes on one plasmid within a limited time. The detection of IS elements or the estimation of the Inc type of the plasmids was not assignable through only one experiment. Multiple experiments needed to be conducted to get enough information to solve the jigsaw of characteristic pieces for each isolate. Although those laboratory methods still represent the gold standard for many analyses and are also often necessary to ensure a valid prediction, DNA-sequencing and its commercialization has evolved to a necessary tool in every laboratory. Multiple technologies have evolved for sequencing genomes, including Roche, Illumina Nanopore and PacBio. Different approaches, as short-read, long-read or hybrid sequencing and plenty of bioinformatics analysis tools were developed in the last decade. Each approach entails its own benefits and

disadvantages. However, they all combine the benefit of rapidly characterising multiple isolates in depth with only one approach. With the correct tool, all necessary information of an isolate can be estimated within hours after sequencing. Thus, the prediction of the corresponding ST, the presence of resistance genes and their influence in the resistance profile, the presence of virulence genes, the location of certain determinants and the mere sequence or the SNP analysis compared to a reference can be investigated with a single command. The degree of information through sequencing expanded excessively. The deep analysis of certain components like gene cassettes can be elucidated on sequence-level through sequencing analysis. SNP analysis can help in outbreak investigations or in the understanding of evolutionary paths [225]. In addition, the application for sequencing for a metagenomics approach or for epigenetic context is thriving. The use of sequencing beyond the area of microorganisms and their characteristics is even far greater.

Although sequencing is a method nowadays indispensable, it still has its limitations or pitfalls. Thus, the choice of sequencing method and the selection of bioinformatics tools can have a severe influence on the outcome of the results. As different sequencing platforms are known to have different error rates, the detection of certain genes can be influenced by error-prone approaches. However, those pitfalls begin on an experimental level. The handling of the sample as well as the selected DNA extraction methods can influence the outcome of the analysis. The impact of DNA yield and DNA quality or in metagenomics approaches, a biased extraction regarding Gram-negative or Gram-positive microorganisms does play a role in the analysis of the generated sequences. As discussed in publication I, the choice of long-read versus short-read sequencing does have an immense influence on the assignment of respective areas within the genome. Thus, short-read sequencing, generating shorter raw reads, has a higher tendency to ascribe falsely some sequence-reads to wrong areas. Long-read sequencing generating longer raw reads, spanning over larger areas and therefore do not need to assign reads but rather scan the real composition. Lastly, the choice of the bioinformatics pipeline used does have its impact on the outcome. Studies showed clearly that different algorithms could alter the final result [226, 227]. These biases and pitfalls do also account for the *in-silico* detection and characterization of plasmids and their impact on resistance gene spread and their influence on the resistance profile. Many tools exist, trying to estimate the size, the resistance genes present on a plasmid, the Inc type and the impact of the resistance genes regarding the resistance profile. Although those bioinformatics tools improved over time, they are still just a most likely calculation of the assembling of raw reads and the analysis of the combined contigs. Especially, when working reference based the detection of newly developed areas or altered structures can easily be overlooked or missed. Regarding the estimation of the resistance profile, many factors can influence the correct

output. Thus, the usage of error-prone long-read sequencing can affect the correct detection of certain genes or mutations leading to resistances [228]. As this information is crucial for the correct resistance profile estimation, errors in the nucleotide base sequence can influence the estimated susceptibility against certain antimicrobial agents. This can have an even higher impact if the resistance development depends on multiple factors as with fluoroquinolones and quinolones. Thus, the choice of sequencing and assembling methods is of high importance. All these factors should always be kept in mind when data is analysed.

3.4. Advancements and further possibilities in the usage of WGS for plasmid characterization

As previously discussed, the correct plasmid reconstruction based on sequencing data remains challenging. It is complicated, to determine repetitive regions, often present on plasmids. The correct assignment of contigs to the chromosome or to the plasmid is dependent on multiple *in-silico* calculations and always needs laboratory validation. In addition, the general combination of contigs to a whole structure remains a calculation, often based on reference comparison. Thus, new structures or rearrangement can easily be missed. However, sequencing and assembly strategies revolutionized the plasmid characterization and is being improved continually. During the research time of this doctoral thesis, multiple programmes were developed to facilitate plasmid detection and characterisation. Also, further sequencing approaches emerged.

