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

Transfer and structural alterations of resistance plasmids carrying carbapenemase-encoding genes in a broiler chicken infection model

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

vorgelegt von
Sead Hadziabdic
Tierarzt, Mag.
aus Doboj, Bosnien und Herzegowina

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Dekan:	Univ.-Prof. Dr. Jürgen Zentek
Erster Gutachter:	Univ.-Prof. Dr. Uwe Rösler
Zweiter Gutachter:	PD Dr. Burkhard Malorny
Dritter Gutachter:	Univ.-Prof. Dr. Thomas Alter

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II List of abbreviations

AMR	Antimicrobial resistance
AMU	Antimicrobial usage
BfR	Bundesinstitut für Risikobewertung
bla	Beta-lactamase
bp	Base pair
CIA	Critically important antimicrobials
CPB	Carbapenemase-producing bacteria
CPE	Carbapenemase-producing enterobacteria
CTX	Cefotaxime
DNA	Deoxyribonucleic acid
*d*T+	*d*Tartrate-positive
EC	European Commission
ECDC	European Center for Disease Control
EFFORT	Ecology from Farm to Fork Of microbial drug Resistance and Transmission
ESBL	Extended-spectrum beta-lactamase
EU	European Union
HGT	Horizontal gene transfer
Inc	Incompatibility
kbp	Kilobase pair
KPC	*Klebsiella pneumoniae* carbapenemase
MBL	Metallo-beta-lactamase
MDR	Multidrug resistance
MEM	Meropenem
MGE	Mobile genetic element

NAL	Nalidixic acid
NCP	National control programme
NDM	New Delhi Metallo-beta-lactamase
OXA	Oxacillinases
PBP	Penicillin-binding protein
PFGE	Pulsed-field gel electrophoresis
S.	*Salmonella enterica* subsp. *enterica* serovar
SHV	Sulphydryl variable
SNP	Single-nucleotide polymorphism
ST	Sequence type
TEM	Temoniera
UN	United Nations
VIM	Verona Integron Metallo-beta-lactamase
VRE	Vancomycin resistant enterococci
WGS	Whole genome sequencing
WHO	World Health Organization
XLD	Xylose lysine deoxycholate

1. Introduction

Antimicrobial resistance (AMR) is among most important threats for global public and animal health (1, 2). Although many AMR genes have an environmental origin and resistance is a naturally occurring phenomenon, a main driver for the emergence and rapid spread of AMR was the intensive antimicrobial usage (AMU) in the last few decades (1, 3, 4). A common observation is that AMR emerges soon after a new antimicrobial compound is introduced into the market (5). The inappropriate use of antimicrobials in humans and animal production led to the emergence of resistant bacteria in both, hospital settings and livestock production. With the aim of tackling the AMR, a final ban on the use of antimicrobials as growth promotors in food-producing animals in European Union (EU), came into force on January 1, 2006. The World Health Organization (WHO) urges that all and especially critically important antimicrobials (CIA) are used reasonably in human and veterinary medicine (2). However, a challenge for preventing the global spread of AMR is the diversity in regulations and management options for antibiotic stewardship in developed and developing countries. International trade and travel have facilitated spread of resistance genes from one geographic region to another (4, 6). This spread was mainly driven by highly transferable mobile genetic elements, such as plasmids which led to dissemination of resistance genes in different bacteria (7).

The emergence of extended-spectrum beta-lactamases (ESBL) producing enterobacteria in the animal production in the last two decades was an alarming indication on the trends of AMR in livestock production (8). However, events that provoked broad attention of the scientific community were first reports of carbapenemase-producing enterobacteria (CPE) detected in livestock in recent years (9, 10). This was due to a common understanding that the occurrence of CPE is restricted to human clinical settings and not to livestock, as carbapenem antibiotics are not licensed for the use in food-producing animals (11). However, in Germany for instance sporadic studies reported detection of CPE in wildlife, livestock animals and animal products (12-17). As for carbapenemase-producing *Salmonella* strains, a *Salmonella enterica* subsp. *enterica* serovar (*S.*) Corvallis with a multidrug resistance (MDR) encoding bla_{NDM-1}-carrying IncA/C_2 plasmid in a wild bird (12), *S.* Infantis with a MDR-encoding bla_{VIM-1}-carrying IncHI2 plasmid in poultry, swine and pork products (13, 14) and *S.* Goldcoast with the same MDR-encoding bla_{VIM-1}-carrying IncHI2 plasmid in a swine breeding farm were detected (16).

The sporadic detection of CPE in livestock suggests their prevalence might be low and a knowledge gap regarding the understanding of the ecology and behavior of CPE in a scenario of entry into a livestock setting. As carbapenems are not licensed for use in food-producing animals in EU, it is unknown what the transfer and stability of plasmids carrying

carbapenemase-encoding genes might be in the absence of antibiotic pressure. Therefore, this doctoral thesis which was performed in the frame of an international research project EFFORT (Ecology from Farm to Fork Of microbial drug Resistance and Transmission), investigated these scenarios by conducting *in vivo* broiler chicken infection experiments with avian native NDM-1-producing *S.* Corvallis and VIM-1-producing *S.* Infantis donor strains. This way, a potential real-life scenario was mimicked and a broader view on consequences of an entry of carbapenemase-producing *Salmonella* strains into a broiler flock was obtained.

1.1. Taxonomy of *Salmonella* and their epidemiology

The bacteria of the *Salmonella* genus remain among most important zoonotic foodborne bacteria for public and animal health in the EU (18). The genus *Salmonella* belongs to the family of Enterobacteriaceae (enterobacteria) and contains two species, *Salmonella enterica* and *Salmonella bongori* (19). The medically relevant species *enterica* is further divided into six subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae* and *indica*), whereas *bongori* is a sole species and relevant for cold-blooded animals (19). Depending on the lipopolysaccharide (O antigens), flagellar proteins (H antigens) and capsular polysaccharides (Vi antigens) salmonellae are classified into serovars. The so called White–Kauffmann–Le Minor scheme contains over 2600 known serovars, with the majority of serovars belonging to the subspecies *enterica* (Table 1) (19, 20).

Table 1. Classification of the *Salmonella* genus into species and subspecies with current number of serovars. Adapted from Guibourdenche et al. (20).

Species	Subspecies	Number of serovars
enterica	*enterica*	1547
	salamae	513
	arizonae	100
	diarizonae	341
	houtenae	73
	indica	13
bongori	-	23
Total	-	2610

Beside the common classification of *Salmonella* into serovars, depending on their host range, salmonellae can be classified also into three types. Host generalists or ubiquitous serovars, such as *S*. Enteritidis and *S*. Typhimurium, are found in different hosts. Host-adapted serovars, such as *S*. Dublin found in cattle and *S*. Choleraesuis in swine, although associated to particular hosts, are also found in limited number of other hosts. Third type are host-restricted serovars which cause severe systematic infections, such as *S.* Gallinarum and *S*. Pullorum in poultry and *S*. Abortusovis in sheep (21). The diversity of serovars, their reservoirs and transmission routes constrain the prevention of human and animal salmonellosis (22). As poultry flocks are an often reservoir for different *Salmonella* serovars, national control programmes (NCPs) for poultry at the EU level are directed towards reduction of “public health relevant” serovars, aiming to minimize their occurrence in commercial poultry flocks and food products derived thereof. Therefore, annual percentage of positive flocks was set up for public health relevant serovars Enteritidis, Typhimurium, Infantis, Virchow and Hadar in different categories of poultry in the EU (18). However,

despite immense and undisputable effects of the NCPs which led to gradual decrease of *Salmonella* prevalence in domestic fowl (*Gallus gallus)* at the EU level, serovars Enteritidis, Typhimurium, monophasic Typhimurium and other nontyphoidal serovars still play an important role in foodborne-associated *Salmonella* outbreaks (18). Actually, in recent years an increase in the cases of human salmonellosis in EU due to infections with *S*. Enteritidis was observed (18).

The prevention of salmonellosis and other zoonotic diseases in general is nowadays hampered by the globalization and international trade (23, 24). An illustrative example is the worldwide spread of a MDR *S*. Typhimurium DT104 strain which was initially detected in UK (25). This strain has spread worldwide, due to the international trade with breeding animals, travelling and sale of food products (26). The role of globalization on the spread of different serovars is reflected in many international outbreaks of salmonellosis which were associated to different products, such as eggs (27), sesame seeds (28) and ready-to-eat food (29). Beside threat of exposing risk categories, such as older persons, infants and immunocompromised patients (30), the prevention of salmonellosis is important since many *Salmonella* serovars became resistant to different antimicrobials in recent years (31). The AMR in *Salmonella* is not solely linked to particular antimicrobial compounds, but MDR serovars are frequently detected (32-34).

1.2. Antimicrobials

Antimicrobials are natural or synthetic substances capable of killing or inhibiting the growth of microorganisms (35). On the other hand, the term “antibiotic” was proposed by Selman Abraham Waksman, describing antibiotics as naturally occurring substances with antibacterial properties (36). The historical discovery of sulfonamides and penicillin guided a new era in the treatment of infectious diseases. Infections that were a common cause of deaths in pre-antibiotic era, became treatable and newly available therapeutic options revolutionized medical treatment (26). Although antibiotics were seen as one of the wonders of the 20th century (3), resistance emerged a long time before their clinical use (4). Namely, as antibiotics are naturally occurring substances, the resistance genes are also part of a competitive environment in which bacteria survive by interaction with these environmental bioactive substances (3, 4). This is illustrated in a study which revealed presence of genes which encode resistance to beta-lactams, tetracyclines and glycopeptides, even in ancient environmental samples, such as 30.000-year-old Beringian permafrost (37). Nevertheless, leading driver of AMR as an emerging worldwide health concern nowadays is the increased AMU over the last few decades (4, 38).

Antibiotics target physiological and biochemical mechanisms of bacteria, leading to cell death (bactericidal) or growth inhibition (bacteriostatic effect) (1). Classification of the most common antimicrobials, based on their target is shown in Table 2 (1).

Table 2. Bacterial targets for the most common antimicrobial drugs.

Target	Antimicrobial class	Representative antibiotics
Cell wall	Beta-lactams	Penicillins, Cephalosporins, Carbapenems and Monobactams
	Glycopeptides	Vancomycin and Teicoplanin
Tetrahydrofolate synthesis	Sulphonamides	Sulphamethoxazole
	Diaminopyrimidines	Trimethoprim
DNA synthesis	Quinolones	Ciprofloxacin and Levofloxacin
	Nitroimidazoles	Metronidazole
RNA synthesis	Rifamycins	Rifampicin
Protein synthesis	Aminoglycosides	Gentamicin and Amikacin
	Macrolides	Erythromycin, Clarithromycin and Clindamycin
	Tetracyclines	Doxycycline and Tigecycline
	Oxazolidinones	Linezolid
Permeability	Lipopeptides	Daptomycin

Due to an assumption of improving growth performance in livestock animals, antibiotics were historically used in subtherapeutic doses as growth promotors (39). One of the first reports which linked AMR with AMU was the Swann Report from 1969 (40). This report, which was initiated by an observation of increased resistance in *Salmonella* in 1960s, concluded that the subtherapeutic use of antibiotics in livestock led to the AMR in enteric bacteria and poses a hazard not only to animal but also to human health. Another reason for the future ban of antimicrobials as growth promotors was the observation of a causal relation between non-therapeutic use of a glycopeptide antibiotic avoparcin in pigs and poultry and emergence of vancomycin resistant enterococci (VRE) in animals and healthy ambulatory people. Namely, in several European countries, use of avoparcin in animal feed led to increase of VRE in farm animals and humans (41, 42). As a part of the European Commissions (EC) strategy to tackle AMR, a final EU-wide ban came into force on January 1 2006 (Regulation No 1831/2003), stating that it is not allowed to add any antibiotics as growth promotors into animal feed.

Following conclusion that the AMR is an important concern for public and animal health, the WHO urges that antimicrobials are used reasonably in human and veterinary medicine. Therefore, the WHO has developed criteria to rank antimicrobials according to their relative importance for human medicine into a CIA list (2). This list was initially published in 2005 and for the sixth time revised in 2018. According to this document, antimicrobials are categorized depending on two criteria: i) the antimicrobial class is the sole, or one of limited available therapies, to treat serious bacterial infections in people and ii) the antimicrobial class is used to treat infections in people caused by either bacteria that may be transmitted to humans from non-human sources, or bacteria that may acquire resistance genes from non-human sources. The medically important antimicrobials are categorized according to these two criteria into three groups: critically important (meet both criteria), highly important (meet one of the criteria) and important antimicrobials (do not meet any of the criteria) (2).

Nevertheless, despite different efforts aiming to slow down the development and spread of AMR in developed countries by reducing AMU, the AMU in developing countries remains high and not fully regulated (43). In the era of globalization, international trade and intercontinental travel, broad dissemination of important resistance genes is inevitable. In a study conducted by Luebbert et al. (6) it was observed that 30.4% of stool samples from examined travelers returning to Germany were positive for ESBL-producing *Escherichia* (*E.*) *coli*, with the ones coming back from India having highest colonization levels. Another striking illustration is the worldwide spread of the NDM-1-carbapenemase-encoding $bla_{\text{NDM-1}}$ gene from Indian subcontinent to Europe and North America (4).

1.3. Beta-lactam antimicrobials

Historically and medically the most important class of antimicrobials are beta-lactams as they are most commonly used antibiotics for the treatment of bacterial infections in humans and animals (44). The beta-lactams contain diverse groups of antibiotics (penicillins, cephalosporins, carbapenems and monobactams) which all carry in their structure a beta-lactam ring (9). The selective toxicity of beta-lactams is based on targeting the cell wall synthesis, since all bacteria, except mycoplasmas, have a cell wall. The mammalian cells are free of murein containing bacterial cell wall compounds, making beta-lactams ineffective against these cells (1). By covalent binding to the penicillin-binding proteins (PBPs), beta-lactams inhibit the formation of cell wall. As PBPs are important in the terminal phase of peptidoglycan cross-linking, their inactivation by beta-lactams leads to the deviation of the structural integrity of the cell wall. This alters osmotic gradient and cell wall weakens, leading to cell lysis and subsequent cell death (5, 44). However, the commonality of beta-lactams–a beta-lactam ring (4-membered 2-azetidinone ring) in their structure is also their weak point, since many bacteria produce enzymes which target and inactivate this beta-lactam ring (1). These ancient enzymes, found in different bacteria, are known as beta-lactamases (45). Therefore, important addition to beta-lactams are beta-lactamase inhibitors, which do not have an antimicrobial effect on their own, however due to their activity against beta-lactamases, they are administered together with beta-lactams and are referred as beta-lactam agents (1).

Majority of beta-lactam-containing agents are nowadays synthetic or semi-synthetic molecules. However, their initial origin is owed to naturally-occurring sources (45). Penicillin (penicillin G or benzylpenicillin) emerged after Alexander Fleming's observation of an untypical growth inhibition of a staphylococci culture on a Petri dish contaminated with *Penicillium notatum* (44, 46). Later one, followed the isolation of Cephalosporin C (*Acremonium* fungi) (47), carbapenems (from soil samples) (48) and monobactams (from soil and water samples) (49).

1.3.1. Penicillins

The initial formulation of penicillin was a mixture of penicillins F, G, K and X (1). Nowadays, when speaking about penicillin, the penicillin G (benzylpenicillin) is understood. The benzylpenicillin was the first discovered and clinically effective antibiotic for the treatment of infections with gram-positive bacteria. The historical discovery of benzylpenicillin and its large-scale production after Florey´s visit to the United States in 1941 where he obtained support by the American authorities and drug companies, has revolutionized

medicine by making this antibiotic broadly available (1, 50). Despite many advantages, penicillins have several drawbacks, such as restricted antibacterial spectrum, hypersensitivity, inactivation by gastric acid, fast elimination and hydrolysis by beta-lactamases. Yet, these drawbacks initiated search for new variants of penicillins, such as penicillin V (for oral usage) and ampicillin with a broad-spectrum of activity against gram-negative bacteria (1). Nowadays, major toxicity of penicillins is the allergic reaction they might provoke in small number of patients (44). Penicillins have a synergistic effect with aminoglycosides, enabling entrance of aminoglycosides into bacterial cell and inhibition of the protein synthesis (51). The resistance to penicillins is mainly due to activity of penicillinase. Therefore, their use in monotherapy is limited, however this can be improved by addition of beta-lactamase inhibitors (44). The structure of penicillins contains a beta-lactam ring fused with a five-membered thiazolidine ring (52) (Figure 1).

1.3.2. Cephalosporins

After 1950s and discovery of naturally occurring and penicillinase stable cephalosporins, numerous variants of cephalosporins have emerged (44). The cephalosporins are a larger group than penicillins. Similar to penicillins, they contain a beta-lactam ring, yet here an additional six-membered dihydrothiazine ring is fused (52) (Figure 1). Cephalosporins lack cross-sensitivity and are stable against staphylococcal beta-lactamases. Their mode of actions is same as for penicillins–irreversible binding to PBPs. Beside classification into oral and parenteral cephalosporins, they can be further grouped into five generations. The first-generation contains i.e. cefalotin and cefalexin, second cefuroxime and cefoxitin, third cefotaxime and ceftazidime, fourth cefepim and cefpirome and by now the fifth-generation compounds, so called anti-MRSA cephalosporins (ceftaroline and ceftobiprole) is existing (1, 53). Resistance to cephaloporins can be intrinsic or acquired by spontaneous mutation or horizontal gene transfer (5).

1.3.3. Carbapenems

Among currently available beta-lactams, due to their stability against most beta-lactamases, carbapenems have the broadest spectrum of antibacterial activity. Their structure is similar to penicillins, yet they have carbon instead of sulfur at C-1 position and double bond between C-2 and C-3 in their five-membered ring (52) (Figure 1). The most common carbapenems are imipenem, ertapenem, meropenem and doripenem (9). Among these, imipenem is the most unstable one (44). The historical discovery of carbapenems was initiated by increased beta-lactam resistance and search for new therapeutic alternatives.

First parent or model carbapenem antibiotic was thienamycin (54). However, clinical use of thienamycin was limited due to its instability in liquid solutions and sensitivity to mild base hydrolysis (44). This initiated search for stable variants and finally imipenem–first carbapenem for complex infections was discovered, followed by ertapenem, meropenem and doripenem (54). Major advantage of carbapenems is their ability to bind to several different PBPs (55). Carbapenems are CIA for the treatment of severe life-threatening bacterial infections in humans (2). Due to their importance for human therapy, they are not licensed for the use in food-producing animals in the EU (11). However, a worrying observation in recent years is the emergence of the plasmid-associated enzymes carbapenemases in bacteria from human and veterinary sector.

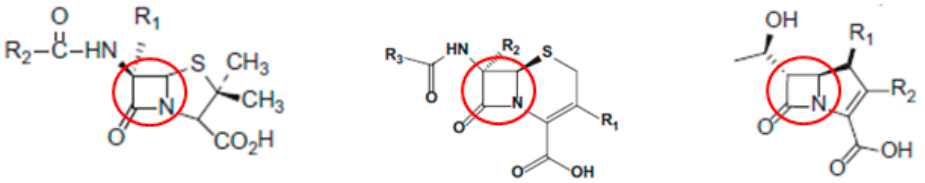

Figure 1. Main structure of penicillins, cephalosporins and carbapenems. The beta-lactam rings are marked in red color. Adapted and approved for reproduction by Bush et al. (44).

1.3.4. Monobactams

The monobactams contain only beta-lactam ring in their structure, without additional fused ring as in penicillins, cephalosporins and carbapenems (52). Due to emergence of carbapenem resistance, only clinically available monobactam–aztreonam is being reconsidered for use either as sole therapeutic option or in combination with other beta-lactamase inhibitors (56).

1.3.5. Other beta-lactam agents

The search for beta-lactamase inhibitors was initiated by the emergence of transferable and plasmid-associated TEM-1 penicillinase (44). This led to search for natural sources of beta-lactamase inhibitors and clavulanic acid from *Streptomyces clavuligerus* was isolated (1). Nowadays, clavulanic acid is used in synergistic effect with penicillins and cephalosporins to inhibit the activity of beta-lactamases (44). Other beta-lactam agents are sulbactam which can be combined with ampicillin and tazobactam in combination with

piperacillin (1). The beta-lactamase inhibitors are not intended for the use as monotherapy as few of these compound have a very weak antibacterial effect (44).

1.4. Antimicrobial resistance

The AMR remains not only an important health issue for human and veterinary medicine but also an important economic concern worldwide (57, 58). According to European Centre for Disease Control (ECDC), annually 33000 of deaths are a direct consequence of an infection with resistant bacteria (59). The AMR can be either natural or acquired (4). The natural or intrinsic property of a bacterium does not permit an antibiotic to have its effect, whereas in acquired resistance, bacteria becomes resistant to a previously susceptible antibiotic (60). The AMR genes can be located on chromosome or MGEs, leading to either vertical or horizontal spread of particular AMR genes, respectively (61). In case of MGEs, the AMR genes can change their position within or between DNA sequences (replicons) of the same cell (i.e., through insertion sequences, transposons harboring AMR gene cassettes/integrons), or they can be transferred to other bacteria by conjugative plasmids or conjugative transposons (62, 63). Successful strategies to tackle the spread of AMR are hampered not only by the existence of MGEs, but also by the diversity of dissemination routes, such as direct or indirect contact, food, water, animal waste and even wild birds (64, 65). Therefore, AMR is a multi-faceted problem which requires preventive approaches from different scientific disciplines (66).

In gram-negative bacteria, the production of hydrolytic enzymes, known as beta-lactamases, is the main mechanism for the deactivation of beta-lactams (45). Depending on which class of beta-lactams they target, beta-lactamases can be classified into penicillinases, cephalosporinases, ESBLs and carbapenemases (9).

1.5. Beta-lactam resistance

The most important drivers of beta-lactam resistance are enzymes which emerged from environmental sources millions of years ago, known as beta-lactamases (45). Nowadays, over 1000 naturally occurring beta-lactamases are known and they are the most studied enzymes (45, 67). In the environment, beta-lactamases enable bacteria to survive in the presence of naturally occurring beta-lactams (45). Resistance to beta-lactams can be induced by different resistance mechanisms, such as alteration of PBPs, having a cell wall that does not allow entrance of antimicrobials, active efflux of antimicrobials and production of beta-lactamases (1). Resistance genes which encode production of beta-lactamases are denoted with *bla*, followed by the name of specific enzyme [e.g. bla_{KPC} (*Klebsiella* (*K.*)

pneumoniae carbapenemase), *bla*$_{VIM}$ (Verona Integron-borne Metallo-beta-lactamase), *bla*$_{NDM}$ (New Delhi Metallo-beta-lactamase)]. Main classification of beta-lactamases is based on biochemical mechanisms of hydrolysis and the characteristic active site into serine and metallo-beta-lactamases (45) (Figure 2). Furthermore, beta-lactamases are classified into molecular class A, B, C and D (68). Class A,C and D have in common that they utilize serine for the hydrolysis of beta-lactams and are referred to as serine-beta-lactamases, whereas for molecular class B one or two zinc (Zn^{2+}) ions are needed, therefore referred to as metallo-beta-lactamases (MBL) (45, 67) (Figure 2).