Long-read sequencing has evolved and is easily available nowadays. A common way to determine plasmidic structures is a hybrid approach, as discussed in our research. However, some long-read approaches are quite error-prone. A general way to reduce this error is to increase the sequencing depth. This often leads to a costly overall approach. We suggest the long-read sequencing with a lower sequencing depth to generate a backbone, working as reference and adding short-read data to this information. Thus, the biased reference-based assemblies, established on a reference database, can be overcome. Larger repetitive regions, and altered structures compared to references will thus be detected with the long-read approach and the short-read-based contigs will erase the error resulting from the long-reads [229-231].

Another promising approach is the method of synthetic long-read sequencing. This technique only requires short-read sequencing and therefore includes the correct sequence detection of an Illumina approach. Here, the sample preparation allows for longer, synthetically generated reads. For example, Tru-seq synthetic long-read (SLR) sequencing is a provider for generating sequences of ten thousand base pairs on an Illumina platform. The library

preparation uses specific indexes for the fragmented DNA-sequences to assemble them after the sequencing step to longer reads [232]. Other SLR-approaches include 10X linked reads, Hi-C, and Bionano optical mapping [233]. These methods are rising and promising approaches for the establishing of reference genomes or reference plasmids and therewith the detailed and correct reconstruction of mobile genetic elements and their resistance determinants.

Another important utility of sequencing is the metagenomics sequencing approach. This does not only investigate one sample in depth but is able to give an overview about the overall situation in a complete sample with all its compositions. Especially considering the dynamics of antibiotic resistances, an overall observation of a sample is more informative than the analysis of a single organism. WGS methods do restrict the investigation of complex uncultured communities. With metagenomics, where DNA can be directly extracted from a sample and not from only one microorganism, the complex composition of microbial communities and their antibiotic resistance genes, often located on plasmids, can be understood better. Overall, metagenomics seems a promising tool for the determination of antibiotic resistance dynamics [234].

Sequencing of microorganisms or whole communities is nowadays an indispensable method to determine plasmids and antimicrobial resistance determinants as well as their dissemination path. However, multiple processes are still in the making, revolutionizing the perspective of our understanding of the genome in a constant manner. It is crucial to stay up to date with these tools, to use all the benefits that sequencing offers in the analysis of plasmids and their resistance determinants.

3.5. Concluding remarks

The dissemination of resistance genes is the main contributor for the increasing AMR development worldwide. Only with a One Health approach, including the environment, humans and food and livestock, the achievement of a thorough understanding of resistance dynamics is guaranteed. The use of antimicrobial agents in the livestock area is a factor, contributing to this resistance development and resistance gene spread. With the work conducted in the presented research, which is part of the ARDIG project, we contributed to the understanding of resistance genes, leading to quinolone and fluoroquinolone resistance. We investigated the overall situation for *qnr* prevalence in Germany in 2017 and published a comprehensive evaluation of the generated data. We conducted an in-depth study of the most prevalent plasmid types, carrying *qnr* and were able to publish plasmid backbones responsible for carrying *qnrB* in ESBL *E. coli* and *qnrS* in commensal *E. coli*, respectively.

Therewith, we outlined an evaluation of these mobile genetic elements, carrying these resistance genes. The investigation for these mobile elements for the present structures, revealed certain associations for *qnr* and other resistance genes and virulence genes. These outcomes will help others to detect similarities when inspecting comparable structures. Moreover, the outcome of different sequencing methods for detecting plasmid was scrutinized. This study allows other researchers to choose the optimal method in investigating mobile genetic elements and represents a platform for further research into this area of plasmid sequencing, assembling and characterization.

It would be of interest, to study the prevalence of quinolone and fluoroquinolone resistance also for different years, areas and matrices. Thus, a more comprehensive, EU wide understanding would be achieved. In addition, the comparison of prevalent plasmid types in different countries would allow a greater understanding in the evolution and development of these mobile genetic elements. Moreover, as WGS is a rapidly evolving technique, new methods for plasmid sequencing and assembling would allow for an even deeper inside into structures and even epigenetic behaviour of resistance determinants on plasmids. Furthermore, the investigation of and comparison with other surroundings, as the environment or human source, would allow for a more complete understanding of these dynamics within a One-Health approach.

However, antimicrobial resistance dynamics is a constantly evolving progress, the constant research is necessary to intervene in the spread of resistance determinants. With this study, we contributed to the understanding of the influence of mobile genetic elements for the dissemination and dynamics of important resistance determinants in commensal *E. coli*.