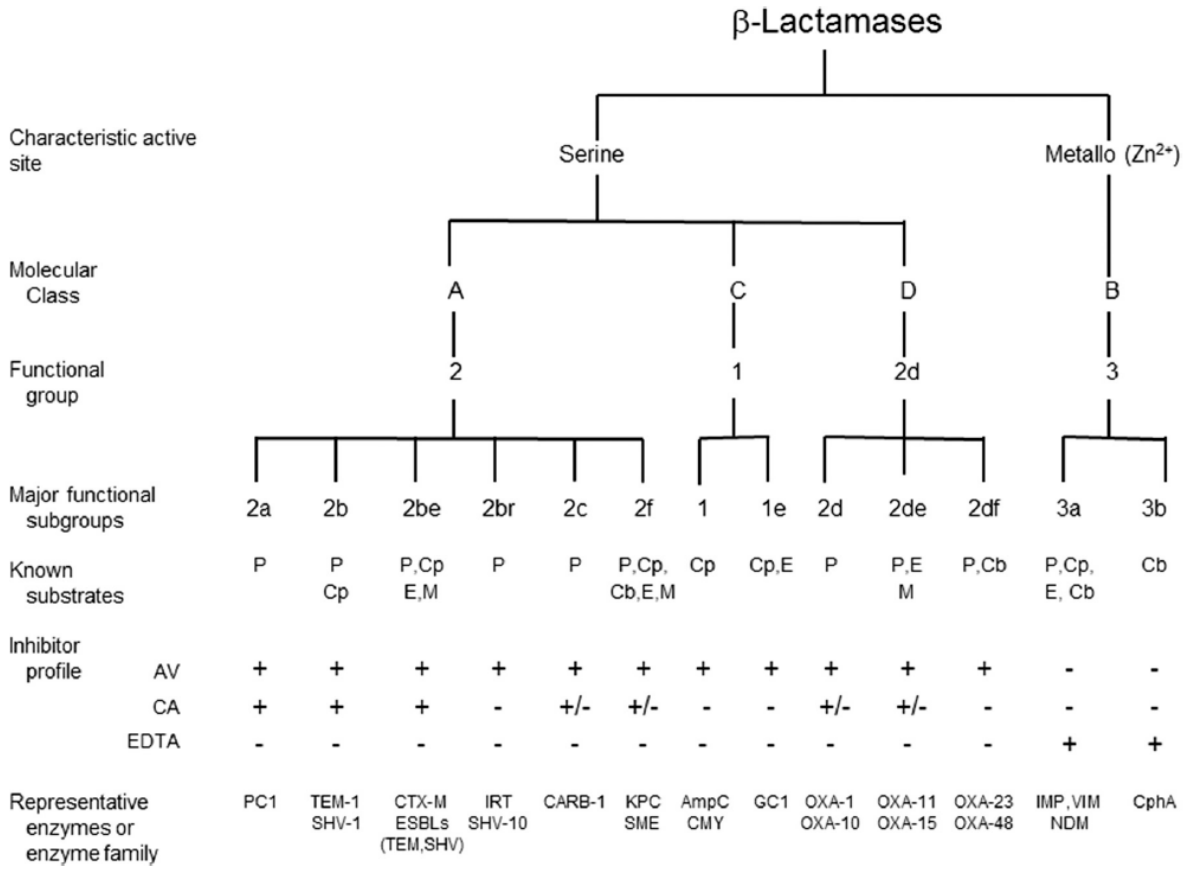

Figure 2. Classification of beta-lactamases. Abbreviations for substrates: AV (avibactam), CA (clavulanic acid), Cb (carbapenem), Cp (cephalosporin), E (extended-spectrum cephalosporin), M (monobactam) and P (penicillin). Adapted and approved for reproduction by Bush et al. (45).

1.5.1. Class A beta-lactamases

The first plasmid-mediated beta-lactam resistance gene of the molecular class A beta-lactamases was *bla*$_{TEM-1.}$ This gene was detected in an *E. coli* strain from the blood culture sample of a patient named Temoniera (therefore TEM abbreviation) in Greece (45, 69). Another known gene of the molecular class A is *bla*$_{SHV-1}$ gene (sulphydryl variable) which, similarly to *bla*$_{TEM-1}$ encodes resistance to penicillins and early-stage cephalosporins (45). As to combat TEM and common beta-lactamases, pharmaceutical industry developed new, beta-lactamase stable compounds (oxyimino third-generation cephalosporins) in 1970s and 1980s (5). However, very quickly, resistance towards these new beta-lactam compounds emerged and new variant, the *bla*$_{SHV-2}$ gene was detected in a *K. ozaenae* strain in Germany (70). Nowadays, several hundred of *bla*$_{TEM}$ and *bla*$_{SHV}$-variants are known and the majority of them have an extended-spectrum of resistance to beta-lactams, which is abbreviated as ESBL (71). The ESBLs are clavulanate-inhibited transferable enzymes produced by certain bacteria capable to hydrolyze penicillins and extended-spectrum cephalosporins (72).

A large group of ESBLs which belong to class A beta-lactamases are CTX-M ESBLs. They were initially detected in Germany (73). Nowadays, CTX-M ESBLs have replaced TEM and SHV enzymes as most prevalent ESBLs in many European countries and *E. coli* and *K. pneumoniae* as dominant hosts with the CTX-M-15 beta-lactamases being prevalent in western Europe (74). The study of Bevan et al. (75) which investigated the epidemiology of CTX-M beta-lactamases revealed that the CTX-M-15 and CTX-M-14 are nowadays most prevalent variants in many countries. Although it is difficult to precisely identify origin of a particular resistance gene, most common is the assumption that resistance genes escaped from environmental bacteria and settled in another clinically relevant bacteria (76). For CTX-M ESBLs, a common hypothesis is that they evolved from a clinically less relevant genus *Kluyvera* (74, 75). Main contributor to the wide spread of CTX-M ESBLs is their association to conjugative plasmids. A study from Germany revealed that the incompatibility (Inc) group IncF has an important role in the *bla*$_{CTX-M-15}$ dissemination among *E. coli* strains isolated from food products (77). Nevertheless, the *bla*$_{CTX-M-15}$ variant is also associated to other plasmids, such as IncF, IncN, IncX4 and IncI1 (7). Worrying is the transposon-mediated integration of carbapenemase-encoding genes, such as *bla*$_{OXA-46}$ (78) and *bla*$_{NDM-1}$ (7) into *bla*$_{CTX-M-}$carrying plasmids which additionally leads to carbapenem resistance in such strains.

Carbapenemases are present in molecular class A (SME, NMC, IMI, GES, KPC), B (IMP, VIM, NDM, KHM) and D of beta-lactamases (OXA-types of enzymes) (9). The most important members of molecular class A carbapenemases are KPC (*K. pneumoniae* carbapenemases). First KPC was detected on a non-conjugative plasmid of a *K. pneumonieae* strain and is therefore referred as *K. pneumoniae* carbapenemase-1 (KPC-

1) (79). Nowadays, the KPC enzymes are the most prevalent members of the molecular class A, found in Asia, North America, Europe and Africa (80). Although KPC enzymes have spread primarily by clonal dissemination of particular *K. pneumoniae* strain (67), these carbapenemase-encoding genes are also associated to different plasmids (81, 82). A frequently studied KPC-encoding gene is the *bla*$_{KPC-2}$ variant (67). An investigation from Spain revealed that the *bla*$_{KPC-2}$ gene was frequently associated with IncP plasmids, was however found in IncN, IncX3, IncU and non-typeable plasmids from enterobacteria isolated in wastewaters (83). The emergence of class A and B carbapenemases is worrying due to their enzymatic activities against carbapenems and broad-spectrum cephalosporins and the observation that their encoding genes have spread globally (84).

1.5.2. Class B beta-lactamases

The class B beta-lactamases, known as MBL are characterized by hydrolysis which is dependent on zinc ions (Zn^{2+}) which are located in the active center of the enzyme (45). They have a broad-spectrum of activity against majority of beta-lactams, except monobactams (52). Carbapenem resistance is nowadays detected in gram-negative non-fermenters, such as *Acinetobacter* (*A.*) *baumannii* and *Pseudomonas* (*P.*) *aeruginosa* and in fermenters, such as different enterobacteria (84). Resistance to carbapenems in enterobacteria is associated to either acquisition of carbapenemase-encoding genes or decrease in the uptake of antibiotic by deficient expression of porins in combination with beta-lactamase overexpression (9). The wide distribution of particular MBL is due to clonal spread of carbapenemase-producing bacteria (CPB) and dominantly mediated by broad host conjugative plasmids (9).

Most important MBL representatives are IMP (imipenemase), VIM (Verona Integron-borne MBL) and NDM (New Delhi MBL) enzymes (9) (Figure 2). First detected IMP was chromosomally-encoded and detected in clinical strain of *Serratia* (*S.*) *marcescens* in Japan (85). Afterwards followed the detection of plasmid-associated IMP also in *S. marcescens* in Japan (86). Nowadays, different IMP variants are detected worldwide (87). A study from Germany revealed first detection of a *bla*$_{IMP-8}$ variant in a *Citrobacter* (*C.*) *freundii* strain of human origin, which was the first report of an IMP-8 in enterobacteria (88). Another study has revealed detection of another variant, the *bla*$_{IMP-35}$ gene in a human *P. aeruginosa* strain from a patient in Dutch-German border region (89).

The VIM MBL were initially detected in a clinical carbapenem-resistant *P. aeruginosa* isolate with an integron-associated chromosomal *bla*$_{VIM-1}$ gene from a patient in Verona (90). These VIM-1-carbapenemase-encoding genes are generally rare in enterobacteria (80), yet sporadic reports revealed their occurrence also in different enterobacteria. In Germany, the

bla$_{VIM-1}$ was linked to *E. coli* strains found in swine and VIM-1-producing *S.* Infantis strains which were associated to swine and poultry (13) and minced pork meat (14). Recently, a *bla*$_{VIM-1}$-carrying IncHI2 plasmid was detected in a *S*. Goldcoast strain from a pig breeding farm, together with VIM-1-producing *S*. Infantis and *Enterobacter* (*E.*) *cloacae* (16). The VIM-1-producing *S*. Infantis strains from previous studies, *S.* Goldcoast and *E. cloacae* from recent study shared the same *bla*$_{VIM-1}$-carrying IncHI2 plasmid. However, understanding of the plasmids transmission routes remains to be elucidated (16). The *bla*$_{VIM-1}$ gene was previously detected on IncY plasmid from an *E. coli* strain isolated from retail seafood (*Venus clam*) (15). A study from France revealed detection of VIM-1-producing *E. coli* in gulls in southern France (91). The VIM and IMP families have in common that their resistance genes are associated to different integrons (80).

The initial detection of NDM and subsequent spread of this carbapenemase revealed dissemination potential of a MBL-encoding gene. Namely, first NDM-1 variant was detected 2008 in a Swedish patient of Indian origin who traveled to New Delhi and acquired a urinary tract infection with carbapenem-resistant *K. pneumoniae* (92). A study by Walsh et al. (93), revealed presence of NDM-1 positive bacteria in drinking and seepage water samples in New Delhi, revealing the spread of this resistance gene into the environment. Nowadays, different *bla*$_{NDM}$ variants are being detected worldwide (94-97). Due to their broad dissemination and occurrence in different gram-negative bacteria, the *bla*$_{NDM}$ genes are becoming the most commonly detected and distributed carbapenemase-encoding genes worldwide (98). The successful spread of different *bla*$_{NDM}$ variants is due to their association to different plasmids and not solely to a particular bacterial clone (9, 94). The *bla*$_{NDM}$ genes are found in *Salmonella* serovars of human (99-101), animal origin (102) and in other enterobacteria (103-105). Recently, Zhang et al. (94) reported an increase of CPE with a *bla*$_{NDM-5}$-carrying IncX plasmid in swine, poultry and beef meat products in China.

As for Germany, in 2012, an avian-native NDM-1-producing *S*. Corvallis strain from a black kite (*Milvus migrans*) was detected (12). This *bla*$_{NDM-1}$ gene was detected on a broad host range MDR-encoding IncA/C_2 plasmid (33). Particularly this finding raised concerns on further dissemination of this MDR-encoding plasmid into livestock production and potential human exposure downstream the production chain.

1.5.3. Class C beta-lactamases

First bacterial enzyme reported to destroy penicillin was AmpC beta-lactamase of an *E. coli* strain. Nowadays, these enzymes are found in several enterobacteria and can be encoded on chromosomes or plasmids (106). For instance, the variants CMY-1, -8, -9, -10, -11, and -19) are associated to chromosomally-encoded AmpC enzymes from *Aeromonas*, while the most common variant CMY-2 is a plasmid-mediated variant related to the AmpC beta-lactamases found in *C. freundii* (106). Although is the expression of AmpC-type enzymes low, this can be inducible by the exposure to particular stimuli, such as beta-lactams (107). Plasmid-associated AmpC beta-lactamase are often found on MDR-encoding plasmids (108-110) and this worrying as it might lead to their broader dissemination. Another variant of bla_{CMY} genes, the bla_{CMY-16} was detected on the aforementioned MDR-encoding bla_{NDM-1}-carrying IncA/C_2 plasmid of a *S*. Corvallis strain (12).

1.5.4. Class D beta-lactamases

The molecular class D beta-lactamases are also known as oxacillinases (OXA) beta-lactamases. Among enterobacteria, the OXA-46 variant is the most common one (58). Although different OXA enzymes are known, few of them have carbapenemase activity (9). First OXA-variant was detected 2001 in Turkey. This plasmid-encoded bla_{OXA-48} gene was detected in a *K. pneumoniae* strain, expressing resistance to penicillins and carbapenems (111). Although OXA-48 is highly prevalent in Mediterranean region and North African countries, this enzyme has by now spread globally (9, 45). The OXA-48 expresses resistance to penicillins and carbapenems, is however as majority of OXA enzymes susceptible to cephalosporins, what hampers their detection with classical CPE isolation methods which are based on media containing cefotaxim. Thus, the OXA-48 producers are among carbapenemase producers which are most difficult to detect (80). This is additionally due to weak expression of the carbapenemase gene resulting in low activity in common phenotypical tests, which might lead to the underestimation of their prevalence (9). Another variant of OXA beta-lactamases, which hydrolyses expanded-spectrum cephalosporins, and weakly carbapenems, is the OXA-163 (112). A concern regarding OXA carbapenemases is their mutation potential which might lead to the expansion of their enzymatic activity (80).

1.6. Spread of antimicrobial resistance by horizontal gene transfer

The AMR can spread vertically or horizontally (4). In vertical transmission, as a result of genetic point mutation which can lead to AMR, the genetic information is passed after cell division to progeny. Horizontal spread, also referred to as horizontal (lateral) gene transfer (HGT) is a dominant mechanism which enables bacteria to acquire new properties and to adapt to a changing environment (26). The HGT plays an important role in evolution of bacteria and enables bacteria to acquire genetic elements from outside of their own clonal lineage (113, 114). The acquisition of these genetic elements can occur by transformation, transduction or bacterial conjugation (114).

1.6.1. Transformation

The discovery of bacterial transformation laid down the foundations for molecular biology and molecular genetics. First observation of this mechanism was made possible by experiments conducted by British bacteriologist Frederick Griffith. Namely, in the year 1928 Frederick Griffith conducted experiments, known as Griffiths experiment, in which, by using *Streptococcus* (*S.*) *pneumoniae* as a model strain, he demonstrated transformation of the R-type (non-virulent) into S-type (virulent) colonies of *S. pneumoniae* which led to increased mortality in a mouse infection model (115). Concerning the role of transformation in the spread of AMR between bacteria in clinical environments, there is still a lack of reliable data due to difficulty to detect uptake and recombination of exogenous DNA (114). The DNA uptake in competent cells (condition of cells capable of DNA uptake) is mediated by a cell surface DNA-binding protein. In such scenario a double-stranded or single-stranded DNA is being taken into the recipient cell. After uptake, the DNA is bonded to a specific protein which inhibits further degradation of DNA by intracellular nucleases, until this DNA element reaches chromosome and by recombination incorporates into the cell (116, 117).

1.6.2. Transduction

The transduction is a mechanism of the DNA transfer between bacterial cells by bacterial viruses, known as bacteriophages (116). Bacteriophages can be virulent or temperate. Virulent bacteriophages have a lytic cycle which includes attachment of the bacteriophage by tail fibers to surface of the host-cell, DNA injection, hydrolyzation of hosts DNA, replication of the bacteriophages DNA and production of bacteriophages proteins, resulting in the immediate cell death (117). The temperate bacteriophages can undergo lytic and lysogenic cycle. The lysogenic mode of action is characterized by the incorporation of

bacteriophage into the host genome, which can lead to further vertical spread. Such bacteriophage is referred as prophage (117). Under natural or inducible conditions, the lysogenic cycle can convert into lytic, leading to the release of bacteriophages (118). The use of bacteriophages is being reconsidered as an additional option aiming to combat the spread of AMR. However, this use is broadly debated due to their potential role in the spread of AMR (119, 120).

1.6.3. Bacterial conjugation

The exchange of genetic material between bacteria occurs dominantly by a mechanism which is known as bacterial conjugation. This is the main way for transporting large DNA fragments and a driving factor which contributes to the commonality of the gene pool (121). Furthermore, is one of the most important mechanisms by which bacteria acquire AMR (122). The acquisition and spread of AMR is mediated by MGEs known as plasmids, which are transferable not only between bacteria of the same genus, but also between bacteria of larger taxonomic distances (123). Plasmids usually carry non-essential, however useful genes which can contribute to phenotypical and evolutionary properties of the host itself (121). For conjugation to occur, close contact between a donor cell which carries a conjugative plasmid and a recipient cell is essential (114). This is possible by formation of a complex were these two cells physically contact, known as mating pair formation (Mpf) which proceeds through synthesis of a type IV secretion system (T4SS). The T4SS generates conjugation pili which extend from donor to recipient cell enabling the exchange of plasmid DNA (62, 121, 124). In a further step, the conjugative double-stranded plasmid is cleaved by a plasmid-encoded endonuclease at the plasmids origin of transfer (*oriT*). In next step, the relaxosome is formed and contains single-stranded plasmid DNA, relaxase and other proteins needed for the DNA transfer. Finally, a cognate coupling protein mediates docking of the relaxosome into the T4SS, and further uptake in recipient strain. The relaxosome subsequently enables generation of the second strand of plasmid DNA in donor and recipient cell (121). As a result of the plasmid transfer between donor and recipient cells, a population of recipient cells with a newly acquired plasmid, also known as transconjugants emerges.

1.7. Transfer of resistance genes by mobile genetic elements

Mobile genetic elements (MGEs) are genetic elements capable of changing their position within as well between different replicons, i.e. genomes. The MGEs can be classified into intercellular MGEs (plasmids and phages) and MGEs which do not transfer independently but their transfer is dependent on their incorporation into plasmids or bacteriophages–intracellular MGEs (transposons, integrons and insertion sequences) (124, 125). In Figure 3 MGEs and their incorporation into a conjugative plasmid is shown (121). As in the frame of this doctoral thesis spread and stability of plasmids carrying carbapenemase-encoding genes was investigated, a more detailed insight into gene cassettes, integrons, transposons, insertion sequences and plasmids will be given.

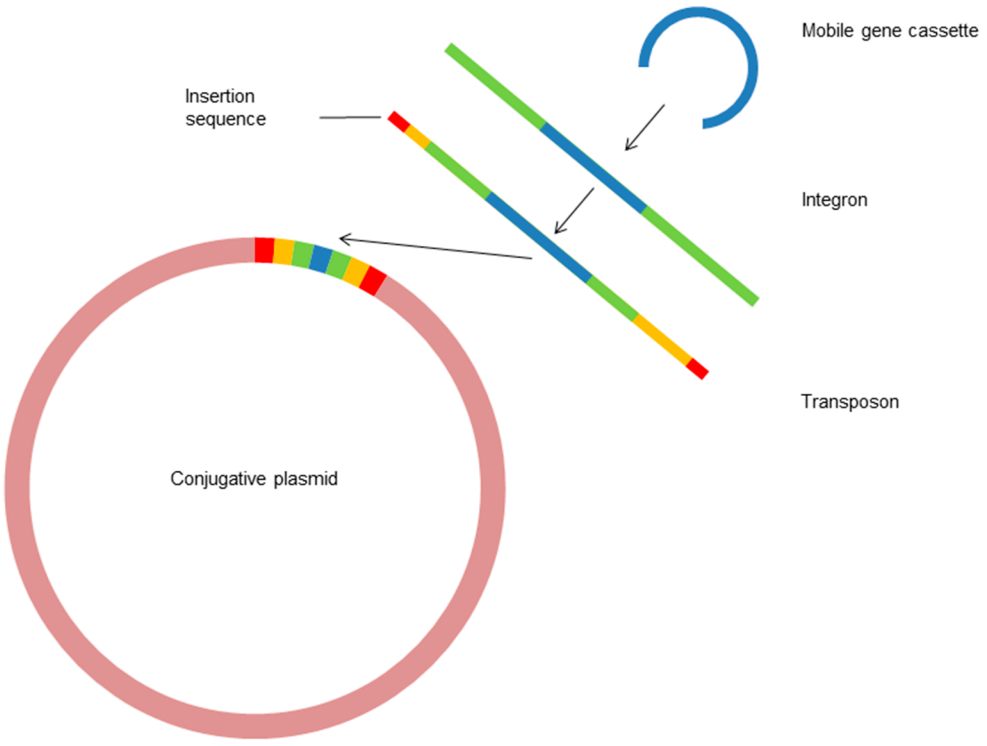

Figure 3. Mobile genetic elements. With blue (mobile gene cassette), green (integron), orange (transposon), red (insertion sequences) and brown color (conjugative plasmid) are marked. Adapted and approved for reproduction by Norman et al. (121).

1.7.1. Gene cassettes and integrons

Gene cassettes are small and simple genetic elements which can exist either in sole circular form or be incorporated into larger genetic structures known as integrons (126). The gene cassettes are not mobile by themselves, can however be transferred due their association with other self-transmissible genetic elements. The incorporation of a gene cassette into an integron is mediated by carriage of a recognition site *attC* which is recognized by an integrase and integrated into an *attI* specific site of the integron itself. Integrons are genetic elements which, by incorporation of gene cassettes, contribute to the genomic complexity of bacteria (126). They can contain either one or more gene cassettes. The gene cassettes are integrated in a specific site (*attI*) which is adjacent to the integrase-encoding *intI* gene of the integron (126). The third and most important part of an integron is the integron-associated promoter (Pc), which allows the expression of the 5´ adjacent incorporated gene cassettes, which itself do not possess an own promotor (126). The mobility of integrons depends on their incorporation into transposases or recombinases for interchromosomal mobility (126). According to the amino-acid sequence of the IntI protein integrons are classified into class 1, 2, 3, 4 and 5 integrons (126). The carbapenemase-encoding genes bla_{NDM-1} and bla_{VIM-1} are often associated to class 1 integrons (33, 127, 128).

1.7.2. Transposons and insertion sequences

A transposable element (TE) is a DNA sequence, capable of changing the position in a genome (129). This can occur within a DNA sequence (different positions within chromosome or plasmid) or between (from one plasmid to another, or from plasmid to chromosome and the other way around). Therefore, they are actually seen as jumping gene systems that can facilitate incorporation of genes, such as resistance genes, into another genetic environment (130). Simplest TEs are insertion sequences (125). They can be found in chromosomes, plasmids and bacteriophages and they carry genes which are needed for their transposition, namely a *tnp* gene which encodes a transposase (125). Transposons are larger than IS and carry genes (*e.g.* resistance genes), which encode for the host important functions (*e.g.* AMR). Hence, they differ from an IS element as they encode at least one function that changes the phenotype of the cell (130). Transposons are flanked by insertion sequences on both sides, that are itself flanked by inverted tandem repeats (ITR), needed for the transposition mechanism. The size of these ITRs can vary from 20 bp to >1 kb. Transposition can be non-replicative (conserved, c*ut and paste*) and replicative transposition. In the first case, the TE is excised from the genome and integrated into new target DNA

resulting only in its dislocation, whereas in replicative transposition another copy of a genetic element is generated (131).

1.7.3. Plasmids

Although described in 1952 by Joshua Lederberg (132) the interest in plasmids increased in last few decades, due to the important role they have in the spread of AMR (7, 121). Plasmids are extrachromosomal autonomously replicating DNA elements (114) which can carry different AMR genes, such as genes conferring resistance to beta-lactams, aminoglycosides, tetracyclines, chloramphenicol, sulfonamides, trimethoprim, macrolides and quinolones (133). Plasmids are found in bacteria from soil, water and clinical environment (124). Although they do not carry genes which encode core cell functions such as growth and multiplication, plasmids can carry genes which might be useful in certain conditions, such as exposure to antibiotics, heavy metals or other biocides (130). The size of plasmids can vary from several kilobases to several hundred kilobases pairs (kbp). Plasmids which encode MDR are usually large (>50 kb), conjugative and control their own copy number (121, 134, 135). This means that plasmids replicate rapidly if their number is too low and replication stops if the plasmid number is too high (121). An important feature for plasmid persistence is that plasmids encode addiction systems which are based on toxin-antitoxin factors. These factors support persistence of a bacterial population which carries a particular plasmid by post-segregationally elimination of progeny that did not inherit the plasmid during cell division (136). In a bacterial cell, average number of plasmids copy per cell, known as copy number, can vary from several to over 100 copies. Large plasmids have usually low copy number due to the metabolic burden plasmid carriage poses to the host (124). The larger the copy number, the higher is the chance that both daughter cells will receive a plasmid (137). Bacteria can carry several plasmids of different Inc groups and their acquisition can be sequential or simultaneous (138).

As for replication of plasmids, the majority of plasmids from gram-negative bacteria have a bidirectional replication which leads to formation of a theta structure. In some plasmids, unidirectional replication also occurs. The majority of plasmids belonging to gram-positive bacteria replicate in a process known as rolling circle replication where a one-stranded plasmid as by-product is generated (117). In the next step, the origin of replication is looped allowing RNA polymerase to access the newly formed single-stranded DNA to form a primer where hosts DNA polymerase binds leading to final double-stranded DNA (117).

Depending on their transferability, plasmids can be conjugative, non-conjugative or they can be mobilized by the presence of another helper plasmid. The acquisition of a plasmid expands the genome of the recipient cell by acquisition of genes which encode new

functions (130). On the other hand, non-conjugative plasmids can spread by co-integrating and transferring with conjugative plasmids and so called helper plasmids (62, 139, 140). The complete structure of a plasmid is composed by core genes and evolutionary acquired accessory genetic elements (62, 121). In a typical AMR-encoding plasmid, the accessory genetic elements are mainly resembled by multiple AMR genes and mobile elements, such as insertion sequences and transposons (62). Main genetic regions of a typical conjugative plasmid are shown in Figure 4.

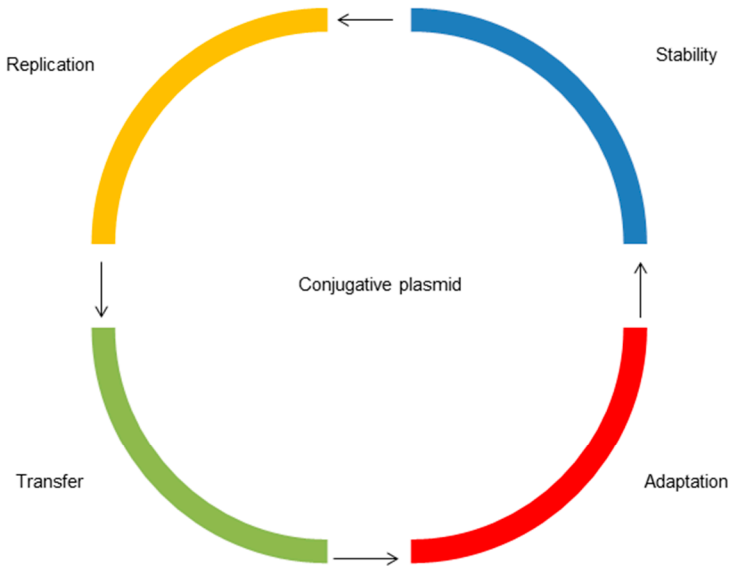

Figure 4. Main genetic regions of a typical conjugative plasmid. With orange (replication), green (transfer), red (adaptation) and blue color (stability region) are marked. Adapted and approved for reproduction by Norman et al. (121).

1.7.3.1. Molecular typing of plasmids

Despite core genetic regions, accurate evolutionary history of a particular plasmid can be challenging, due to the insertion of genetic elements, recombination and alteration of plasmids structure (121). First classification scheme for plasmids was conjugation-based, on stable coexistence of plasmids in a bacteria and was proposed by Datta and Hedges in 1971 (141). Nowadays, a commonly used typing scheme is based on Inc/rep typing and classification of plasmids into Inc groups (142). A single bacterium can carry several plasmids, however their persistence depends on their compatibility. As plasmids can share similar replication and partition systems, they cannot be propagated stably in same host bacteria. This is referred as plasmid incompatibility (7). The initial typing scheme for plasmids

was based on Southern hybridization for 19 replicons and was proposed by Couturier et al. (143). Nowadays, the commonly used typing method is PCR-Based Replicon Typing (PBRT) scheme, developed by Carattoli et al. (144), which serves as a rapid tool to screen large number of strains and investigate their role in the spread of AMR. The PBRT scheme was updated by addition of new Inc groups and optimized by speeding up the procedure (145-147). A drawback of PBRT scheme is the possibility to identify only known replicons and failure to identify newly emerged replicons (133). Currently, 27 Inc types in enterobacteria, 14 in *Pseudomonas* and 18 in *Staphylococcus* are described (148). Furthermore, Bousquet et al. (149) proposed another scheme which relies on detection of different partition systems in MDR-encoding plasmids as an addition to PBRT scheme. This scheme was used for *E. coli*, *K. pneumoniae* and *Salmonella enterica* strains with high specificity for majority of replicons, except the IncX group. Another PCR typing method is mobility (MOB) typing based on conjugative and mobilization relaxase *mob* genes (150). In order to further characterize plasmids belonging to the same Inc group, high resolution methods were developed. Such method is the plasmid multi-locus sequence typing (pMLST) which is based on a web tool available at http://pubmlst.org/plasmid/ and is however only available for certain Inc groups.

As to gain insight into plasmid content and estimate size of plasmids in particular bacterial strains, a commonly used laboratory technique is pulsed-field gel electrophoresis (PFGE) where enzymatic digestion with S1 nuclease (S1-PFGE) is performed (151). This enzyme removes background single-stranded RNA and DNA and the supercoiled plasmid DNA is linearized, making the size estimation after the plasmids DNA migration in a PFGE gel more accurate. Without this linearization, the migration of supercoiled plasmids would not resemble their molecular weight, hindering accurate estimation of the plasmids size (151). The approximate plasmid size is determined after staining and comparing to a molecular size standard *S*. Braenderup H9812 strain. The negatively charged plasmid DNA fragments from such S1-PFGE gel can be transferred onto a positively charged nylon membrane and hybridized with a specific DNA probe (e.g. resistance genes) that, after its visualization gives information if particular resistance gene is located on a plasmid or not (152).

A significant advancement which enabled deeper insight into molecular typing of bacteria as well evolutionary changes of bacterial genomes and plasmids came with the introduction of next-generation sequencing used for whole genome sequencing (WGS) analysis. From 2010, WGS technology started to migrate from research into public health laboratories (153). With decrease of costs and improvement in rapidity and reliability of WGS, it is foreseen that WGS will replace traditional phenotypic methods for surveillance of AMR and other hazards to food safety in near future (154, 155). Depending on the length of

generated sequences (reads), short and long-read WGS technologies are currently available (156).

Most commonly used short-read WGS technology is the Illumina sequencing-by-synthesis approach, where fluorescently labeled reversible-terminator nucleotides, on a clonally amplified DNA template, are immobilized on a flow cell (157). Nowadays, Illumina sequencing is becoming a method of choice for typing of bacterial strains in outbreak investigations (158, 159). Although high accuracy and throughput are advantages of Illumina technology, a common drawback is the assembly of complete genomes due to occurrence of repetitive regions (*e.g* insertion sequences) and multiple plasmids (61). This is due to the generation of short reads (150-300 bp paired reads) while Illumina sequencing, which, despite different bioinformatic assembling approaches, makes assembly of closed genomes, such as plasmids, difficult (153). This issue was overcome with third-generation WGS platforms which target single DNA molecules (153). Such platform is the single-molecule real time (SMRT) sequencing technology from Pacific Biosciences (PacBio) which has the advantage of generating longer reads, whose size can vary from 10 to 60 kb, making assembly of complete genomes easier (160). The PacBio is a commonly used technology for generating reference sequences of bacterial genomes which can be used for different analysis, such as phylogenetic analysis. Another promising long-read and real-time technology is the MinION, developed by Oxford Nanopore Technologies, which is the smallest, first commercial and portable sequencing machine, with still a drawback of generating high error rates (153, 161). A drawback of PacBio technology is a higher error rate per continuous long-reads in comparison to Illumina short-read sequencing. Therefore, for high quality DNA sequences, an innovative approach known as hybrid assembly which combines long and short-read sequencing is usually performed. This enables correction of errors of long reads by additional high-throughput and high-accuracy short read data (160).

Today, with the rise of next-generation sequencing technologies, different *in silico* tools for typing of plasmids (162), resistance (163) and virulence genes (164) as well for serotyping of *Salmonella* (165) are available. They are based on detection of particular genes by comparing raw or assembled sequences to a selection of gene sequences deposited in a reference database (61). These *in silico* tools are gathered in a platform which is freely offered by the Technical University of Denmark (DTU) named Center for Genomic Epidemiology (CGE) (http://www.genomicepidemiology.org/). Also, different freely available software tools for the visualization and comparison of genomes are available. Such one is the BRIG (BLAST Ring Image Generator) which enables circular comparison of plasmids or whole bacterial genomes (166). Linear comparison of genomes can be visualized by Easyfig (Easy genome comparison figures) (167) which is also an user-friendly software solution for the visualization of sequencing data. The BRIG is usually used to compare whole genomes,

whereas Easyfig is used for the comparison of particular regions of different genomes. In this doctoral thesis, BRIG was used as a tool for the visualization of different plasmid sequences.

A major advantage of WGS is the possibility to re-run *in silico* analysis of sequenced genomes without the need to perform additional laboratory work. This enables for instance rapid screening of deposited sequences for newly emerged resistance genes. Such approach leads to a more rapid and precise source identification and can serve as a guideline where specific intervention measures, aiming to reduce or at least slow down the spread of AMR might be directed.

1.8. AMR plasmids

Plasmids which carry AMR-encoding genes are referred to as AMR plasmids (57, 133). Although particular AMR genes and plasmids are often associated to certain hosts, such as *bla*$_{CTX-M-1}$-associated IncI1 and IncN plasmids in food-producing animals and *bla*$_{CTX-M-15}$-associated IncF plasmids in companion animals and humans, this is still debatable for certain geographical regions (57). Concerning is that not one, but several AMR genes can be carried by a plasmid, enabling persistence of such MDR-encoding plasmid by exerting antibiotic pressure on a single AMR gene (57).

Another concern is that AMR genes are not only restricted to certain plasmids but can be found in plasmids of different Inc groups. This is illustrated by successful spread of *bla*$_{NDM-1}$ gene, which is nowadays found in IncA/C, IncHI1, IncL/M and IncF plasmids (7, 168). Although it was presumed that the carbapenemase-encoding *bla*$_{VIM-1}$ gene has a more restricted range, this gene is found on IncN, IncA/C, IncR plasmids (7), IncHI2 (169) and IncY plasmids (15).

Although 27 different Inc groups of plasmids in enterobacteria are known, particular Inc groups, such as IncF, IncI, IncA/C, IncH, IncL/M and IncN are more frequently associated to AMR and carry the greatest variety of AMR genes (148). As in this doctoral thesis transfer and stability of MDR-encoding *bla*$_{NDM-1}$-carrying IncA/C$_2$ and MDR-encoding *bla*$_{VIM-1}$-carrying IncHI2 plasmids was investigated, a more detailed insight into these two Inc groups will be given.

1.8.1. IncA/C plasmids

Plasmids of the incompatibility group IncA/C were among first plasmids reported to be implicated in the spread of AMR (170). Research of the IncA/C plasmids was initiated due to transferrable tetracycline and sulfonamide resistance detected in a fish pathogen *Aeromonas liquafaciens* (171). This incompatibility group contains low-copy, conjugative broad host range plasmids. The definition broad host range is generally used for a genetic element capable of transferring between different bacteria (130). Work of Carattoli et al. (172) revealed two variants of IncA/C plasmids (IncA/C$_1$ and IncA/C$_2$) which differ in 26 single-nucleotide polymorphisms (SNPs) of the *repA* gene. The IncA/C$_2$ type contains two types (type 1 and 2). An important feature of the IncA/C$_2$ plasmids is that they carry two resistance islands (ARI-A and ARI-B). The ARI-A is present only in type 1, whereas the ARI-B resistance island is present in type 1 and 2 of the IncA/C$_2$ plasmids (170). The size of IncA/C plasmids varies from 40 to 230 kb (142).

In recent years, the interest in IncA/C plasmids arose due to their worldwide spread and carriage of AMR genes which encode resistance to beta-lactams (carbapenems and cephalosporins) and other antibiotics such as aminoglycosides, tetracyclines, chloramphenicol and trimethoprim (7, 142). As described in Fischer et al. (12), an avian native carbapenemase-producing *S.* Corvallis (12-01738) strain carrying a MDR-encoding bla_{NDM-1}-carrying IncA/C_2 plasmid was isolated from a black kite (*Milvus migrans*) in Germany in 2012 (12, 33). This plasmid had 82% of nucleotide identity to the bla_{NDM-1}-carrying IncA/C plasmid from a human *Providencia* (*P.*) *stuartii* strain of a patient in Afghanistan (173). Recently, Paskova et al. (135), reported detection of a bla_{NDM-1}-carrying ~300 kb multireplicon (R and A/C) plasmid in an *E. coli* strain from human urine, isolated in Czech Republic. Remarkably, this multireplicon plasmid had 99% of nucleotide identity to the pRH-1238 plasmid of *S*. Corvallis (12, 33) confirming its broad host range, its co-existence in human clinical settings and an assumption of bla_{NDM-1} gene spill-over from or into the human clinical sector (174).

1.8.2. IncH plasmids

The IncH plasmids are low-copy broad host range plasmids, whose size can vary from 75 to 400 kb (142, 175). The IncH plasmids are further divided into IncHI and IncHII plasmids. Based on the homology of nucleotide sequence, the IncHI group is further divided into IncHI1-IncHI5 (176). An important prerequisite for the conjugation of IncHI1 plasmids is the temperature. Namely, for IncHI1 plasmids, 27-33°C is the optimal temperature range and temperatures above 37°C inhibit the transfer. The work conducted by Alonso et al. (177) suggests that these plasmids have a role in the HGT in soil and aqueous environments (142, 177). Nowadays, the IncHI1 and IncHI2 plasmids are detected in bacteria from different matrices across Europe (142) with the IncHI2 plasmids being frequently detected in enterobacteria (133). Many of these plasmids carry genes which encode resistance to different antimicrobials, such as beta-lactams, sulphonamides, aminoglycosides, tetracyclines and streptomycin (142). As for carbapenemase-producing *Salmonella* serovars in Germany, MDR-encoding bla_{VIM-1}-carrying IncHI2 plasmid was detected in a *S*. Infantis strain isolated in livestock farms (swine and poultry) (13) and pork meat (14) and *S*. Goldcoast from a pig breeding farm (16). Another feature of IncHI2 plasmids is carriage of genes which encode resistance to heavy metals (such as mercuric ions, copper, silver ions, tellurite, arsenate and arsenite). In a study by Fang et al. (178) it was shown that beside AMR genes, also genes encoding resistance to heavy metals, such as copper (*pco*) and silver (*sil*) were detected in different IncHI2 plasmids.

1.9. Potential sources for an infection with CPE in humans

1.9.1. Hospitals, wastewaters and companion animals as a source of CPE

Due to their unique structure and effect against different beta-lactamases, including ESBLs, carbapenems are considered as one of the most reliable antibiotics for the treatment of severe life-threatening bacterial infections in humans (179). However, their therapeutic use in humans led to the emergence and spread of CPB (174). Especially infections with CPE in humans are frequently associated with high mortality (180). As carbapenemase-encoding genes are mostly associated to MDR bacteria, only few therapeutic options remain in case of an infection with such bacteria (76). A common assumption is that long-term healthcare facilities are among main reservoirs of MDR bacteria, with long-term stay in hospitals, exposure to antibiotics, mechanical ventilation and organ transplantation as important risk factors for acquiring an infection with CPB (80). Hospital-associated outbreaks with bacteria producing important carbapenemases, such as KPC (181-183), VIM (182, 184, 185), NDM (186-188) and OXA (189, 190) have been reported in numerous studies.

The spread of carbapenemase-encoding genes from hospitals into the ecosystem can be driven by release of wastewaters into the environment (76). A study from seven European countries, where influents and effluents of the urban wastewater treatment plants were sampled, revealed that the diversity of the AMR genes occurring in treatment plants mirrors the AMR genes found in respective clinics (191). In a study by Marathe et al. (192), where effluents in Mumbai were screened, a new imipenemase variant, the IMP-81 was detected suggesting also role of the effluents as reservoirs for new AMR genes. In a study by White et al. (193) it was observed that CPB from wastewaters do not completely resemble strains detected in hospital settings, suggesting either other sources, or that some CPB remained undetected in one of these environments. The persistence of MDR bacteria in wastewaters can be promoted by residues of pharmaceutical products. Although wastewaters of pharmaceutical industries are treated in developed countries, in a country such as India, which is among the largest pharmaceutical producers such treatment measures often lack, what increases the risk of MDR bacteria persisting and being freely released into the environment (66).

Potential source for a human infection with CPB are companion animals. In a study by Shaheen et al. (194), NDM-1-producing *E. coli* strains were detected in cats and dogs. In a study by Reynolds et al. (195), a canine *E. coli* strain with a sequence of the $bla_{\text{NDM-5}}$-carrying IncF plasmid integrated into the chromosome was detected. An explanation for the occurrence of CPB in companion animals is the off-label use of antibiotics which are usually not allowed for use in animals. This applies also for carbapenems which under certain conditions, such as to prevent unacceptable suffering, can be used for the treatment of cats

and dogs (174, 196). The occurrence of CPB in animals can therefore jeopardize not only successful therapy of pet animals but also humans. Despite CPE being occasionally detected in companion animals, a direct transmission of CPE between animals and humans is not frequently described (197). In a study from China, by using WGS, it was shown that a common NDM-producing *E. coli* strain was shared among farms, flies, dogs and farmers, giving evidence on transmission of carbapenem-resistant *E. coli* between these populations including environmental contamination (198). The close contact of pets and their owners as well as association of carbapenemase-encoding genes to MGEs might result in cross-species transmission (199). Therefore, it is important to minimize potential spread of CPE from companion animals to humans by an evidenced-based use of antibiotics and surveillance of AMU and AMR in companion animals (199).

1.9.2. CPE in poultry and wild birds

The use of antimicrobials in commercial poultry production is often linked to production pressure and aim to treat and prevent the spread of a bacterial disease in a flock (200). The occurrence of ESBL-producing enterobacteria in poultry is particularly worrying as commensal *E. coli* strains have potential to carry and further disseminate resistance genes along the food chain (201). Different studies have investigated occurrence and spread of ESBL-/pAmpC-producing enterobacteria in broiler chickens (202-204). In a study by Projahn et al. (205) a pseudo-vertical transmission from parent flocks to hatchery was observed. The authors observed that day-old broiler progeny from ESBL/AmpC-positive parent flock was negative for ESBL/AmpC-producing enterobacteria, suggesting low effect of the hatchery and potential role of other factors in the dissemination of ESBL/pAmpC-producing enterobacteria. However, other studies have shown that vertical transmission, from colonized grandparent and parent flocks, plays an important role for the occurrence of ESBL bacteria in broiler progeny (206, 207). A study by Dierikx et al. (208) revealed occurrence of ESBL/AmpC-producing strains in each level of the broiler production pyramid.

The environmental contamination is another factor which contributes to the spread of ESBL-producing bacteria (209). In a study conducted by Hiroi et al. (210) it was observed that the contamination of broiler house played an important role for ESBL-producing strains entering and disseminating in a broiler flock. Since AMR-encoding plasmids can carry genes which encode also resistance to heavy metals, it is hypothesized that the co-selection due to heavy metals exposure can favor persistence of such plasmids. In a study by Pal et al. (211), where genomic data were analyzed, it was concluded that biocide and heavy metal compounds might promote AMR by co-selection effect on the chromosomal biocide and

heavy metal resistance genes. This shows that not only reduced AMU, but also other intervention approaches are needed aiming to slow down the spread of AMR.