4. Summary

Influence of mobile genetic elements on the dissemination of resistance determinants in commensal *Escherichia coli*

The presence of resistance determinants in livestock and food and the possible AMR dissemination is a global threat. It can result in treatment failure when trying to treat infections caused by no longer susceptible microorganisms. Especially, resistance development and spread of resistance to highest priority critically important antimicrobial agents as quinolones is highly undesirable. The pentapeptide repeat proteins encoded by *qnr* genes is a PMQR leading to an increased MIC against quinolones and fluoroquinolones. Tackling the spread of PMQRs needs in-depth investigation of its prevalence, of the vector characteristics and of the main dissemination paths. Therefore, the establishing of appropriate protocols for characterizing the plasmids carrying the PMQR as *qnr* with e.g. different sequencing techniques is highly desirable. The OHEJP-ARDIG project targets the international and integrative examination of the topics evolved around resistance development. Located within ARDIG, this thesis aims to understand the prevalence and characteristics of quinolone- and fluoroquinolone-resistant commensal *E*. *coli* and their mobile genetic elements. A focus lies on *qnr*-carrying plasmids, and their characterization, using an optimized sequencing and assembling approach.

Therefore, we used different sequencing and assembling strategies for assessing their reliability for AMR monitoring in commensal *E. coli*. Isolates were subjected to WGS with Illumina NextSeq, PacBio and ONT for an in-depth characterization of their plasmid content. We further assembled the generated raw reads with different techniques, including long-read only, short-read only and hybrid approach. The established data was compared for validity with data from laboratory-generated experiments. We found long-read sequencing resulting in error prone prediction of the plasmid genome, while short-read sequencing was rather insufficient for linking AMR genes to specific plasmids. Only a hybrid approach allowed for an overall analysis of the whole plasmid genome and its characteristics. With the establishing of the most reliable sequencing technique for detecting plasmids, we scrutinized the prevalence of *qnr* on MGEs in *E. coli* from German livestock and food, as understanding the pathways of PMQR spread begins with monitoring the presence of e.g. *qnr* genes on plasmids. Thus, we investigated the prevalence of the *qnr*-carrying plasmids in commensal *E. coli*. We aimed to detect the common characteristics of *qnr*-carrying plasmids and *E. coli* as well as their association to other risk factors as e.g. virulence genes. We found *qnr* to be widely spread over different livestock and food matrices, detected in different ST of *E. coli*. We frequently detected *qnr* and *qac* co-existing on the same plasmid and in association to other resistance

genes like cephalosporin determinants. In addition, most of the investigated isolates had point mutations in the QRDR, leading to even higher MIC values. Thus, *qnr*-carrying *E. coli* often harboured multiple risk factors that need to be considered when evaluating their impact on resistance development and spread in livestock and food. As we detected *qnrS* and *qnrB* to be the most frequent variants in German livestock and food in *E. coli*, we investigated the plasmids, carrying these resistance genes. We found *qnrS1* to be highly prevalent in the analysed samples, located mainly on IncX plasmids. All here investigated IncX plasmids carried a *bla* resistance determinant and were recognized as transmissible. Thus, it seemed that IncX plasmids are the main vector for the dissemination of *qnrS* resistance genes. While *qnrS* is frequently detected in livestock and food samples, *qnrB* was often reported in samples, isolated from humans, associated with ESBL *E. coli*. This combination of resistance against two important antimicrobial agents is highly undesirable from a clinical point of view. Therefore, we further examined the presence and characteristics of *qnrB*-carrying ESBL *E. coli*. Here, we found a small Col-plasmid to be the main vector of *qnrB19*. In addition, larger IncH and IncN plasmids were detected as carriers for *qnrB*. While the Col-plasmid did not carry any other resistance genes, the other prevalent plasmid types were responsible for a multi-resistance phenotype. In addition, all plasmids were characterized as transmissible. Thus, another vector was characterized, presumably responsible for the spread of *qnrB* in ESBL *E. coli*. However, the *E. coli* harboruing the *qnrB* or *qnrS* genes were highly heterogenic in their STs and O:H-types.

Overall, we found *qnr* genes frequently in combination with other resistance determinants, virulence factors and quaternary ammonium compounds. Moreover, known and unknown point mutations within the chromosome increased the MIC against quinolones and fluoroquinolones. All these factors demonstrate the importance of the resistance determinant *qnr*. As the general characteristics of the *E. coli*, like the resistome, the carried virulence genes or the ability of pathogenicity, was highly diverse, the general risk outgoing from the *qnr*-carrying *E. coli* is also broad. However, we detected prevalent plasmid types carrying *qnrB* and *qnrS*, recognized as a probable driver of *qnr* spread. Furthermore, we have shown that the choice of sequencing and assembly methods is of high importance when investigating MGEs. Only with the correct sequencing and assembly approach, a reliable risk assessment can be ensured. With this study, we contributed to the understanding of the influence of MGEs for the dissemination and dynamics of important resistance determinants in commensal *E. coli.*

5. Zusammenfassung

Einfluss mobiler genetischer Elemente auf die Verbreitung von bedeutenden Resistenzdeterminanten in kommensalen *Escherichia coli*

Das Vorhandensein und die Verbreitung von Resistenzdeterminanten in Nutztieren und Lebensmitteln stellt eine globale Bedrohung dar. Die Behandlung von Infektionen, ausgelöst durch (multi)resistente Mikroorganismen, stellt die Humanmedizin vor ungeahnte Herausforderungen und finanziellen Problemen. Insbesondere die Resistenzbildung und Ausbreitung von Resistenzen gegen sogenannte „highest priority critically important antimicrobial agents“, wie Fluorchinolone, ist als besorgniserregend einzustufen. Das über *qnr*-Gene kodierte Pentapeptid-Repeat-Protein ist ein PMQR, das zu einer Erhöhung der MHK-Werte gegenüber diesen Chinolonen und Fluorchinolonen führen kann. Um diese Ausbreitung nun effektiv zu verhindern, ist eine fundierte Untersuchung der Prävalenz, der Vektormerkmale und der Hauptverbreitungswege notwendig. Die Etablierung geeigneter Protokolle zur Charakterisierung der PMQR-tragenden Plasmide mittels verschiedenen Sequenzierungstechniken ist folglich sinnvoll. Das OHEJP-ARDIG-Projekt hat das Vorhaben, die internationale und integrative Auseinandersetzung mit den Themen rund um die Resistenzentwicklung zu stärken. Diese, im ARDIG-Projekt angesiedelte Dissertation, hat dabei zum Ziel, die Prävalenz und Eigenschaften von Chinolon- und Fluorchinolonresistenten kommensalen *E. coli* und ihren mobilen genetischen Elementen zu verstehen.

Ein Schwerpunkt liegt hierbei auf *qnr*-tragenden Plasmiden und deren Charakterisierung unter Verwendung eines optimierten Sequenzierungs- und Assemblierungsansatzes. Hierfür wurden verschiedene Sequenzierungs- und Assemblierungsstrategien verwendet, um die Zuverlässigkeit für die AMR-Überwachung in kommensalen *E. coli* zu bewerten. Die Isolate wurden mittels Illumina NextSeq, PacBio und ONT WGS Methoden untersucht, um das Vorhandensein von Plasmiden zu charakterisieren. Die so generierten raw-reads wurden mit verschieden Algorithmen assembliert, darin einbezogen waren long-read-only, short-read-only und hybrid-Ansätze. Die ermittelten Daten wurden auf Übereinstimmung mit Daten aus Laborversuchen verglichen. Dabei konnte nachgewiesen werden, dass die long-read-Sequenzierung zu einer fehleranfälligen Vorhersage des Plasmidgenoms führen kann, während die short-read-Sequenzierung unzureichend war, um AMR-Gene mit spezifischen Plasmiden zu verknüpfen. Nur ein hybrider Ansatz ermöglichte eine Gesamtanalyse des vollständigen Plasmidgenoms und seinen Eigenschaften.

Mit der Etablierung der zuverlässigsten Sequenzierungstechnik zum Nachweis von Plasmiden konnten wir die Prävalenz von *qnr* auf MGEs in *E. coli* aus deutschen Nutztieren

und Lebensmitteln untersuchen. Unser Ziel war es hier, die gemeinsamen Merkmale von *qnr*-tragenden Plasmiden in *E. coli*, sowie ihre Assoziation mit anderen Risikofaktoren wie z.B. Virulenzgenen zu untersuchen. Es wurde festgestellt, dass *qnr* über verschiedene Nutztier- und Lebensmittelmatrizen und in verschiedenen *E. coli* ST weit verbreitet ist. Häufig wurde beobachtet, dass *qnr* und *qac* auf demselben Plasmid und in Assoziation mit anderen Resistenzgenen wie Cephalosporin-Determinanten koexistieren. Darüber hinaus wiesen die meisten der untersuchten Isolate Punktmutationen im QRDR auf, was zu noch höheren MHK-Werten führte. Zusätzlich zeigte sich, dass *qnr*-tragende *E. coli* häufig mehrere Risikofaktoren beherbergen, die bei der Bewertung ihrer Auswirkungen auf die Entwicklung von Resistenzen und die Ausbreitung in Nutztieren und Lebensmitteln berücksichtigt werden sollten.