Another challenge for public and animal health emerged with first reports of CPE detected in livestock. Most commonly, CPB are associated to human clinical facilities and were not frequent in food-producing animals (174). However, their detection in livestock is particularly worrying due to their potential dissemination downstream the food chain, leaving limited therapeutic options in case of a human infection. Despite carbapenems not being licensed for use in food-producing animals, the persistence of CPB can be promoted by co-selection with other antibiotics. In a study by Ye et al. (212) it was observed that use of ampicillin and amoxycillin increased number of CPB and promoted *in vivo* transmission of a *bla*$_{NDM-1}$-carrying plasmid. So far, the occurrence of CPB in the EU is still quite low and there are very few studies on plasmid-associated carbapenem resistance in livestock animals (57). As for CPE in poultry in Germany, VIM-1-producing *S*. Infantis ST32 and *E. coli* ST131 were detected previously. These strains were detected in the frame of a national research project RESET, where several longitudinal and cross-sectional studies were performed. The VIM-1-producing *S*. Infantis ST32 strain carrying a *bla*$_{VIM-1}$-carrying IncHI2 plasmid was detected in a broiler farm (13). The VIM-1-producing *E. coli* ST131 was found when the bacterial cultures were re-tested for the presence of CPE (17).

Most studies on the CPB being detected in poultry production are actually from Asian countries (213-217). This reflects a common assumption of Asian continent as an epicenter of AMR, where AMR in different bacteria is rapidly spreading, due to poor AMR stewardship. This is a concerning observation, as 70% of the world population lives in the Asian continent, and therefore the AMR in Asian countries this is far from being only an Asian problem (213). A study by Wang et al. (102) revealed isolation of *S*. Indiana with a *bla*$_{NDM-1}$-carrying plasmid from chicken carcass. Other studies have shown association of different *bla*$_{NDM}$ variants to IncX3 plasmids in other enterobacteria. An NDM-5-producing *E. coli* (ST156) was isolated from a poultry farm in China. Here, the *bla*$_{NDM-5}$ gene was also associated to an IncX3 plasmid (214). Liu et al. (215) reported detection of plasmid-mediated *bla*$_{NDM-17}$ in a poultry native *E. coli* ST48 strain, also associated to an IncX3 plasmid. In a study conducted by Xiang et al. (216) 16 different *E. coli* ST-types and two ST-types of *K. pneumoniae* with a *bla*$_{NDM-5}$-carrying IncX3 plasmid were found in a selection of strains originating from commercial chicken farms in China. A study by Luchao et al. (217), where fecal samples were collected from chickens at slaughtering in China, has revealed two *E. coli* strains co-producing NDM and MCR-1.

As for other VIM-producing bacteria from poultry, *Pseudomonas* strains with chromosomally-associated *bla*$_{VIM}$-variants were detected in samples of a chicken farms in China. Here, beside chickens, one VIM-producing *Pseudomonas* strain was isolated from a

swallow and fly (218). In another study which investigated prevalence of CPE in poultry in Algeria, high prevalence of *bla*$_{OXA-58}$ was observed (219). A study by Ceccarelli et al. (220) which investigated CPB in Dutch food-producing animals, environmental freshwater and imported ornamental fish, revealed detection of *Shewanella* strains with chromosomally located *bla*$_{OXA-48}$-like variants. However, OXA-46 production occurs naturally in *Shewanella* and does not pose relevance for public health (76). As for KPC, a study from Egypt revealed occurrence of *bla*$_{KPC}$, *bla*$_{OXA-48}$ and *bla*$_{NDM}$ among *K. pneumoniae* isolates from chickens, drinking water samples and workers (221).

In other studies, wild birds were identified as carriers of different AMR genes (222-224). As for carbapenemase-encoding genes, beside NDM-1-producing *S*. Corvallis strain from a black kite in Germany (12), a study from France revealed occurrence of VIM-1-producing *E. coli* strains in gulls from southern France (91). A study from Australia revealed presence of different variants of the *bla*$_{IMP}$ gene in enterobacteria isolated from silver gulls (225). Even in geographically remote regions, such as Alaska, plasmid-encoded *bla*$_{KPC-2}$ and chromosomally-encoded *bla*$_{OXA-48}$ genes were detected among *E. coli* strains from gulls (226).

1.10. *In vivo* animal models for the investigation of conjugation-mediated HGT of resistance plasmids

The investigations of the conjugation-mediated HGT of resistance genes *in vivo* in different avian models are relatively sporadic. However, in several studies, chicken models were used to investigate the *in vivo* transfer of AMR and virulence plasmids (227-231). In a study conducted by Guillot et al. (227), transfer of a conjugative MDR-encoding plasmid, which encodes resistance to chloramphenicol, tetracyclines, sulphonamide and trimethoprim between two *E. coli* strains in chickens was investigated. The authors showed that the plasmid transfer occurred rapidly and increases with the administration of tetracycline. In a study by Hart et al. (228) transfer of resistance genes which encode resistance to tetracyclines between different *E. coli* strains was investigated in a mouse and chicken model. The authors observed rapid detection of transconjugants, with increase of transfer rates after the administration of tetracycline. In a study conducted by Marosevic et al. (229), the effect of antibiotic pressure on the *in vivo* spread of macrolide-lincosamide-streptogramin B resistance was investigated in a chicken model. The study revealed that the *Enterococcus* (*E.*) *faecalis* native conjugative *erm*(B)-carrying broad host range plasmid transfers not only under antibiotic pressure but also in the absence of antibiotic pressure. Chicken infection models were used in a study which investigated transfer of a *tet*(O) gene between different *Campylobacter jejuni* strains (230).

In a study conducted by Oladeinde et al. (232), eviscerated broiler ceca were used to investigate the transfer of a bla_{CMY-2}-carrying IncK2 plasmid to *S*. Heidelberg recipient strain. The authors observed acquisition of this plasmid in one *S*. Heidelberg strain, suggesting this might be a potential way of MDR acquisition in this *Salmonella* serovar. An *ex vivo* model of the porcine caecum was used to study the effect of subtherapeutic administration of doxycycline on doxycycline-resistant enterobacteria and transfer of a *tet*(A)-carrying IncI1 plasmid (233). The authors concluded that potential cross-contamination of swine feed with doxycycline could led to the selection of doxycycline-resistant *E. coli* and transconjugant strains. The spread of plasmids contributes not only to the spread of AMR among bacteria, but can also lead to the acquisition of additional virulence properties. An *in vivo* study conducted by Lacey et al. (231) revealed that the acquisition of a *netB*-containing plasmid, which encodes pore-forming toxin NetB, led to the conversion of a nonpathogenic into a disease-causing *Clostridium perfringens* strain in chickens.

As for other animal species, murine models were used in several studies to investigate *in vivo* transfer of resistance plasmids (212, 234-236). In a study conducted by Nijsten et al. (234), *in vivo* transfer of two plasmids carrying genes which encode resistance to amoxycillin, oxytetracycline, streptomycin, chloramphenicol, sulphamethoxazole and

trimethoprim (wild porcine *E. coli* strain) and resistance to amoxycillin, oxytetracycline, streptomycin and sulphamethoxazole, (human *E. coli* strain) to nalidixic acid resistant *E. coli* recipients in the presence of rat, pig and human intestinal microflora was investigated. The authors observed high transfer frequency in rats colonized with human microflora, with the conclusion that the nature of donors and recipient strains and intestinal microflora plays a role in the extent of plasmid transfer. The extent of plasmid transfer increased with the exertion of antibiotic pressure by lincomycin (234). In another study by Dahl et al. (235) comparison of the *in vitro* and *in vivo* transferability of a plasmid carrying genes which encode resistance to glycopeptides in a mouse model in the absence of antibiotic selection was investigated. The authors concluded that the *in vitro* transfer of this plasmid was reproducible *in vivo* and observed even higher transfer rates *in vivo.* The authors hinted on the importance of commensal microflora as recipients of new resistance genes. The effect of antibiotics from different functional categories on the spread of a MDR-encoding plasmid was investigated *in vivo* in a rat model (212). The authors showed that the use of ampicillin and amoxycillin promoted persistence of carbapenem-resistant enterobacteria and transfer of the bla_{NDM-1}-carrying plasmid *in vivo*. In a study conducted by Jacobsen et al. (236) it was observed that the plasmid transfer between different bacteria is possible *in vivo*. Namely, the authors revealed transfer of a resistance plasmid from a food strain of *Lactobacillus plantarum* to an *E. faecalis* strain.

The plasmid-mediated transfer of particular AMR genes was investigated also in different insect models. In a study conducted by Akhtar et al. (237) plasmid-mediated spread of *tet*(M) gene in a house fly digestive tract as a model was investigated. The authors revealed detection of first transconjugants 24 hours after donor strain was administered. As house flies carried the transconjugants for 4-5 days, this might lead to further dissemination of resistance due to the biological nature of the house fly itself (237). The authors concluded on the importance of house flies as vectors of different bacteria and a suitable microenvironment for HGT in *E. faecalis* strains. Study by Petridis et al. (238), revealed that transfer of plasmids which carry genes which encode chloramphenicol resistance between different *E. coli* strains in a house fly model is possible. Another insect, the lesser mealworm beetles which is a common pest in poultry was used as an *in vivo* model (239). The authors investigated transfer of a MDR-encoding plasmid from *S*. Newport to an *E. coli* recipient strain and showed transfer of this plasmid at a high frequency in this model (239).

1.11. The EFFORT project

In 2013, an international research project EFFORT (Ecology from Farm to Fork Of microbial drug Resistance and Transmission) was initiated. The EFFORT project was set to study complex ecology of AMR and interactions between bacteria from animals, food chain and environment. The aim was to provide scientific evidence and high-quality data for decision makers, the scientific community and other stakeholders about the consequences of AMR. Such data could be used to support political decisions and to prioritize risk management options (www.effort-against-amr.eu). The project lasted five years, was set by a scientific consortium of 20 partners from 10 European countries and structured into ten work packages (WPs).

The German Federal Institute for Risk Assessment (BfR) was a partner institution, mainly active in WP2 and WP3. The WP2 explored molecular approaches for determining the molecular ecology and epidemiology of AMR genes. In WP3 ecology and transfer of resistance mechanisms was studied (www.effort-against-amr.eu). Main input of BfR was in WP3 where *in vivo* transfer and stability of plasmids carrying carbapenemase-encoding bla_{NDM-1} and bla_{VIM-1} genes was investigated. Due to an earlier detection of avian native NDM-1-producing *S*. Corvallis and VIM-1-producing *S*. Infantis in Germany, aim was to investigate and understand spread and stability of these MDR-encoding plasmids in a scenario of entry into a broiler flock in the absence of antibiotic pressure. This is important as broiler meat production plays an important role in human nutrition in developed and developing countries worldwide.

Therefore, broiler chicken model was selected as a starting point and the work conducted within the doctoral thesis investigated transfer and stability of bla_{NDM-1}-carrying IncA/C_2 and bla_{VIM-1}-carrying IncHI2 plasmid *in vivo*.

1.12. *In vivo* broiler chicken infection experiments

In order to investigate transfer and stability of MDR-encoding *bla*$_{NDM-1}$-carrying IncA/C_2 and *bla*$_{VIM-1}$-carrying IncHI2 plasmids, broiler chicken infection model was selected as an *in vivo* model for these investigations. As donors, avian-native NDM-1-producing *S*. Corvallis and VIM-1-producing *S*. Infantis strains were selected. Beside the potential transfer of plasmids carrying carbapenemase-encoding genes to gut enterobacteria, aim was to investigate also potential transfer of these plasmids to other *Salmonella* recipients. For this purpose, nalidixic-acid resistant *S.* Paratyphi B (*d*Tartrate-positive, *d*T+), *S*. Infantis and *S*. Enteritidis were selected. Three separate broiler chicken infection experiments were conducted at the BfR. First animal experiment lasted 28 days and a detailed design of this experiment is shown in **publication 1** (240). The second and third animal experiment lasted 29 days. The design of second animal experiment is shown in **publication 3** (241). The third animal experiment differed from the second in that avian native VIM-1-producing *S*. Infantis was administered as donor strain and that an additional control group of broiler chickens was included. The animal experiments were approved by the German State Authority for Health and Social Affairs (Lageso) (no. 0308/15).

1.13. Objectives of the doctoral thesis

- To investigate the *in vivo* transfer of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid and *bla*$_{VIM}$-carrying IncHI2 plasmid to gut enterobacteria in the absence of selective pressure
- To investigate the *in vivo* transfer of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid and *bla*$_{VIM}$-carrying IncHI2 plasmid to selected *Salmonella* recipients in the absence of selective pressure
- To investigate structural alterations and stability of *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid and *bla*$_{VIM}$-carrying IncHI2 plasmid after an *in vivo* passage

2. Published articles

2.1. Publication 1 (including supplemental material)

Hadziabdic, Sead; Fischer, Jennie; Malorny, Burkhard; Borowiak, Maria; Guerra, Beatriz; Kaesbohrer, Annemarie; Gonzalez-Zorn, Bruno; Szabo, Istvan (2018). ***In vivo* transfer and microevolution of avian native IncA/C_2 *bla*$_{NDM-1}$-carrying plasmid pRH-1238 during a broiler chicken infection study.** In: Antimicrobial Agents and Chemotherapy 62(4).

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Own contribution to Publication 1:

In this study, I actively participated in the *in vivo* animal experiments. This included daily observation of the animals, sampling and laboratory analysis of the collected samples. I performed an in-depth molecular analysis of the selected bacterial strains which included PCR amplification for the detection of *bla*$_{NDM-1}$ and *bla*$_{CMY-16}$ genes, PFGE restriction (XbaI and S1-PFGE) for the molecular typing and visualization of the plasmid content. Additionally, I performed the *bla*$_{NDM-1}$ hybridisation and prepared strains for Illumina Miseq WGS analysis. I surveyed the literature, analyzed, interpreted the data, wrote the draft version of the manuscript and revised it.

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MECHANISMS OF RESISTANCE

In Vivo Transfer and Microevolution of Avian Native IncA/C_2 bla_{NDM-1}-Carrying Plasmid pRH-1238 during a Broiler Chicken Infection Study

Sead Hadziabdic,[a] Jennie Fischer,[a] Burkhard Malorny,[a] Maria Borowiak,[a] Beatriz Guerra,[a]* Annemarie Kaesbohrer,[a] Bruno Gonzalez-Zorn,[b] Istvan Szabo[a]

[a]German Federal Institute for Risk Assessment (BfR), Department for Biological Safety, Berlin, Germany

[b]Departamento de Sanidad Animal and Centro de Vigilancia Sanitaria Veterinaria, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain

ABSTRACT The emergence and spread of carbapenemase-producing *Enterobacteriaceae* (CPE) in wildlife and livestock animals pose an important safety concern for public health. With our *in vivo* broiler chicken infection study, we investigated the transfer and experimental microevolution of the bla_{NDM-1}-carrying IncA/C_2 plasmid (pRH-1238) introduced by avian native *Salmonella enterica* subsp. *enterica* serovar Corvallis without inducing antibiotic selection pressure. We evaluated the dependency of the time point of inoculation on donor (*S.* Corvallis [12-SA01738]) and plasmid-free *Salmonella* recipient [D-tartrate-fermenting (D-Ta$^+$) *S.* Paratyphi B (13-SA01617), referred to here as *S.* Paratyphi B (D-Ta$^+$)] excretion by quantifying their excretion dynamics. Using plasmid profiling by S1 nuclease-restricted pulsed-field gel electrophoresis, we gained insight into the variability of the native plasmid content among *S.* Corvallis reisolates as well as plasmid acquisition in *S.* Paratyphi B (D-Ta$^+$) and the enterobacterial gut microflora. Whole-genome sequencing enabled us to gain an in-depth insight into the microevolution of plasmid pRH-1238 in *S.* Corvallis and enterobacterial recipient isolates. Our study revealed that the fecal excretion of avian native carbapenemase-producing *S.* Corvallis is significantly higher than that of *S.* Paratyphi (D-Ta$^+$) and is not hampered by *S.* Paratyphi (D-Ta$^+$). Acquisition of pRH-1238 in other *Enterobacteriaceae* and several events of plasmid pRH-1238 transfer to different *Escherichia coli* sequence types and *Klebsiella pneumoniae* demonstrated an interspecies broad host range. Regardless of the microevolutionary structural deletions in pRH-1238, the single carbapenem resistance marker bla_{NDM-1} was maintained on pRH-1238 throughout the trial. Furthermore, we showed the importance of the gut *E. coli* population as a vector of pRH-1238. In a potential scenario of the introduction of NDM-1-producing *S.* Corvallis into a broiler flock, the pRH-1238 plasmid could persist and spread to a broad host range even in the absence of antibiotic pressure.

KEYWORDS antimicrobial resistance, *Enterobacteriaceae*, *Salmonella*, NDM-1 carbapenemase, *in vivo* transfer, microevolution, broiler chickens

Salmonella infections continue to play an important role in veterinary and public health (1). Their importance is nowadays elevated by increased antimicrobial resistance in bacterial populations throughout different stages of food production (2). As carbapenems are members of a potent class of β-lactams and the last option in the treatment of severe human infections, they are not licensed for use in veterinary medicine (3–5). However, reports revealing the appearance of carbapenem-resistant/nonsusceptible bacteria in livestock (poultry, cattle, and swine), food products, wild animals, and the environment have increased in recent years (6–11). The true extent of

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Address correspondence to Istvan Szabo, istvan.szabo@bfr.bund.de.

* Present address: Beatriz Guerra, European Food Safety Authority, Parma, Italy.

carbapenemase-producing bacteria in livestock might be underestimated in Europe, due to the voluntary basis for screening at the European Union level (12). One of the most common mechanisms leading to carbapenem resistance is the production of carbapenem-hydrolyzing β-lactamases, mainly encoded by the genes bla_{VIM}, bla_{IMP}, and bla_{NDM} (which are responsible for the production of class B metallo-β-lactamases), bla_{KPC} (class A β-lactamases), and bla_{OXA-48} (class D β-lactamases) (13). Worrisome is the worldwide spread of these enzymes by mobile genetic elements, like integrated conjugative elements and plasmids (14). Among carbapenem-resistant/nonsusceptible bacteria, NDM-1-producing bacteria are usually not more virulent. However, due to many nosocomial outbreaks, they are regarded as the most harmful ones. This is linked to the broad geographical reservoirs of NDM-1 in many unrelated bacterial species, due to the location of the bla_{NDM-1} genes on broad-host-range plasmids (15). A recent study has revealed the localization of the bla_{NDM-1} gene on type 1 IncA/C_2 plasmid pRH-1238 (referred to as pRH-1738 by Fischer et al. [11]) in an avian native *Salmonella enterica* subsp. *enterica* serovar Corvallis strain (12-SA01738) isolated from a wild bird (*Milvus migrans*) in 2012 in Germany (11). The discovery of this first completely sequenced bla_{NDM-1}-*fosA3*-IncA/C plasmid (GenBank accession number KR091911.1) (16) is of great value due to its host and potential for dissemination into livestock production. This is additionally emphasized by the broad host range of IncA/C plasmids, allowing replication not only in *Enterobacteriaceae* but also in other bacterial species, such as *Pseudomonas* and *Photobacterium damselae* (14). Genome analysis of the pRH-1238 plasmid revealed the coexistence of several resistance genes [bla_{NDM-1}, bla_{CMY-16}, *fosA3*, *sul1*, *sul2*, *strA*, *strB*, *aac(6′)-Ib*, *aadA5*, *aphA6*, *tet*(A), *mphA*, *dfrA17*, and *floR*], facilitating resistance to carbapenems, fosfomycins, aminoglycosides, co-trimoxazole, tetracyclines, and macrolides (16). The above-mentioned studies and recent reports on VIM-1-producing *Escherichia coli* and *S.* Infantis isolates in swine and poultry farms (17, 18) showed that the spread and persistence of carbapenemase-producing bacteria in wild birds and livestock are a reality. With recent reports of VIM-1-producing *S.* Infantis being simultaneously found in swine and minced pork meat in Germany (19), the concerns of human exposure via the food chain are additionally highlighted.

Commercial poultry production is a continuously evolving livestock branch, characterized by fast turnovers and production pressure, which, combined with poor management, could lead to the misusage of antimicrobials (20). This might also favor commercial broiler production acting as a niche for selection of multidrug-resistant bacteria. Furthermore, the intestinal tract of broiler chicken offers a cohabitat for different *Enterobacteriaceae and E. coli*, which is described as a major opportunistic pathogen in chickens with a potential for zoonotic transfer to humans (21). Therefore, it is of relevance to explore if and to what extent different genera and clonal lines might act as potential recipients of the bla_{NDM-1}-carrying plasmid pRH-1238. This is an important concern due to previous confirmation of the presence of multidrug-resistant and NDM-1-producing *S.* Corvallis bacteria in a wild bird. Knowing the broad host range of bla_{NDM-1}-carrying plasmids, our aim was to obtain an insight into a potential scenario for this introduction under experimental conditions but conditions that still mimic the rearing management practices common to commercial broiler production. Therefore, we aimed to investigate the intraspecies transfer (*Salmonella* to *Salmonella*) and interspecies transfer (*Salmonella* to endogenous gut microflora) capacities of this bla_{NDM-1}-carrying plasmid without inducing antibiotic selection pressure in a broiler chicken infection study. The objective of our study was (i) to determine the excretion dynamics of an avian native donor strain (*S.* Corvallis [12-SA01738]) and a poultry-associated recipient strain [D-tartrate-fermenting (D-Ta^+) *S.* Paratyphi B (13-SA01617), referred to here as *S.* Paratyphi B (D-Ta^+)] at different inoculation time points, (ii) to analyze the *in vivo* broad-host-range capacity of the pRH-1238 plasmid, and (iii) to analyze the microevolution of the pRH-1238 plasmid (GenBank accession number KR091911.1) using whole-genome sequencing (WGS).

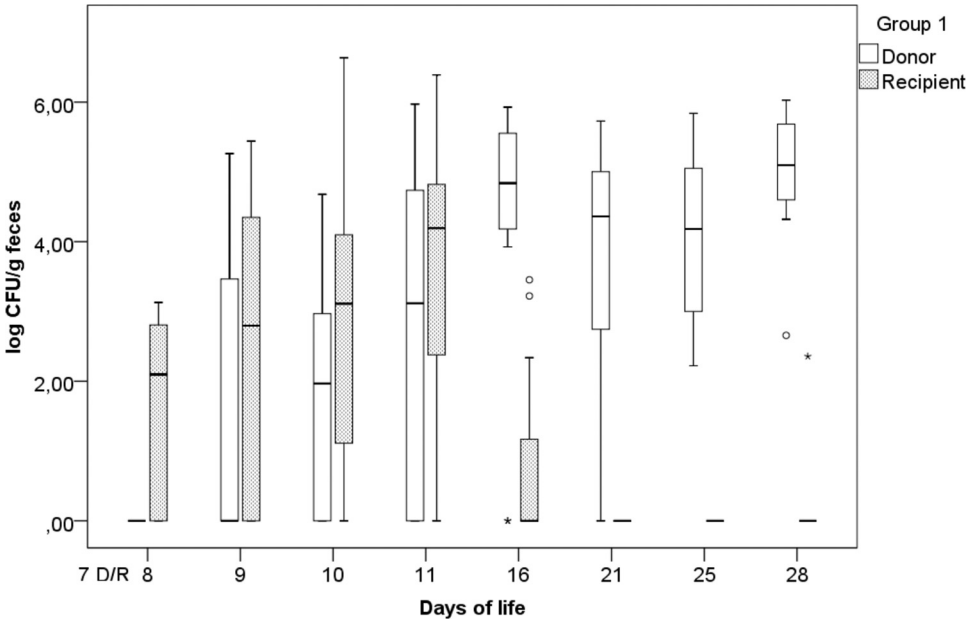

FIG 1 Fecal excretion of the donor (D; *S.* Corvallis) and the recipient [R; *S.* Paratyphi B (D-Ta$^+$)] in group 1 (simultaneous inoculation of donor and recipient strains at day 7), expressed as the log number of CFU per gram of feces, with outliers (°) and extreme outliers (*) included.

RESULTS

Fecal excretion of challenge strains. (i) Group 1. The fecal excretion of the challenge strains (expressed as the log number of CFU per gram of feces) over time in group 1 (simultaneous inoculation of challenge strains) is represented in Fig. 1. The highest excretion rates for *S.* Corvallis (log 5.09 CFU/g) and *S.* Paratyphi B (D-Ta$^+$) (4.19 log CFU/g) were observed on the 28th and 11th days of life, respectively. On the 2nd day postinoculation (p.i.), five animals started to shed *S.* Corvallis, leading up to 11 animals shedding *S.* Corvallis on day 28. As for *S.* Paratyphi B (D-Ta$^+$), on the 1st day p.i., seven animals shed *S.* Paratyphi B (D-Ta$^+$), with various levels of excretion being detected on the 9th day (seven animals) and 16th day (three animals) of life. Statistical analysis revealed a significant difference between challenge strain excretion on the 8th ($P = 0.018$), 16th ($P = 0.008$), 21st ($P = 0.008$), 25th ($P = 0.003$), and 28th ($P = 0.003$) days of life, contrary to the findings for the 9th ($P = 0.161$), 10th ($P = 0.285$), and 11th ($P = 0.169$) days of life.

(ii) Group 2. In group 2 [time-delayed inoculation of *S.* Paratyphi B (D-Ta$^+$)], the highest excretion rates for *S.* Corvallis (log 5.51 CFU/g) and *S.* Paratyphi B (D-Ta$^+$) (3.22 log CFU/g) were on the 12th and 16th days of life, respectively (Fig. 2). On the 1st day p.i., 6 animals shed *S.* Corvallis, and these animals continued shedding *S.* Corvallis through the 28th day of life, when 10 animals were still excreting *S.* Corvallis. After *S.* Paratyphi B (D-Ta$^+$) inoculation on the 10th day of life, four (11th day of life) to nine animals (16th day of life) were excreting *S.* Paratyphi B (D-Ta$^+$). In groups 1 and 2, after the 16th day of life, a decrease in *S.* Paratyphi B (D-Ta$^+$) excretion was observed (Fig. 1 and 2).

(iii) Group 3. In group 3 (time-delayed inoculation of S. Corvallis), the highest excretion rates for *S.* Paratyphi B (D-Ta$^+$) (4.78 log CFU/g) and *S.* Corvallis (log 5.18 CFU/g) were reached on the 14th and 21st days of life, respectively (Fig. 3). In this group, the detection of *S.* Paratyphi B (D-Ta$^+$) varied from 3 animals on the 8th day of life to 11 animals on the 16th day of life. Contrary to the results in groups 1 and 2, where a decrease in *S.* Paratyphi B (D-Ta$^+$) excretion from the 16th day onwards was observed, seven and four animals were still excreting *S.* Paratyphi B (D-Ta$^+$) in this group on the 21st and 28th days of life, respectively. As for *S.* Corvallis, on the 1st day

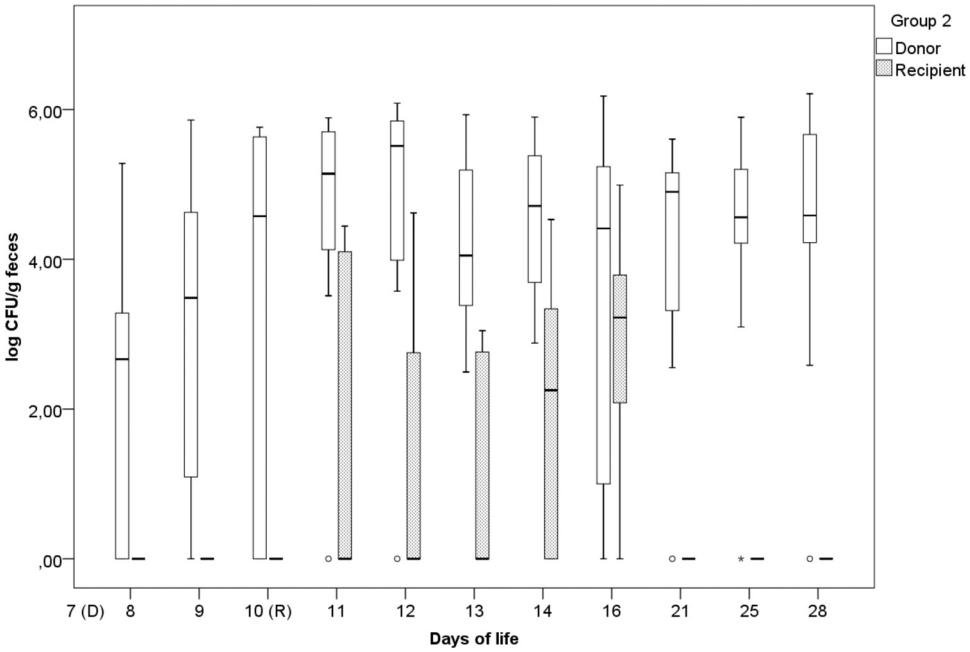

FIG 2 Fecal excretion of the donor (*S.* Corvallis) and the recipient [*S.* Paratyphi B (D-Ta$^+$)] in group 2 (delayed recipient inoculation on day 10), expressed as the log number of CFU per gram of feces, with outliers (°) and extreme outliers (*) included.

p.i., 2 animals were shedding *S.* Corvallis, and on the 28th day of life 11 animals were shedding *S.* Corvallis.

Fecal excretion of transconjugants. In groups 1 and 3, the earliest *Enterobacteriaceae* transconjugants were detected at 3 days p.i., whereas in group 2, the earliest

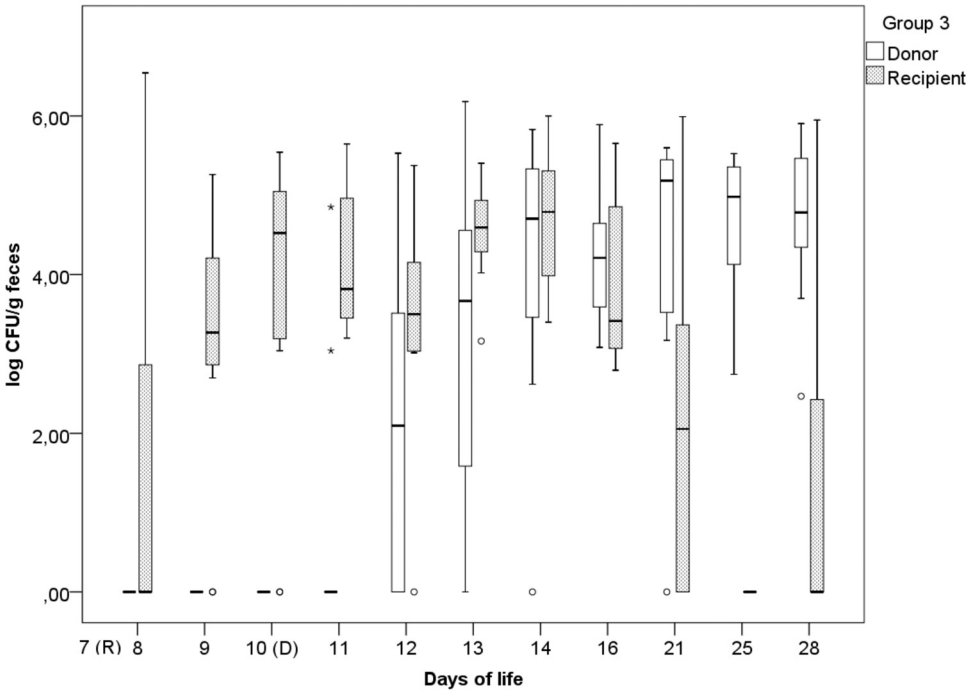

FIG 3 Fecal excretion of the donor (*S.* Corvallis) and the recipient [*S.* Paratyphi B (D-Ta$^+$)] in group 3 (delayed donor inoculation on day 10), expressed as the log number of CFU per gram of feces, with outliers (°) and extreme outliers (*) included.

TABLE 1 Number of animals shedding NDM-1-producing *Enterobacteriaceae*

Day of life	No. of positive animals		
	Group 1[a]	Group 2[a]	Group 3[b]
8th	0	0	—[d]
9th	0	1	—
10th	1	3	—
11th	3	3	0
12th	ND[c]	4	0
13th	ND	3	2
14th	ND	1	2
16th	1	3	1
21st	9	4	6
25th	4	4	6
28th	5	2	5

[a]Inoculation of *S.* Corvallis on the 7th day of life.
[b]Inoculation of *S.* Corvallis on the 10th day of life.
[c]ND, not determined.
[d]—, days prior to *S.* Corvallis inoculation.

detection was after 2 days p.i. (Table 1). *E. coli* transconjugants were detected in all groups, whereas *Klebsiella* transconjugants were detected only in group 2. During the 21 days p.i., *S.* Paratyphi B (D-Ta$^+$) transconjugants were not detected.

Variability of native plasmid content and plasmid acquisition in challenge strains. S1 nuclease-restricted pulsed-field gel electrophoresis (S1-PFGE) analysis revealed a higher variability in the native plasmid content of *S.* Corvallis reisolates in groups 1 and 2 than in the native plasmid content of *S.* Corvallis reisolates in group 3 (see Fig. S4 in the supplemental material) as well as the loss of the ~310-kb IncHI2 and <20-kb ColRNAI plasmids from *S.* Corvallis reisolates in group 1 and 2 (Fig. S2 and S3). In *S.* Corvallis reisolates from all groups, slight deviations (group 1 reisolates G1-28d-T10 and G1-28d-T10 [postmortem], group 2 reisolate G2-16d-T1, and group 3 reisolates G3-28d-T3 and G3-28d-T3 [postmortem], where the reisolates are denoted on the basis of the group [G1 to G3], day of life [1d to 28d], and chick [T1 to T11]) to larger deviations (in group 1 reisolate G1-11d-T10, group 2 reisolates G2-8d-T1 and G2-12d-T1, and group 3 reisolate G3-16d-T3) in the size of the ~180-kb pRH-1238 plasmid progeny were observed (Fig. S2 to S4). One *S.* Paratyphi B (D-Ta$^+$) reisolate (G1-16d-T5) acquired an ~100-kb plasmid (Fig. S1) lacking bla_{NDM-1}. Furthermore, hybridization of the *S.* Corvallis reisolates with the NDM-1 probe revealed that the bla_{NDM-1} gene was carried by a 110- to 130-kb plasmid (reisolates G1-11d-T10, G2-8d-T1, G2-12d-T1, and G3-16d-T3) and a >400-kb (reisolate G2-28d-T1) plasmid (Fig. S2 to S4) and not the ~180-kb plasmid.

Molecular characterization of NDM-1-producing *Enterobacteriaceae* transconjugants. In order to assess clonal relatedness as well plasmid(s) acquisition, *E. coli* and *Klebsiella* transconjugants were selected for further molecular typing by XbaI restriction analysis and S1-PFGE. In all three experimental groups, macrorestriction with the XbaI endonuclease revealed identical PFGE patterns for NDM-1-producing *E. coli* transconjugants of phylogenetic group A. At the individual-animal level, different PFGE patterns were observed for NDM-1-producing *E. coli* transconjugants on a particular sampling day, e.g., the 21st day of life (group 1 reisolates G1-21d-T7-I [*E. coli* phylogroup A] and G1-21d-T7-II [*E. coli* phylogroup B1]), as well as different genera of *Enterobacteriaceae* (resisolates G2-25d-T2-II [*E. coli* phylogroup D] and G2-25d-T2 -III [*Klebsiella pneumoniae*]) (Fig. S5). Digestion with S1 nuclease revealed that all selected NDM-1-producing *Enterobacteriaceae* transconjugants acquired the ~180-kb pRH-1238 plasmid, with the plasmid contents in *E. coli* strains that belonged to the same phylogenetic group (e.g., phylogenetic groups D and A) differing (Fig. S6). All strains except one *E. coli* isolate (G3-21d-environment [subcolony I]) encoded the bla_{NDM-1} gene on an ~180-kb plasmid (Table 2 and Fig. S6).

WGS analysis. With whole-genome sequencing (WGS) analysis, we confirmed the *in vivo* transfer of the pRH-1238 plasmid to different *E. coli* sequence types (ST) (ST-117,

TABLE 2 Molecular characteristics of selected challenge strain reisolates and transconjugants

Species or serovar	Designation[a]	Sequence type	Inc designation(s)	% sequence identity[b] to pRH-1238 (size of pRH-1238 progeny [kb[c]])	Presence of bla_{NDM-1} and bla_{CMY-16}	Additional resistance gene(s) present
S. Corvallis	G1-11d-T10	ST-1541	IncHI2, IncA/C_2, ColpVC	66.03 (~100)	Only bla_{NDM-1}	bla_{TEM-1B}
S. Corvallis	G1-16d-T10	ST-1541	IncHI2, IncA/C_2, ColRNAI	99.40 (~180)	Both	
S. Corvallis	G1-21d-T10	ST-1541	IncHI2, IncA/C_2, ColpVC	99.66 (~180)	Both	bla_{TEM-1B}
S. Corvallis	G1-28d-T10	ST-1541	IncHI2, IncA/C_2, ColpVC	95.55 (~170)	Both	bla_{TEM-1B}
S. Corvallis	G1-28d-T10[d]	ST-1541	IncHI2, IncA/C_2, ColRNAI	98.69 (~180)	Both	
S. Corvallis	G2-8d-T1	ST-1541	IncHI2, IncA/C_2, ColpVC, ColRNAI	79.17 (~140)	Both	
S. Corvallis	G2-10d-T1	ST-1541	IncHI2, IncA/C_2, ColRNAI	99.54 (~180)	Both	
S. Corvallis	G2-12d-T1	ST-1541	IncHI2, IncA/C_2, ColpVC, ColRNAI	73.46 (~130)	Both	
S. Corvallis	G2-16d-T1	ST-1541	IncA/C_2, ColRNAI	95.25 (~170)	Both	
S. Corvallis	G2-25d-T1	ST-1541	IncHI2, IncA/C_2	99.66 (~180)	Both	
S. Corvallis	G2-28d-T1	ST-1541	IncHI2, IncA/C_2	99.54 (>400)	Both	
S. Corvallis	G3-16d-T3	ST-1541	IncHI2, IncA/C_2, ColRNAI	75.69 (~140)	Both	
S. Corvallis	G3-28d-T3	ST-1541	IncHI2, IncA/C_2, ColRNAI	99.45 (~180)	Both	
S. Corvallis	G3-28d-T3[d]	ST-1541	IncHI2, IncA/C_2, ColRNAI	99.65 (~180)	Both	
E. coli	G1-21d-T7 (I)	ST-2040	IncX1, IncA/C_2, ColpVC, ColRNAI	99.76 (~180)	Both	*qnrS1*, bla_{TEM-1B}
E. coli	G1-21d-T7 (II)	ST-156	IncA/C_2	99.83 (~180)	Both	*tet*(B), bla_{TEM-1B}
E. coli	G1-21d-T1	ST-2040	IncX1, IncA/C_2, ColpVC, ColRNAI	99.76 (~180)	Both	*qnrS1*, bla_{TEM-1B}
E. coli	G2-21d-T5	ST-117	p0111, IncA/C_2	99.68 (~180)	Both	
E. coli	G2-21d-T9 (I)	ST-2485	IncA/C_2	99.85 (~180)	Both	
E. coli	G2-25d-T2 (II)	ST-2485	IncA/C_2	98.62 (~180)	Both	
K. pneumoniae	G2-25d-T2 (III)	ST-1106	Col(MGD2), IncA/C_2, ColRNAI	99.53 (~180)	Both	*oqxA*, *oqxB*, bla_{SHV-1}
E. coli	G3-21d-environment (I)	ST-2040	IncX1, IncA/C_2, ColpVC, ColRNAI	89.62 (~170)	Only bla_{CMY-16}	*qnrS1*, bla_{TEM-1B}
E. coli	G3-21d-T3	ST-2040	IncX1, IncA/C_2, ColpVC, ColRNAI	99.74 (~180)	Both	*qnrS1*, bla_{TEM-1B}
E. coli	G3-21d-T6 (I)	ST-2040	IncX1, IncA/C_2, ColpVC, ColRNAI	95.31 (~180)	Both	*qnrS1*, bla_{TEM-1B}
S. Paratyphi B (D-Ta$^+$)	G1-16d-T5	ST-28	IncI1	None	Neither	

[a]Designations indicate the group (G1 to G3), day of life (1d to 28d), animal (T1 to T11), and subcolony (I to IV) origin.
[b]Based on consensus sequence length (CLC Genomics Workbench [v9.5] software).
[c]Based on S1-PFGE restriction.
[d]Postmortem (cecal content isolates).

ST-156, ST-2040, and ST-2485) as well as to a *K. pneumoniae* strain (ST-1106). Furthermore, we reconfirmed the loss of an ~310-kb IncHI2 plasmid in one *S.* Corvallis reisolate (G2-16d-T1) and the ColRNAI plasmid in several *S.* Corvallis reisolates from group 1 and group 2 (Table 2). The ColRNAI plasmid was detected in *E. coli* (ST-2040) and *K. pneumoniae* (ST-1106) strains. On the other hand, the ~310-kb IncHI2 plasmid was not detected in any of the NDM-1-producing *Enterobacteriaceae* transconjugants (Table 2). The position of the bla_{NDM-1} gene in all *S.* Corvallis reisolates on the pRH-1238 plasmid progeny could be confirmed, whereas one strain (G1-11d-T10) did not harbor the bla_{CMY-16} gene (Table 2). One *S.* Corvallis reisolate (G2-28d-T1) harbored a bla_{NDM-1}-carrying >400-kb plasmid (Fig. S3) (based on Southern blotting and hybridization of S1 nuclease-restricted PFGE). Mapping of this strain to reference plasmid pRH-1238 yielded a consensus sequence identity of 99.53%, and the >400-kb plasmid for this strain might resemble a fusion of IncA/C_2 (pRH-1238) and IncHI2 (~490 kb).

Further to the resistome of pRH-1238, additional resistance genes conferring resistance to β-lactams (bla_{TEM-1B}) were detected in three *S.* Corvallis reisolates (G1-11d-T1, G1-21d-T1, G1-28d-T1), whereas one *S.* Paratyphi B (D-Ta^+) reisolate (G1-16d-T5) did not harbor additional resistance genes, other than the ~100-kb IncI1 plasmid. Additional resistance genes for NDM-1-producing *Enterobacteriaceae* are shown in Table 2.

On the basis of consensus sequence mapping of the pRH-1238 plasmid progeny to the reference sequence of pRH-1238 from the *S.* Corvallis reisolates in all groups, a deletion in transfer (Tra) region 1 (Tra1) (~50 to 60 kb in size) was observed (Fig. 4). In one strain (G1-11d-T10), this led to the loss of bla_{CMY-16}. Another noteworthy result was the high percent sequence identity among the pRH-1238 progeny from selected *Enterobacteriaceae* transconjugants, in contrast to *S.* Corvallis reisolates (Table 2 and Fig. 5).

DISCUSSION

Recent publications have reported the occurrence of carbapenem-nonsusceptible *Enterobacteriaceae* in wild birds, livestock, and food products and their spread, related to plasmid-mediated carbapenemases (11, 17–19). As carbapenems are not licensed for use in livestock, it is assumed that the occurrence of carbapenemase-producing bacteria is triggered by coselective pressure, since plasmids carrying bla_{NDM-1}, like the plasmid chosen for use in this study, commonly harbor multiple but variable resistance determinants (16, 22). Still, current research shows that the spread of certain plasmid-mediated resistance genes in broiler chickens is also possible without antibiotic selective pressure (20, 23). Therefore, for understanding the mechanisms contributing to the spread of carbapenem resistance or carbapenem-nonsusceptible isolates *in vivo*, the objective of our animal trial was to explore the broad-host-range capacity and stability of a conjugative bla_{NDM-1}-carrying plasmid, IncA/C_2 plasmid pRH-1238, hosted by an *S.* Corvallis strain in chickens without antimicrobial selection pressure, representing the nonuse of carbapenems in livestock. With the help of WGS, such a setup enabled us to obtain an insight into the microevolution of the plasmid *in vivo*.