Da *qnrS* und *qnrB* als die häufigsten Varianten in deutschen Nutztieren und Lebensmitteln in *E. coli* identifiziert wurden, wurden die Plasmide, die diese Resistenzgene tragen, eingehend untersucht. Es konnte gezeigt werden, dass *qnrS1* in den analysierten Proben stark verbreitet ist und sich hauptsächlich auf IncX-Plasmiden befindet. Alle hier untersuchten IncX-Plasmide trugen zusätzlich eine *bla*-Resistenzdeterminante und wurden als übertragbar eingestuft. Somit scheint es, dass IncX-Plasmide ein Hauptvektor für die Verbreitung von *qnrS*-Resistenzgenen sind. Während *qnrS* häufig in Nutztier- und Lebensmittelproben nachgewiesen wird, wurde *qnrB* vermehrt in Proben gemeldet, die aus Menschen isoliert wurden und mit ESBL *E. coli* assoziiert sind. Diese Kombination von Resistenz ist aus klinischer Sicht höchst unerwünscht. Daher haben wir das Vorhandensein und die Eigenschaften von *qnrB*-tragenden ESBL-*E. coli* weiter untersucht. Hier zeigte sich ein kleines Col-Plasmid als Hauptvektor von *qnrB19*. Außerdem wurden größere IncH- und IncN-Plasmide als Träger für *qnrB* nachgewiesen. Während das Col-Plasmid keine anderen Resistenzgene aufwies, waren die anderen häufigen Plasmidtypen für einen multiresistenten Phänotyp verantwortlich. Außerdem wurden alle Plasmide als übertragbar charakterisiert. Damit wurde ein weiterer Vektor aufgezeigt, der vermutlich für die Ausbreitung von *qnrB* in ESBL *E. coli* verantwortlich ist. Zusätzlich waren die *E. coli*, die die *qnrB*- oder *qnrS*-Gene enthielten, in ihren STs und O:H-Typen hochgradig heterogen.

Insgesamt fanden wir *qnr*-Gene häufig in Kombination mit anderen Resistenzdeterminanten, Virulenzfaktoren und quartären Ammoniumverbindungen. Darüber hinaus konnten mehrere charakterisierte und unbekannte Punktmutationen innerhalb des Chromosoms identifiziert werden, die die MHK-Werte gegenüber Chinolonen und Fluorchinolone erhöhten. Die Summe dieser Faktoren demonstriert die Bedeutung des *qnr*-Resistenzgens. Da die allgemeinen Eigenschaften der *E. coli*, wie das Resistom, die übertragenen Virulenzgene oder die Fähigkeit zur Pathogenität, sehr vielfältig ist, ist auch das allgemeine Risiko, das

von den *qnr*-tragenden *E. coli* ausgeht, breit gefächert. Wir haben jedoch vorherrschende Plasmidtypen entdeckt, die *qnrB* und *qnrS* tragen, die wahrscheinlich als Verantwortlicher der *qnr*-Ausbreitung gesehen werden können. Darüber hinaus wurde gezeigt, dass die Wahl der Sequenzierungs- und Assemblierungsmethoden bei der Untersuchung von MGEs von großer Bedeutung für eine verlässliche Risikobewertung ist.

Die Entwicklung und Verbreitung antimikrobieller Resistenzen ist ein sich ständig weiterentwickelnder Prozess, der dauerhaft verfolgt werden sollte. Mit dieser Studie wurde in einem Teilbereich zum Verständnis des Einflusses von MGEs auf die Verbreitung und die Dynamik wichtiger Resistenzdeterminanten in kommensalen *E. coli* beigetragen.

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VI. Conflict of interest

No conflict of interest to declare.

VII. Selbstständigkeitserklärung

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

Berlin, den 20.10.2022 Katharina Anna Juraschek