Challenge strain excretion. During our study, we observed prolonged fecal excretion of NDM-1 carbapenemase-producing *S.* Corvallis (12-SA01738), contrary to that of *S.* Paratyphi B (D-Ta^+) (13-SA01617). Statistical analysis of data from group 1 [simultaneous inoculation of *S.* Corvallis and *S.* Paratyphi B (D-Ta^+)] revealed that the fecal excretion of *S.* Corvallis was significantly higher toward the end of the trial (the 16th, 21st, 25th, and 28th days of life) (Fig. 1) and was not hampered by the later inoculation of *S.* Paratyphi B (D-Ta^+), as in group 2 (the difference between *S.* Corvallis excretion in groups 1 and 2 was statistically significant only by 8th day of life) (Fig. 1 and 2). Because of previous studies reporting the invasiveness of *S.* Paratyphi B (D-Ta^+) toward epithelial cells and macrophages and their presence in ceca, the liver, and the spleen (24), the decreased excretion of *S.* Paratyphi B (D-Ta^+) observed in our *in vivo* trial is a noteworthy finding. Although this serovar is reported to be broiler associated, we have not observed a competitive advantage, contrary to the findings for *S.* Corvallis. On the other hand, the prolonged excretion of NDM-1-producing *S.* Corvallis in the absence of

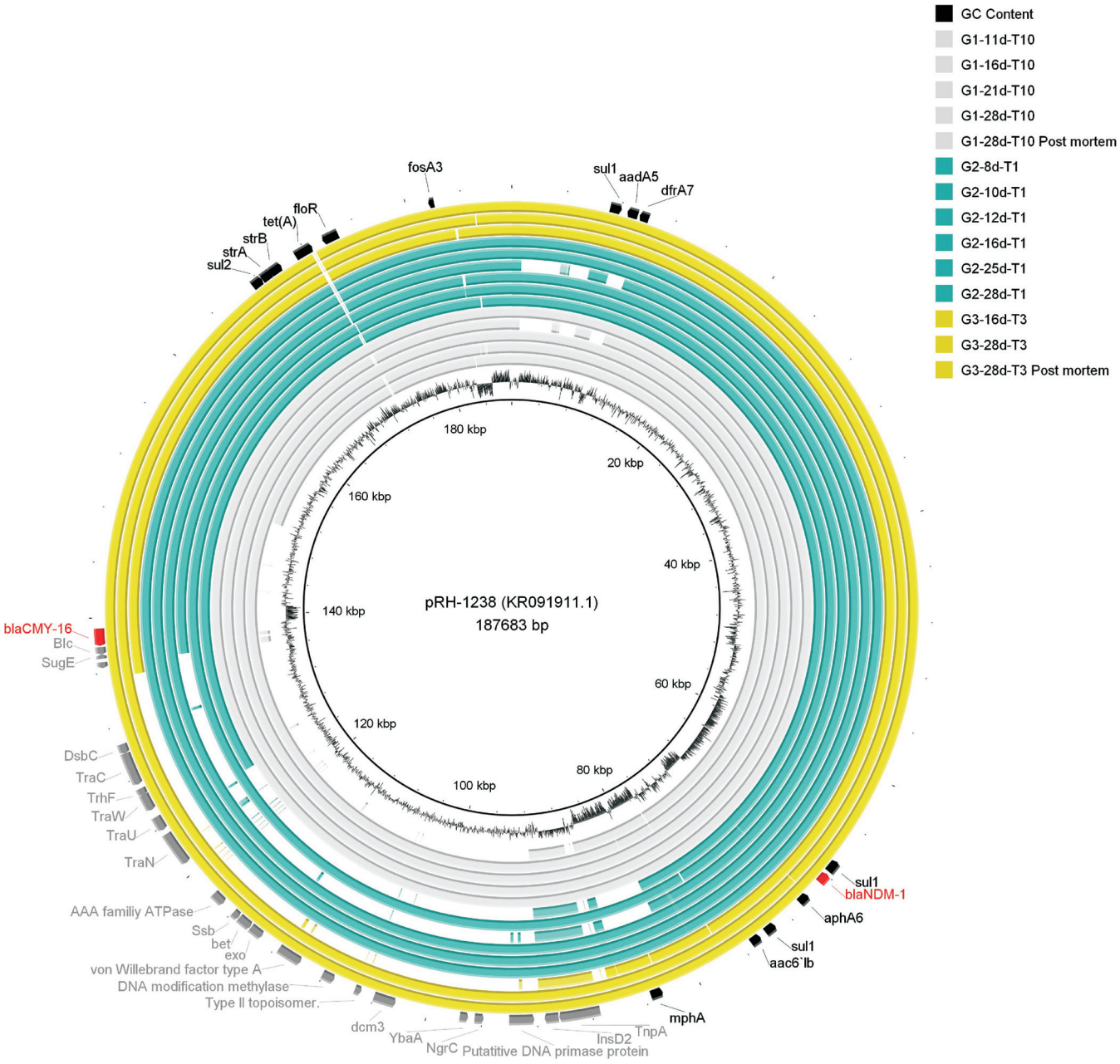

FIG 4 Visualization of assemblies of 14 pRH-1238 consensus sequences from *S.* Corvallis reisolates mapped to the reference pRH-1238 plasmid (GenBank accession number KR091911.1). Innermost circle, pRH-1238 coordinates; second-innermost circle, GC content of pRH-1238 reference plasmid; gray circles, group 1; green circles, group 2; yellow circles, group 3; outermost annotations, the resistome (black), *bla* genes (red), and deletions (dark gray) in Tra1 and the adjacent region, obtained using BRIG (50). Note in strain G1-11d-T10 pRH-1238 progeny the loss of the bla_{CMY-16} gene.

antibiotic pressure is an important concern due to its resistome and the broad host range of the pRH-1238 plasmid.

***In vivo* transfer of bla_{NDM-1}-harboring plasmid pRH-1238.** In our study, we demonstrated the *in vivo* transfer of IncA/C_2 bla_{NDM-1}-carrying conjugative plasmid pRH-1238 from avian native *S.* Corvallis to *E. coli* strains belonging to phylogroups A, B1, and D, represented by four *E. coli* multilocus sequencing types (ST-117, ST-156, ST-2040, and ST-2485) and a *K. pneumoniae* isolate (ST-1106) (Table 2). At the individual level, on particular sampling days, we observed pRH-1238 acquisition not only in different *E. coli* strains but also in different *Enterobacteriaceae* genera (Table 2). This, together with their rapid onset of excretion (Table 1), demonstrates the broad host range and the high

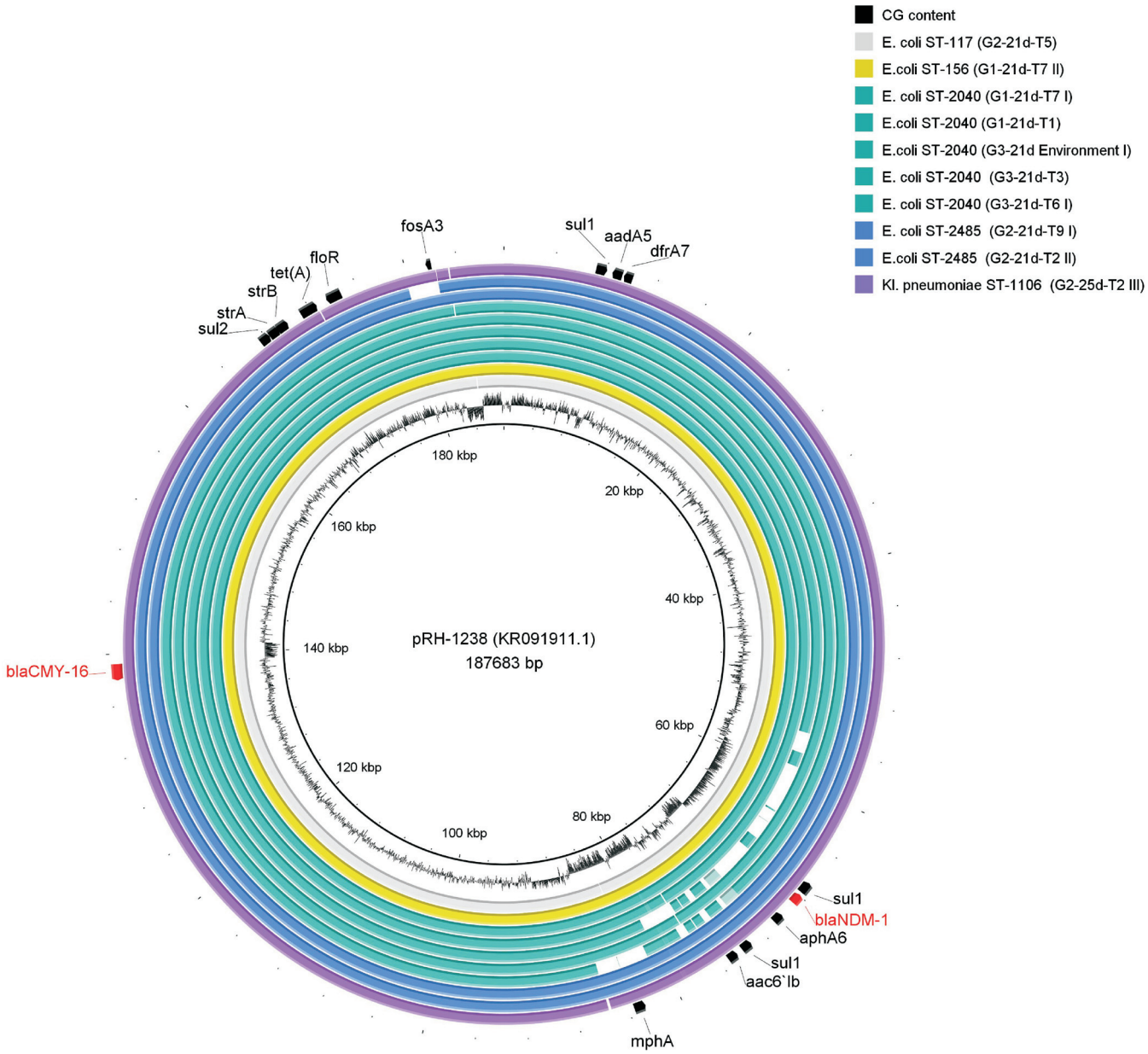

FIG 5 Visualization of assemblies of 10 pRH-1238 consensus sequences from carbapenemase-producing *E. coli* (ST-117, ST-156, ST-2040, and ST-2485) and *K. pneumoniae* (ST-1106) transconjugants mapped to the reference pRH-1238 plasmid (GenBank accession number KR091911.1). Innermost circle, pRH-1238 coordinates; second-innermost circle, GC content of pRH-1238 reference plasmid; gray circle, ST-117 strain; yellow circle, ST-156 strain; green circles, ST-2040 strain; blue circles, ST-2485 strain; purple circle, ST-1106 strain; outermost annotations, resistome (black) and *bla* genes (red), obtained using BRIG (50). Note in strain G3-21d-environment (I) pRH-1238 progeny the loss of the bla_{NDM-1} gene.

transferability of this multidrug resistance-conferring plasmid, leading to multidrug resistance acquisition in one horizontal gene transfer event. The affected species and genus (*E. coli* and *Klebsiella*) underline the importance of this concern due to their clinical relevance and ubiquitous distribution in the environment, acting as potential reservoirs of bla_{NDM-1} (25). This deserves attention, especially in commercial broiler production, where contamination pressure due to continuous rearing cycles as well as short interservice breaks could lead to the continuous propagation of pRH-1238 within a mixed bacterial population. With the previous detection of avian native NDM-1 carbapenemase-producing *S.* Corvallis in wild birds, such an entry scenario in commercial broiler production would presumably lead to rapid and diverse bla_{NDM-1} dissemination within a broiler flock even without antibiotic pressure. This might also lead to environmen-

tal contamination, as has been observed for extended-spectrum β-lactamase (ESBL)/AmpC-producing *E. coli* strains (26). The broad host range and the high level of transferability without antibiotic pressure should be kept in mind with the implementation of preventative measures. Instead of relying only on selective and coselective pressure as a measure to minimize carbapenemase-producing bacteria, further approaches assessing quantification of resistance gene dissemination with and without selective antibiotic pressure should also be considered.

The detection of enterobacterial transconjugant strains until the end of the trial (in group 1, from the 10th day of life onwards; in group 2, from the 9th day of life onwards; and in group 3, from the 13th day of life onwards) (Table 1) underlines that fact the plasmid acquisition has a presumably low or negligible fitness cost (27). Intestinal bacteria serve as reservoirs or even vectors for antibiotic resistance plasmids (28), findings which are further emphasized by the plasmid and resistance gene acquisition from the gut microflora observed in challenge strains (Table 2).

As we did not detect NDM-1-producing *S.* Paratyphi B (D-Ta$^+$) transconjugants, we assume that the host's *E. coli* population has an important influence on the reception and further spread of the pRH-1238 plasmid. This might be linked to the dense and diverse population and host gut adaptation of *E. coli*, serving as native recipients of pRH-1238. Although our *in vitro* filter mating conjugation experiments indicated a high transfer rate of the pRH-1238 plasmid to *S.* Paratyphi B (D-Ta$^+$) (see Table S2 in the supplemental material), its absence *in vivo* might be linked to (i) serovar colonization dynamics, (ii) the abundance, diversity, and interference of *E. coli* strains, and (iii) the detection limit of the method used in this study (~100 CFU/g). The majority of *E. coli* NDM-1 producers belonged to phylogroup A (represented by ST-2040); however, ST-117 and ST-156 strains were also detected. Besides being associated with poultry, strains of these STs are also described to be a potential source of not only β-lactam resistance genes but also polymyxin resistance genes (29–31). In a recent publication, a human-acquired *mcr-1*-carrying ST-117 strain of avian origin was characterized, highlighting the capability of this ST for resistance gene acquisition (30). The observed dominance of *E. coli* strains belonging to phylogroup A might resemble their occurrence in the gut or their ability to serve as native recipients for pRH-1238, as described for certain clonal lines dominant in the spread of the plasmid-mediated *oqxAB* gene encoding quinolone resistance (23).

Furthermore, bla_{CMY-16} is a variant of the bla_{CMY-2} lineage, which has been described to be the most common plasmid-mediated AmpC enzyme common to different *Enterobacteriaceae* worldwide (32). Therefore, the introduction of the pRH-1238 plasmid into a broiler flock should be assessed as well in light of the potential further dissemination of not only bla_{NDM-1} but also bla_{CMY-16}, which might be additionally propagated due to the use of cephalosporins in commercial poultry production. For future understanding, it is of interest to predict the dissemination potential of plasmid-mediated resistance genes relevant to public health and questioning the genera or serovars dominant in this exchange. Such data could contribute to a wider picture, broaden our knowledge for carbapenem resistance risk assessment, and serve as an asset for future approaches minimizing the spread of antimicrobial resistance *in vivo*.

Plasmid content variability in *S. Corvallis* reisolates. The observed native plasmid variability (plasmids of ~310 kb [IncHI2], 180 kb pRH-1238 [IncA/C$_2$], and <20 kb [ColRNAI]) was predominant in *S.* Corvallis reisolates from groups 1 and 2 (Fig. S2 and S3). This observation leads us to the assumption that the simultaneous and initial inoculation of *S.* Corvallis led to certain rearrangement mechanisms in native plasmid content, observed as the complete loss of the ~310-kb IncHI2 and <20-kb ColRNAI plasmids or partial region deletions in the ~310-kb IncHI2 plasmid (up to ~100 kb) and in the ~180-kb pRH-1238 plasmid (up to ~50 to 60 kb) (Fig. S2 to S4). We speculate that the earlier (7th day of life) inoculation of *S.* Corvallis in experimental groups 1 and 2 led to this occurrence. In a recent study by Card et al. (33) with a chemostat which mimicked the broiler microbiome, it seemed that the bacterial community stabilized by

day 6. In our case, this unstable microbial population might support mobilome restructuring as well the interaction and subsequent acquisition of bla_{TEM-1B} in *S.* Corvallis reisolates from group 1 in later stages of the trial (Table 2). Plasmid exchange and certain structural deletions might also be an important part of host adaptation regulation. Previous studies have reported that the acquisition and loss of certain genetic elements in bacteria are stimulated by the adaptation to the new environment, which influences their pathogenicity and might have subsequent consequences for human and animal health (34, 35). As our study focused on NDM-producing *Enterobacteriaceae* detectable on xylose-lysine-deoxycholate (XLD) and chromID Carba agar and we did not conduct metagenomics analysis, we presume that bla_{TEM-1B} might have originated from an *E. coli* ST-2040 strain. Furthermore, it seems that this sequence type played a significant role in plasmid exchanges (acquisition of pRH-1238 and ColRNAI and transfer of the ColpVC plasmid) with *S.* Corvallis (Table 2).

Microevolution of pRH-1238 in *S.* Corvallis and enterobacterial transconjugants. Besides the plasmid content variability observed after S1 restriction for the IncHI2 (~310-kb) and ColRNAI (<20-kb) plasmids in *S.* Corvallis reisolates, the large-scale structural changes in pRH-1238 progeny were determined as deletions in Tra1 and downstream (~50 to 60 kb in size) between two resistance islands: ARI-A [harboring *sul1*, bla_{NDM-1}, *aph6*, *mphA*, and *aac6′Ib*] and ARI-B [harboring *sul2*, *strA*, *strB*, *tet*(*A*), *floR*, *fosA3*, *sul1*, *aadA5*, *dfrA7*] (16). These deletions did not lead to a significant alteration of the pRH-1238 β-lactam resistome, as only one strain did not harbor bla_{CMY-16} (Table 2 and Fig. 4), due to its position adjacent to Tra1 of pRH-1238. This occurrence is in line with observations indicating the large-scale structural changes often observed in neighboring areas of transposons and insertion sequence elements, indicating that these elements contribute to plasmid genome evolution (36). In a recent *in vitro* study by Porse et al. (37), deletions in the IncN plasmid (also constituting Tra regions) in recipient *E. coli* strains were observed, contrary to the findings for native *K. pneumoniae* and recipient *Klebsiella* strains. The authors stated that this occurrence might possess a potential competitive benefit for recipient *E. coli* strains. In contrast to the findings of our *in vivo* study, the deletions in the pRH-1238 progeny were dominant in *S.* Corvallis reisolates and not *E. coli* and *K. pneumoniae* strains (Fig. 4 and 5), suggesting that these deletions might be host or incompatibility group dependent. Generally, the observed losses of the IncHI2 and ColRNAI plasmids as well as deletions in the pRH-1238 progeny might indicate an evolutionary background in *S.* Corvallis adaptation which enables maintenance of the pRH-1238 resistome even without antibiotic pressure in wild birds.

A noteworthy observation was a >400-kb plasmid in sample G2-28d-T1 (Table 2 and Fig. S3) which seemed to be a fusion of IncHI2 and IncA/C_2 plasmid pRH-1238. This mobilome restructuring might be triggered by intrinsic *S.* Corvallis mechanisms and also linked with the persistence of bla_{NDM-1} in *S.* Corvallis. Namely, plasmid fusion and cointegration are frequent phenomena in plasmid evolution and adaptation and prevent, e.g., plasmid incompatibility and facilitate the interaction with a broad range of hosts (38). For a better understanding, it is of interest to explore if these occurrences are triggered by certain metabolic processes in the gut, bacterial stress, or a possible interaction with competitive gut microflora. Interestingly, pRH-1238 progeny from two strains sampled from the cecal contents showed a high percentage of sequence identity to the pRH-1238 backbone (Table 2 and Fig. 4). Such an occurrence indicates that the *S.* Corvallis reisolates harboring native pRH-1238 exist in the intestinal tract and continuously disseminate pRH-1238 *in vivo*. Previous findings have reported on the higher level of colonization of *Salmonella* in the ceca, leading to higher rates of conjugation, which has been observed for a conjugative extended-spectrum cephalosporin resistance gene-harboring plasmid from *S.* Newport to *E. coli* strains and vice versa (39). Furthermore, deletions in pRH-1238 among *Enterobacteriaceae* transconjugants were minor and not attributed to Tra1, and the sequences of pRH-1238 progeny with these deletions revealed a higher degree of identity to the reference backbone of pRH-1238 (Table 2 and Fig. 5). This indicates that the pRH-1238 acquisition or transfer

process itself might not lead to a significant alteration of pRH-1238 in transconjugant strains and that these strains might also serve as long-term reservoirs of pRH-1238 *in vivo*.

In conclusion, we demonstrated the prolonged fecal excretion of an avian native NDM-1 carbapenemase-producing *S.* Corvallis strain (12-SA01738) with microevolutionary deletions in the pRH-1238 backbone that preserved the bla_{NDM-1} gene during a broiler chicken *in vivo* study. The conjugative pRH-1238 IncA/C_2 bla_{NDM-1}-carrying plasmid was transferable to different *Enterobacteriaceae*, expanding its resistance gene pool among gut microflora in the absence of antibiotic pressure throughout the trial. This study shows at the molecular level how the rapid and diverse dissemination of bla_{NDM-1}-harboring IncA/C_2 plasmids in commercial broiler production can occur even in the absence of selective pressure. Furthermore, it highlights the need for understanding the mechanisms of the interaction of the host microflora and *Salmonella* serovars and calls for additional efforts in future intervention approaches to avoid the further spread of multidrug resistance plasmids in commercial broiler production.

MATERIALS AND METHODS

Challenge strains. Avian native *Salmonella enterica* subsp. *enterica* serovar Corvallis (strain 12-SA01738) of ST-1541 harboring the bla_{NDM-1}-carrying IncA/C_2 plasmid pRH-1238 (GenBank accession number KR091911.1) was selected as the donor strain. Native D-tartrate-fermenting (D-Ta^+) *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (13-SA01617), referred to here as *S.* Paratyphi B (D-Ta^+), of ST-28, isolated in 2013, with intrinsic resistance to nalidixic acid was selected as the recipient. The pRH-1238 plasmid is the first completely sequenced bla_{NDM-1}-*fosA3*-IncA/C plasmid. It is 187,683 bp in size, has a GC content of 51.7%, and contains 173 predicted coding sequences (CDSs). It contains two resistance islands (ARI-A and ARI-B) and two transfer (Tra) regions (Tra1 and Tra2), with bla_{NDM-1} being located in ARI-A and bla_{CMY-16} being located in Tra1 (16). Besides pRH-1238, the donor strain harbors two additional plasmids of incompatibility group IncHI2 (~310 kb) and ColRNAI (<20 kb), whereas *S.* Paratyphi B (D-Ta^+) was selected as plasmid-free recipient strain. The phenotypic and genotypic properties of the donor and recipient strains are listed in Table S1 in the supplemental material. The selection of *S.* Paratyphi B (D-Ta^+) was based on its high prevalence in commercial poultry production in Germany (40) as well as optimal *in vitro* conjugation transfer frequency (CTF) at 42°C (which corresponds to the average body temperature of birds) with *S.* Corvallis as the donor strain (Table S2). All strains were obtained from the strain collection of the National Reference Laboratory (NRL) for *Salmonella* in Germany.

***In vitro* filter mating conjugation experiments.** Prior to our *in vivo* study, *in vitro* filter mating conjugation experiments with selected *Salmonella* strains (Table S2) were conducted to determine the average conjugation transfer frequency (CTF) for four potential recipient strains with *S.* Corvallis (12-SA01738) as the donor. After aerobic growth with gentle shaking at 37°C to obtain an optical density at 560 nm (OD_{560}) value of 0.25, a mixture of the *Salmonella* donor and recipient at a ratio of 1 to 2 was centrifuged (20,000 × *g* for 2 min), inoculated on 0.45-μm -pore-size filter membranes (Merck Millipore, Germany) that had previously been placed on lysogeny agar (LBA; Thermo Fisher Scientific, Germany), and incubated for 4 h at room temperature (RT), 37°C, or 42°C. Following incubation, the filter membranes were suspended in 4 ml of lysogeny broth (LBL; Thermo Fisher Scientific, Germany), decimally diluted, and plated on transconjugant selective plates (as described in Table S3). All filter mating conjugation experiments were conducted in triplicate in order to determine the average CTF rate (Table S2).

Broiler chicken infection study. For the *in vivo* trial, 33 broiler chicks (Ross 308) were randomly selected as 1-day-old chicks, without prior determination of the chick sex. Housing, clinical examination, individual labeling, and sampling followed. Animals were randomly divided into three experimental groups (group 1 [G1], G2, and G3), each containing 11 animals (animals T1 to T11) and housed in the facilities for animal experiments at the German Federal Institute for Risk Assessment, Berlin, Germany. In order to evaluate the dependency of the time point of inoculation on excretion of the challenge strains for the 28 days of the experiment, three experimental setups (groups 1, 2, and 3) were assembled. In group 1, the donor and recipient were simultaneously inoculated on the 7th day of life, whereas in group 2 (inoculation on the 7th day of life for the donor and the 10th day of life for the recipient) and group 3 (inoculation on the 7th day of life for the recipient and the 10th day of life for the donor), time-delayed inoculations were used. At the end of the experiment (at the 28th day of life), all animals were handled carefully following electrical stunning before being sacrificed for postmortem cecum extirpation. The experimental design containing the time frame and the related activities is shown in Fig. 6. During the experiment, microambient conditions complied with the hybrid management guide, and the animals were checked daily for evaluation of criteria for health and well-being. The animal trials were approved by the German State Authority for Health and Social Affairs (Lageso; no. 0308/15).

To prevent unintentional cross-reaction with intestinal microbiota, 1-day-old chicks were tested for possible colonization with (i) ESBL/pAmpC- or carbapenemase-producing *E. coli* using the laboratory protocol provided and recommended by the EURL for antimicrobial resistance (41) and (ii) *Salmonella*

FIG 6 Experimental design of the test groups (groups 1 to 3), with inoculation (red) and sampling (blue) days, as well as the end day of the experiment (blue with black numbers), being marked.

spp. following standard ISO 6579:2002/Amd 1:2007 (International Organization for Standardization, Switzerland). The procedure was repeated on the day of inoculation to reconfirm the absence of interfering background flora.

Inoculation challenge and sampling plan. On the day of inoculation, both challenge strains were grown aerobically in LBL at 37°C with gentle shaking to obtain an OD_{560} value of 0.35, which corresponded to a bacterial count of 4×10^6 CFU per 100 μl for both strains used as the inoculum. On day 7, animals were orally inoculated (for group 1, with the donor and recipient strains; for group 2 with the donor strain; for group 3, with the recipient strain), followed by a second inoculation (for group 2, with the recipient strain; for group 3, with the donor strain) on the 10th day of life. After inoculation, a 4-day consecutive sampling was performed, and further sampling was performed two times per week toward the end of trial (Fig. 6). Animals were always sampled individually in a particular time frame with preweighed cotton cloacal swabs (Deltalab, Spain) in order to determine the counts of the excreted challenge strains, expressed as the number of CFU per gram of feces.

Bacterial strain isolation. After suspending the fecal material (~0.2 g) in 5 ml of 0.85% (wt/vol) NaCl, the suspension was subjected to decimal dilution and a 100-μl deposition volume per plate was plated with an automatic spiral plater in duplicate on selective agar plates using the spiral colony counting technique with a Whitley automatic spiral plater (Don Whitley Scientific, UK). On the 1st day postinoculation (p.i.), dilutions of 1:10 and $1:10^3$ were plated, and these were later adjusted to 1:10 and $1:10^2$ on the basis of excretion dynamics. Challenge strain and transconjugant detection was based on growth on xylose-lysine-deoxycholate (XLD) agar (Thermo Fisher Scientific, Germany) with antibiotic supplementation (meropenem [0.125 mg/liter], cefotaxime [1 mg/liter], and/or nalidixic acid [50 mg/liter]), depending on the target strain (donor, recipient, or *Salmonella* transconjugants), and chromID Carba (bioMérieux, France) for detection of carbapenemase-producing *Enterobacteriaceae* (CPE) (Table S3). Colonies suspected of being *Salmonella* were detected on XLD agar as red-yellow colonies with a black center, and CPE (e.g., *E. coli*, *Klebsiellae*) were detected on chromID Carba as purple and blue colonies. In order to further characterize the challenge strain [e.g., to characterize the variability in the plasmid content in *S.* Corvallis and plasmid acquisition in *S.* Paratyphi B (D-Ta$^+$)], reisolates from particular chicks within each group [*S.* Corvallis reisolates in group 1 (chick T10; see below), group 2 (chick T1), and group 3 (chick T3) and *S.* Paratyphi B (D-Ta$^+$) reisolates in group 1 (chick T5), group 2 (chick T1), and group 3 (chick T3)] were preserved, whereas when possible four subcolonies (marked I to IV) of presumptive NDM-1 carbapenemase-producing *Enterobacteriaceae* transconjugants were selected for molecular characterization. Selected strains were denoted on the basis of the group (G1 to G3), day of life (1d to 28d), chick (T1 to T11), and subcolony (I to IV) origin.

Confirmation of presence of pRH-1238 in transconjugants. Transconjugants were screened by PCR amplification of bla_{NDM-1} and bla_{CMY-16} using a 1:10-diluted overnight culture as the template as described in previous publications (42, 43). PCR mixtures (25-μl reaction volume) contained 17.5 μl of master mix (4 μl primer mix [each at 400 nM], 2.5 μl deoxynucleoside triphosphate [dNTP] mix [each 200 μM], 2.5 μl 10× buffer, 1.25 μl $MgCl_2$ [2.5 mM], 7.05 μl PCR-grade water, 0.2 μl *Taq* DNA polymerase [Invitrogen, USA]) and 7.5 μl DNA template under the following conditions: initialization for 5 min at 94°C; denaturation, annealing, and extension for 30 s at 94°C, 30 s at 54°C, and 1 min at 72°C for 30 cycles; elongation for 5 min at 72°C; and a final hold at 4°C.

Genus/species identification of transconjugants through MALDI-TOF MS. A random selection of phenotypically different (purple and blue) colonies of *Enterobacteriaceae* transconjugants grown on chromID Carba was tested by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS; Bruker Daltonik, Germany) to confirm the taxonomic classification at the genus and species levels. After subculture on LBA, a small amount of a bacterial colony was transferred in duplicate onto the target wells of an MSP 96 polished steel barcode (BC) plate (Bruker Daltonik, Germany) and suspended in 1 μl of HCCA (α-cyano-4-hydroxycinnamic acid) matrix (Bruker Daltonik, Germany) according to the manufacturer's instructions. After the bacteria were air dried at room temperature, mass spectrometry was performed using MALDI flexControl software (Bruker Daltonik, Germany), and microorganisms were identified according to the values obtained, where values of 2.3 to 3.0, obtained by use of the MALDI Biotyper (Bruker Daltonik, Germany) database, indicated a very sure species identification, values of 2.0 to 2.29 indicated a sure genus identification and a probable species identification, values of 1.7 to 1.99 indicated a probable genus identification, and values of 0.0 to 1.69 indicated a not reliable identification.

Molecular characterization of transconjugants. (i) Phylotyping of *E. coli* transconjugants. A multiplex PCR assay for phylotyping of 104 selected *E. coli* transconjugants (for group 1, $n = 43$; for group 2, $n = 19$; for group 3, $n = 42$) into phylogenetic groups A, B1, B2, and D was conducted (44). All PCRs

were done in a 25-μl reaction volume containing 18 μl of master mix (5 μl primer mix [10 pmol each primer], 2.5 μl dNTP mix [each 200 μM], 2.5 μl 10× buffer, 7.5 μl PCR-grade water, 0.5 μl *Taq* DNA polymerase [Invitrogen, USA]) and 7 μl of the DNA template (a 1:10-diluted overnight culture) under the following conditions: an initialization step of 5 min at 94°C; denaturation, annealing, and extension for 30 s at 94°C, 30 s at 65°C, and 30 s at 72°C for 30 cycles; elongation for 5 min at 72°C; and a final hold at 4°C.

(ii) XbaI PFGE analysis. Macrorestriction with the XbaI endonuclease (Roche Applied Sciences, Switzerland) was performed for 104 NDM-1-producing *Enterobacteriaceae* transconjugant strains according to the PulseNet standardized protocol (www.pulsenetinternational.org) (45) using a contour-clamped homogeneous electric field (CHEF-DRIII) system (Bio-Rad Laboratories, Madrid, Spain) for separation of the fragments. As a molecular size standard, *Salmonella* serovar Braenderup strain H9812 (restricted with XbaI) was used. Gel imaging was conducted in a GenBox apparatus (Syngene, UK), and gel documentation was conducted with GeneSnap software (Syngene, UK).

Molecular characterization of the pRH-1238 plasmid. (i) S1-PFGE plasmid profiling. In order to evaluate the variability in native plasmid content (the IncHI2 plasmid [~310 kb], IncA/C_2 plasmid pRH-1238 [187,683 bp], and the ColRNAI plasmid [<20 kb]) among the *S.* Corvallis strains and plasmid acquisition in *S.* Paratyphi B (D-Ta$^+$), in total 20 reisolates (for group 1, animal T10 [$n = 6$]; for group 2, animal T1 [$n = 8$]; for group 3, animal T3 [$n = 6$]) and 18 reisolates (for group 1, animal T5 [$n = 4$]; for group 2, animal T1 [$n = 6$]; for group 3, animal T3 [$n = 8$]), respectively, were typed by S1-PFGE. Additionally, 104 NDM-1-producing *Enterobacteriaceae* transconjugants were typed by S1 nuclease (TaKaRa, USA) restriction in order to visualize the transferred ~180-kb pRH-1238 plasmid. For S1-PFGE gels intended for Southern blotting, a MidRange pulsed-field gel marker (Biolabs, USA) was used as a size marker. The generated fragments were separated using the CHEF-DRIII system (Bio-Rad Laboratories, Spain) with S-1 running conditions (1 s to 25 s, 17 h, 6 V/cm, 120 V), as previously described (46).

(ii) Southern blotting of an S1-PFGE gel hybridized with a *bla*$_{NDM-1}$ probe. To map the position of a single carbapenem resistance marker (the *bla*$_{NDM-1}$ gene on pRH-1238), Southern blotting and hybridization of an S1-PFGE gel with a digoxigenin-labeled NDM-1 probe of 20 selected *S.* Corvallis reisolates and 16 NDM-1-producing *Enterobacteriaceae* isolates (group 1, $n = 5$; group 2, $n = 6$; group 3, $n = 5$) belonging to different *E. coli* phylogroups as well as one *K. pneumoniae* were conducted as previously described (46).

(iii) Whole-genome sequencing analysis. On the basis of the variability of the plasmid content detected with S1-PFGE analysis, the genomes of 25 strains, including 15 challenge strain reisolates [*S.* Corvallis, $n = 14$; *S.* Paratyphi B (D-Ta$^+$), $n = 1$] and 10 NDM-1-producing *Enterobacteriaceae* transconjugants, were sequenced. All strains selected for whole-genome sequencing are listed in Table 2.

Genomic DNA was extracted using a PureLink genomic DNA minikit (Invitrogen, USA), followed by fluorometric DNA concentration (in nanograms per microliter) measurement by Qubit fluorometric quantitation (Invitrogen). Sequencing libraries were prepared using a Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. Paired-end sequencing was performed on an Illumina MiSeq benchtop apparatus (MiSeq reagent [v3] 600 cycle kit; 2 × 300 cycles). Raw reads were assembled *de novo* using CLC Genomics Workbench (v9.5) software (Qiagen, Denmark), and STs, plasmid content, and resistance genes were detected using the services BatchUploader (47), PlasmidFinder (48), and ResFinder (49), available at the Center for Genomic Epidemiology (CGE; http://www.genomicepidemiology.org).

In order to evaluate microevolutionary changes in the *bla*$_{NDM-1}$-carrying pRH-1238 plasmid among *S.* Corvallis reisolates and to assess possible structural deletions in the plasmid backbone due to the transfer in *Enterobacteriaceae* transconjugants, assembled genomes were mapped against the reference sequence of pRH-1238 (size, 187,683 bp; GenBank accession number KR091911.1) using CLC Genomics Workbench (v9.5) software. The percent sequence identity was calculated on the basis of the size of the consensus sequence in reference to that of the sequence of pRH-1238, whereas their visualization was done using BLAST Ring Image Generator (v0.95; BRIG) software (50).

Statistical analysis of challenge strain excretion. For statistical analysis of challenge strain fecal excretion, SPSS21 (v2.0) software (SPSS Inc., USA) was used. Due to the nonnormal distribution of bacterial count excretion data, a log transformation was conducted. In experimental group 1 (simultaneous inoculation of donor and recipient strains), excretion of challenge strains was compared using the Wilcoxon matched-pairs test on the basis of the related data assumption, and comparisons between groups were conducted by the Mann-Whitney U test. Differences were considered significant if the *P* value was less than or equal to 0.05. Challenge strain excretion is graphically presented by box-whisker plots. The boxes indicate the medians (horizontal lines) and the lower and upper quartiles (lower and upper sides of the boxes, respectively). Outliers, which are values numerically distant from the rest of the data, were included for determination of the statistical significance.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.02128-17.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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I.S., J.F., and B.G. designed the *in vivo* experiments. B.M. supervised the project. I.S. and S.H. collected the samples and coordinated the *in vivo* study. S.H. performed the experiments, analyzed and interpreted the results, and wrote the draft manuscript. S.H., J.F., and M.B. analyzed the whole-genome sequencing data. B.M., B.G., A.K., and B.G.-Z. revised the draft version of the manuscript.

B. Guerra is currently employed with the European Food Safety Authority (EFSA) in its BIOCONTAM Unit, which provides scientific and administrative support to EFSA's scientific activities in the area of microbial risk assessment.

The positions and opinions presented in this article are those of the authors alone and are not intended to represent the views or scientific works of EFSA.

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TABLE S1. Phenotypic and genotypic properties of challenge strains

	Challenge strains	Origin/ Year of isolation	Plasmids	Phenotypic resistance profile (by MIC)a)	Genotypic resistance profile of pRH-1238	Genotypic resistance of others	Reference
Donor	NDM-1 producing *S*. Corvallis (12-SA01738)	Black kite (*Milvus migrans*) 2012	IncHI2 (~310 kb) IncA/C_2 pRH-1238 (187,683 bp) ColRNAI (< 20 kb)	AMP, CHL, CIP, FFN, FOT, KAN SMX, STR, TAZ, TET, TMP	*bla_{CMY-16}, bla_{NDM-1}, floR, accA4* [*aac(6´)-Ib*], *tet(A), drfA1/7/17, strA/B, sul1, sul2, aadA5, fosA3, aphA6, mphA*	*qnrS*	(1, 2)
Recipient	*S*. Paratyphi B (*d*Ta+) (13-SA01617)	Chicken meat 2013	none	CIP, NAL, STR, TMP	-	*aadA1, dfrA1*	-

[a] MIC carried out following CLSI guidelines, CLSI M07-A9 and using EUCAST epidemiological cut-off values (http://www.eucast.org)

Definition of abbreviations:

AMP (Ampicillin), CHL (Chloramphenicol), CIP (Ciprofloxacin), NAL (Nalidixic acid), FFN (Florfenicol), FOT (Cephotaxime),

KAN (Kanamycin), SMX (Sulphamethoxazole), STR (Streptomycin), TAZ (Cephtazidime), TET (Tetracycline), TMP (Trimethoprim)

TABLE S2. Average CTF rates based on filter mating conjugation experiments for donor and recipient strains

Donor	**Recipients**	**Strain no.**	**Conjugation transfer frequency (CTF) rate**		
			RT	37°C	42°C
NDM-1 producing *S*. Corvallis (12-SA01738)	*S*. Enteritidis [with 60 kb *pSEV* virulence plasmid (3)]	07-SA03428	$5{,}08 \times 10^{-5}$	$8{,}71 \times 10^{-3}$	$1{,}88 \times 10^{-2}$
	S. Enteritidis (without 60 kb *pSEV* virulence plasmid)	04-SA02117	$8{,}56 \times 10^{-5}$	$1{,}50 \times 10^{-2}$	$1{,}57 \times 10^{-2}$
	S. Infantis	14-SA03263	$6{,}64 \times 10^{-5}$	$9{,}04 \times 10^{-3}$	$1{,}10 \times 10^{-2}$
	S. Paratyphi B (*d*Ta+)	13-SA01617	$3{,}50 \times 10^{-4}$	$8{,}07 \times 10^{-3}$	$1{,}06 \times 10^{-2}$

TABLE S3. Antibiotic supplementation of selective plates for target microorganisms

Target microorganisms	**Medium**	**Antibiotic supplementation**		
		Cefotaxime (1 mg/l)	Meropenem (0.125 mg/l)	Nalidixic acid (50 mg/l)
S. Corvallis	XLD agar	+	+	-
S. Paratyphi B (*d*Ta+)	XLD agar	-	-	+
NDM-1-producing *S*. Paratyphi B (*d*Ta+)	XLD agar	+	+	+
Carbapenemase-producing Enterobacteriaceae (CPE)	chromID® CARBA agar	unknown	unknown	unknown

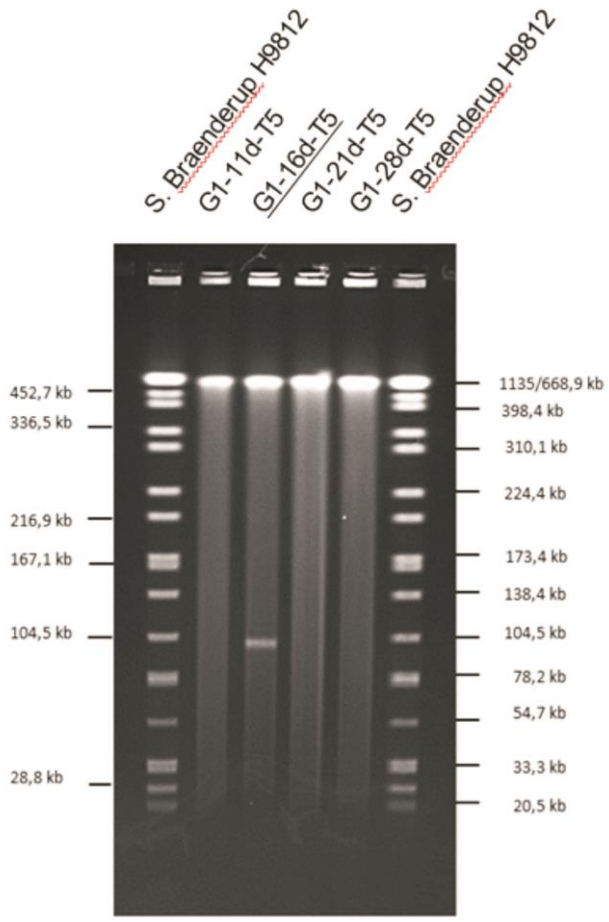

FIGURE S1. S1-PFGE analysis of *S*. Paratyphi B (*d*Ta+) reisolates from Group 1 in lanes 2-5 with molecular-sized standard, *Salmonella* Braenderup strain H9812 (restricted with XbaI) in lanes 1 and 6. Underlined strain selected for WGS.

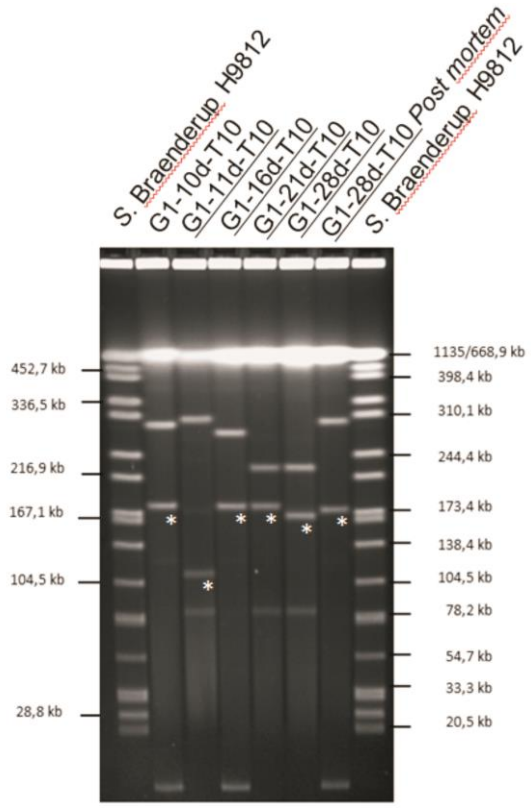

FIGURE S2. S1-PFGE analysis of *S*. Corvallis reisolates from Group 1 in lanes 2-7 with molecular-sized standard, *Salmonella* Braenderup strain H9812 (restricted with XbaI) in lanes 1 and 8. Asterix (*) marked $bla_{\text{NDM-1}}$ hybridisation. Underlined strains selected for WGS.

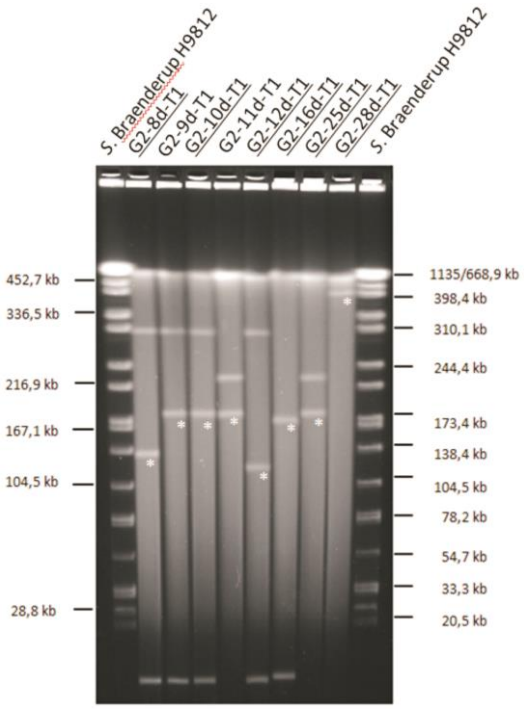

FIGURE S3. S1-PFGE analysis of *S*. Corvallis reisolates from Group 2 in lanes 2-9 with molecular-sized standard, *Salmonella* Braenderup strain H9812 (restricted with XbaI) in lanes 1 and 10. Asterix (*) marked $bla_{\text{NDM-1}}$ hybridisation. Underlined strains selected for WGS.

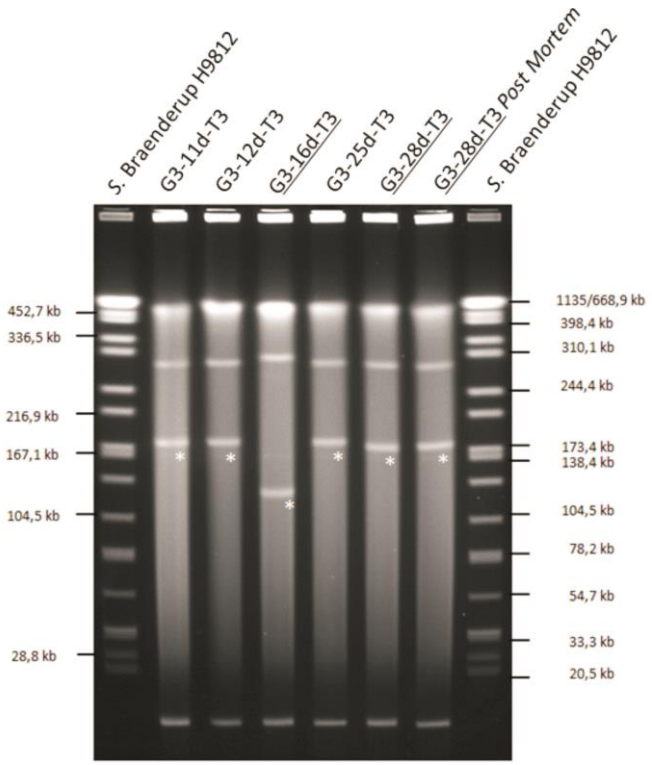

FIGURE S4. S1-PFGE analysis of *S*. Corvallis reisolates from Group 3 in lanes 2-7 with molecular-sized standard, *Salmonella* Braenderup strain H9812 (restricted with XbaI) in lanes 1 and 8. Asterix (*) marked bla_{NDM-1} hybridisation. Underlined strains selected for WGS.

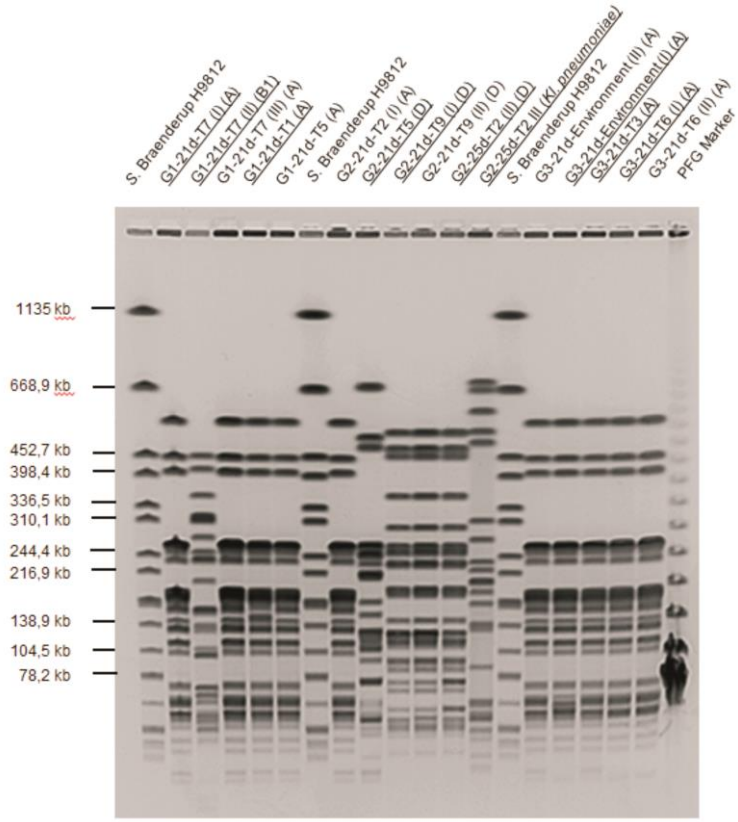

FIGURE S5. XbaI-PFGE analysis of NDM-1 producing Enterobacteriaceae transconjugants from Group 1 (lanes 2-6), Group 2 (lanes 8-13), Group 3 (15-19) with *E. coli* phylogroups (A, B1 and D) designation in brackets and one *K. pneumoniae* strain. Molecular-sized standard *Salmonella* Braenderup strain H9812 (restricted with XbaI) in lanes 1, 7, 14 and MidRange PFG Marker in lane 20. Underlined strains selected for WGS.

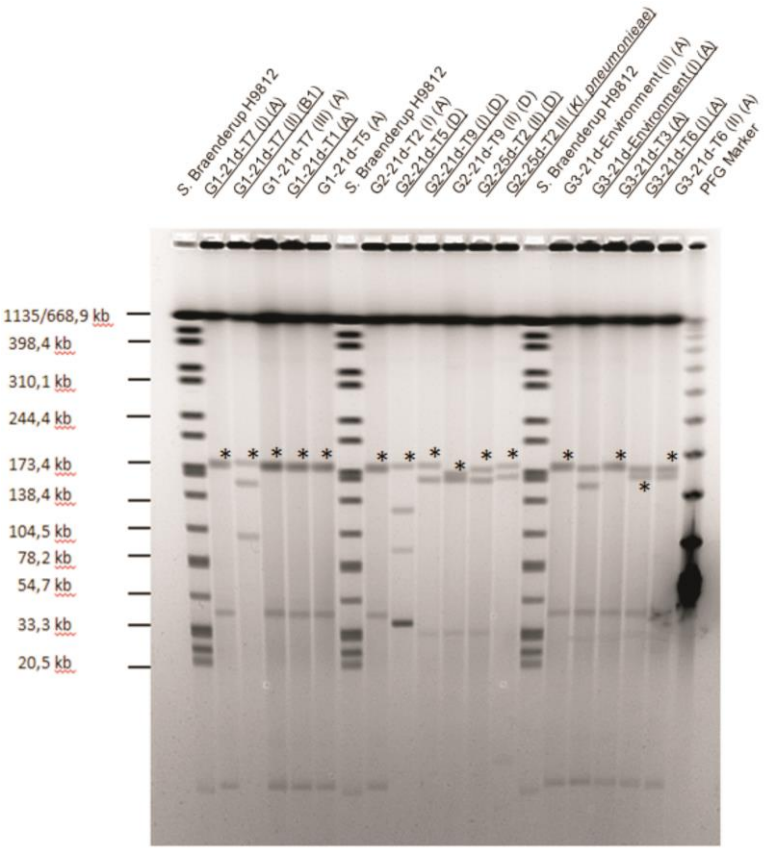

FIGURE S6. S1-PFGE analysis analysis of NDM-1 producing Enterobacteriaceae transconjugants from Group 1 (lanes 2-6), Group 2 (lanes 8-13), Group 3 (15-19) with *E. coli* phylogroups (A, B1 and D) designation in brackets and one *K. pneumoniae* strain. Molecular-sized standard *Salmonella* Braenderup strain H9812 (restricted with XbaI) in lanes 1, 7, 14 and MidRange PFG Marker in lane 20. Asterix (*) marked bla_{NDM-1} hybridisation. Underlined strains selected for WGS.

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2.2. Publication 2

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Own contribution to Publication 2:

For this publication, I performed an in-depth molecular analysis (PCR amplification, S1-PFGE restriction) and extracted the DNA for subsequent PacBio RSII long-read sequencing. I surveyed the literature and wrote the draft version of the manuscript.

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PROKARYOTES

Complete Genome Sequence of an Avian Native NDM-1-Producing *Salmonella enterica* subsp. *enterica* Serovar Corvallis Strain

Sead Hadziabdic,[a] Maria Borowiak,[a] Angelina Bloch,[a] Burkhard Malorny,[a] Istvan Szabo,[a] Beatriz Guerra,[b] Annemarie Kaesbohrer,[a] Jennie Fischer[a]

[a]Department for Biological Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany
[b]European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT Carbapenems are an important class of β-lactams and one of the last options for treating severe human infections. We present here the complete genome sequence of avian native carbapenemase-producing *Salmonella enterica* subsp. *enterica* serovar Corvallis strain 12-01738, harboring a bla_{NDM-1}-carrying IncA/C_2 plasmid, isolated in 2012 from a wild bird (*Milvus migrans*) in Germany.

Antimicrobial resistance in bacterial populations among food-producing animals presents an important concern for public health (1). Therefore, carbapenems are classified as "critically important" antimicrobials and one of the last options for treating severe human infections caused by multidrug-resistant bacteria (2). In recent years, carbapenemase-producing *Enterobacteriaceae* from different nonhuman matrices were sporadically detected in Germany (3–5). Through routine diagnostics in 2012, the German National Reference Laboratory for *Salmonella* received an avian native NDM-1-producing *Salmonella enterica* subsp. *enterica* serovar Corvallis strain (12-01738) harboring the bla_{NDM-1} gene on an ~180-kb IncA/C_2 plasmid (6, 7).

In recent *in vivo* infection studies, we demonstrated the persistence of this strain in broiler chickens and dissemination of its IncA/C_2 bla_{NDM-1}-carrying plasmid to different *Enterobacteriaceae*, both without antibiotic pressure (8). Such observations are noteworthy due to a possible scenario of bla_{NDM-1} introduction into commercial broiler production and downstream in the production chain, posing a subsequent risk for human exposure. In order to obtain the full-genome sequence, this *S.* Corvallis strain (12-01738) was submitted to PacBio RS II long-read sequencing.

DNA extraction using the PureLink genomic DNA minikit (Invitrogen, Carlsbad, CA, USA) was followed by PacBio RS II system-based genome sequencing (GATC Biotech AG, Constance, Germany). *De novo* genome assembly was performed using the SMRT Analysis software (version 2.3.0; Pacific Biosciences, USA).

Through additional whole-genome sequencing analysis by Illumina MiSeq technology, we demonstrated the presence of the bacterial chromosome (4,887,378 bp) and, as confirmed by S1-PFGE (S1–pulsed-field gel electrophoresis) plasmid profiling, the presence of three plasmids in this isolate (average coverage, 153.38-fold per consensus base).

Plasmid analysis using the tools available at the Center for Genomic Epidemiology (CGE; http://www.genomicepidemiology.org/) revealed the presence of an IncA/C_2 NDM-1-encoding plasmid (pSE12-01738-2; 177,190 bp), two additional plasmids of incompatibility group IncHI2 (sequence type 1 [ST-1]; pSE12-01738-1; 284,485 bp), and a ColE-like (ColRNAI) plasmid (pSE12-01738-3; 10,047 bp). The pSE12-01738-2 plasmid is a derivative of the bla_{NDM-1}-carrying IncA/C_2 multiresistance plasmid pRH-1238 (GenBank accession number KR091911), previously described by Villa et al. (7) but lacking a genetic element including genes for chromate and macrolide resistance as well as a

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Address correspondence to Jennie Fischer, Jennie.Fischer@bfr.bund.de.

class I integron carrying *dfrA7-aadA5-sul1* resistance gene cassettes. This, along with previously observed structural deletions in pRH-1238 after an *in vivo* passage, indicates certain evolutionary plasticity, still enabling maintenance of the bla_{NDM-1} gene (8).

CGE-based resistome analysis revealed that the IncHI2 plasmid pSE12-01738-1 harbors two resistance genes [*aac(6')Ib-cr* and *aacA4*], also located on the IncA/C_2 plasmid pSE12-01738-2, which itself harbors 16 resistance genes. The ColE-like (ColR-NAI) plasmid pSE12-01738-3 harbors only a *qnrS1* gene. The genome was annotated using the automated Prokaryotic Genome Annotation Pipeline (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/), where the presence of 5,177 coding sequences, 235 pseudogenes, and 123 RNA genes (22 rRNAs, 84 tRNAs, and 17 noncoding RNAs) was observed on the bacterial chromosome.

Accession number(s). These sequences were deposited in GenBank under the accession numbers CP027677 (chromosome), CP027678 (pSE12-01738-1), CP027679 (pSE12-01738-2), and CP027680 (pSE12-01738-3).

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B. Guerra is currently employed with the European Food Safety Authority (EFSA) in its BIOCONTAM Unit that provides scientific and administrative support to EFSA's scientific activities in the area of microbial risk assessment. The positions and opinions presented in this article are those of the authors alone and are not intended to represent the views or scientific works of EFSA.

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2.3. Publication 3 (including supplemental material)

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Own contribution to Publication 3:

In this study, I actively participated in the *in vivo* animal experiments. This included daily observation of the animals, sampling and laboratory analysis of the collected samples. I performed an in-depth molecular analysis of the strains which included S1-PFGE analysis for the visualization of plasmid content and alterations of the plasmids size. I conducted additional *in vitro* filter mating conjugation experiments. Additionally, I prepared the strains for Illumina Miseq WGS analysis. I surveyed the literature, analyzed, interpreted the data, wrote the draft version of the manuscript and revised it.

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MECHANISMS OF RESISTANCE

The bla_{NDM-1}-Carrying IncA/C_2 Plasmid Underlies Structural Alterations and Cointegrate Formation *In Vivo*

Sead Hadziabdic,[a] Jennie Fischer,[a] Maria Borowiak,[a] Burkhard Malorny,[a] Katharina Juraschek,[a] Annemarie Kaesbohrer,[a] Beatriz Guerra,[b] Carlus Deneke,[a] Bruno Gonzalez-Zorn,[c] Istvan Szabo[a]

[a]Department for Biological Safety, German Federal Institute for Risk Assessment (BfR), Berlin, Germany
[b]European Food Safety Authority, Parma, Italy
[c]Departamento de Sanidad Animal and Centro de Vigilancia Sanitaria Veterinaria, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain

ABSTRACT In 2012, a carbapenemase-producing *Salmonella enterica* serovar Corvallis isolate carrying a bla_{NDM-1} multiresistance IncA/C_2 plasmid, apart from IncHI2 and ColE-like plasmids, was detected in a wild bird in Germany. In a recent broiler chicken infection study, we observed transfer of this bla_{NDM-1}-carrying IncA/C_2 plasmid to other *Enterobacteriaceae*. Here, we focused on the stability of this plasmid and gained insight into the type and frequency of its structural alterations after an *in vivo* passage in a broiler chicken infection study.

KEYWORDS NDM-1 carbapenemases, *Salmonella*, broiler chicken infection study

Antimicrobial resistance is described as the most urgent threat to global public health and food safety today (1). In a previous broiler chicken infection study, we demonstrated that the multidrug-resistance bla_{NDM-1}-carrying IncA/C_2 plasmid (pRH-1238) is transferable, without antibiotic pressure, to intestinal *Escherichia coli* strains and a *Klebsiella pneumoniae* strain (2). In this *in vivo* study, we aimed to investigate and understand the dynamic of structural alterations in the bla_{NDM-1}-carrying IncA/C_2 plasmid pSE12-01783-2 with *Salmonella enterica* serovar Corvallis as host. We evaluated if these alterations are sporadic or frequently occurring events and whether they influence further *in vitro* transfer. With the use of Illumina and Nanopore whole-genome sequencing (WGS) analysis, we aimed to detect structural alterations occurring in a bla_{NDM-1}-carrying pSE12-01783-2 plasmid and reveal the full structure of the cointegrated bla_{NDM-1}-carrying IncHI2-IncA/C_2 megaplasmid.

Broiler chicken infection study. Each experimental group (G1 to G4), consisting of 10 1-day-old Ross 308 broiler chicks (T1 to T10), was placed separately in the Facilities for Animal Experimentation at the German Federal Institute for Risk Assessment. The experimental design is shown in Fig. 1. Challenge strains were inoculated orally, with inoculum containing ~5×10^6 CFU of the respective challenge strain in 100 μl. Animal experiments were approved by the German State Authority for Health and Social Affairs (Lageso) (no. 0308/15).

Donor and recipient strains. As a model strain, an avian native *Salmonella* Corvallis (GenBank accession number CP027677) strain carrying three plasmids, 284,485-bp IncHI2 (pSE12-01738-1, CP027678), multiresistance NDM-1-encoding 177,190-bp IncA/C_2 (pSE12-01738-2, CP027679), and 10,047-bp ColE-like ColRNAI (pSE12-01738-3, CP027680), was selected (3). As potential recipients of the bla_{NDM-1}-carrying pSE12-01738-2 plasmid, nalidixic acid (NAL)-resistant avian native *Salmonella enterica* serovar Paratyphi B (*d*Ta+) (13-01617), *Salmonella enterica* serovar Enteritidis (07-03428), and *Salmonella enterica* serovar Infantis (14-03263) were selected. This investigation describes structural alterations and not transfer of the bla_{NDM-1}-carrying IncA/C_2 pSE12-01738-2 plasmid *in vivo*. For the study of genome and plasmid content alteration

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Address correspondence to Istvan Szabo, istvan.szabo@bfr.bund.de.

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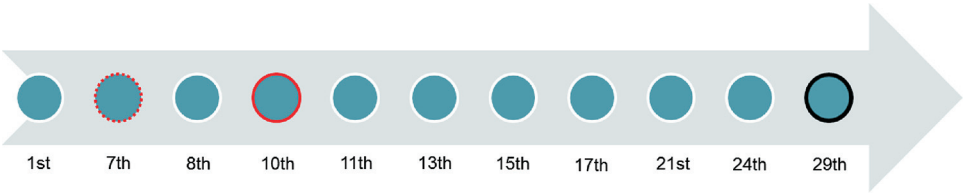

FIG 1 Experimental design containing sampling days (blue circles); recipient [*S.* Paratyphi B (*d*Ta+), *S.* Enteritidis, and *S.* Infantis] inoculation in groups 2, 3, and 4 (red dotted outer ring) on 7th day of life; and donor (*S.* Corvallis) inoculation in groups 1 to 4 (red solid outer ring) on 10th day of life. The black outer ring indicates the end of the experiment with cecum removal postmortem at the 29th day of life.

among *S.* Corvallis isolates, reisolates from three chicks belonging to four groups were selected. The selection of strains is shown in Table 1. To evaluate the *in vivo* stability of the plasmid content without selective pressure in isolation, 7 *S.* Corvallis reisolates detected on xylose lysine deoxycholate (XLD) agar from different chicks of group 1 were included. These are shown in Fig. S2 in the supplemental material. In total, 97 *S.* Corvallis reisolates were characterized in depth for the purpose of this investigation.

Isolation of *S.* Corvallis reisolates. *S.* Corvallis reisolates were isolated by suspending fresh fecal droppings from each chick in 4.5 ml of 0.85% (wt/vol) NaCl, from which a 100-μl deposition volume was plated in duplicates onto XLD plates (Thermo Fisher Scientific, Germany) supplemented with 1 mg/liter cefotaxime (CTX) and 0.125 mg/liter meropenem (MEM). The addition of cefotaxime as a second antibiotic was to inhibit overgrowth by *Pseudomonas* spp. with intrinsic carbapenem resistance, which could hamper detection and quantification of the donor strain. Seven *S.* Corvallis strains from group 1 were isolated by plating onto XLD without selective supplementation. Strains were preserved at −80°C for later molecular analysis. Prior to molecular analysis, strains were serotyped. The designation is based on group (G1 to G4), day of isolation (1st to 29th day of life), and chick identifier (ID) (T1 to T10).

S1-PFGE plasmid profiling and *bla*$_{NDM-1}$ hybridization of *S.* Corvallis reisolates. All 97 reisolates of *S.* Corvallis were subjected to S1 pulsed-field gel electrophoresis (PFGE). Generated fragments were separated by the CHEF-DRIII system (Bio-Rad Laboratories, Spain) under running conditions as previously described (4). The S1-PFGE gels

TABLE 1 Distribution of 90 *S.* Corvallis strains which were selected for in-depth molecular analysis, including the strains isolated from the cecal content (29d*)[a]

Group/s	ID	Sampling days							
		11d	13d	15d	17d	21d	24d	29d	29d*
Group 1	T1								
	T5								
	T9					n.a	n.a		
Group 2	T1	n.a							
	T4		n.a						
	T7	n.a							
Group 3	T3								
	T7								
	T9								
Group 4	T1								
	T5								
	T7							n.a	

	Strains available for an in-depth molecular analysis
n.a	Strain not available for the analysis
	Strains selected for WGS SNP analysis
*	*Post mortem* cecal content isolates

[a]Darker gray, strains selected for in-depth molecular analysis (all isolates, except n.a.); lighter gray, strains selected for WGS SNP analysis (see "Whole-genome SNP analysis" paragraph below). n.a, strain not available for analysis; *, postmortem cecal content isolates.

of strains from group 1 and group 2 were further used for Southern blotting and bla_{NDM-1} hybridization.

***In vitro* conjugation experiments.** Filter mating conjugation experiments with *S.* Corvallis strains harboring pSE12-01738-2 variants (D1, D2, D3, and D4) from the *in vivo* trial were conducted. Plasmid profiles of these are shown in Fig. S1 in the supplemental material. As recipients, nalidixic acid-resistant *S.* Paratyphi B (*d*Ta+), *S.* Enteritidis, and *S.* Infantis were selected. From overnight cultures of selected strains, 500 μl was inoculated into 25 ml (1:50) of Luria Bertani Bouillon-Miller liquid (LBL) and grown aerobically at 37°C with shaking (200 rpm), until optical density at 600 nm (OD_{600}) reached a value of 0.2. Afterward, donor and recipient strains were mixed 1:2 (100 μl: 200 μl) and centrifuged (16,000 rpm for 5 min). Here, 200 μl of supernatant was discarded and the pellet was resuspended in the remaining 100 μl and plated on an 0.22-μm-pore-size mixed-cellulose ester membrane filter (Merck Millipore, Germany) placed on LB agar (Thermo Fisher Scientific, Germany). Conjugation experiments lasted 4 h and were repeated at room temperature (RT), 37°C, and 41.5°C. NDM-1-producing *Salmonella* transconjugants were selected on XLD containing 1 mg/liter CTX, 0.125 mg/liter MEM, and 50 mg/liter nalidixic acid (NAL) and confirmed by serotyping, and conjugal transfer frequency (CTF) was calculated per donor.

WGS analysis. The whole-genome sequencing (WGS) analysis was conducted with Illumina MiSeq technology. Strains were grown overnight at 37°C in 4 ml of LBL with 1 mg/liter CTX, from which 1 ml was processed for DNA extraction using the PureLink genomic DNA minikit (Invitrogen, USA). DNA concentration (ng/μl) was measured with the Qubit fluorometric quantitation (Invitrogen) system. Sequencing libraries were prepared with the Nextera XT DNA sample preparation kit (Illumina, San Diego, CA, USA). Paired-end sequencing was performed with the Illumina MiSeq benchtop (MiSeq Reagent v3 600-cycle kit, 2 $\times$ 251 cycles). Raw reads were assembled *de novo* using CLC Genomics Workbench 9.5.2 (Qiagen, Hilden, Germany), and sequence types (STs), plasmid types, and resistance genes were detected using BatchUpload (5). Comparison of the pSE12-01738-2 variants (D3 and D4) was performed by mapping the raw reads to the reference pSE12-01738-2 plasmid (GenBank accession number CP027679) and visualizing them using BRIG (6).

Oxford Nanopore MinION sequencing. The *S.* Corvallis reisolate G2-21d-T4 (D2) carrying an ~450-kb cointegrate of pSE12-01738-1 IncHI2 and pSE12-01738-2 IncA/C_2 plasmid was sequenced with MinION technology. The sequencing library was prepared from genomic DNA using the Rapid Sequencing kit (Oxford Nanopore Technologies, Oxford, United Kingdom) and sequenced for approximately 16 h using the Flow-MIN106 R9 flow cell.

For genome assembly, the hybrid assembly software Unicycler (v0.4.4) was used (7). It starts from an initial SPAdes short-read assembly and simplifies the assembly using information from short and long reads, thereby achieving a complete and accurate assembly (8). Assemblies were polished using Pilon (9). The cointegrated megaplasmid is represented using CLC Genomics Workbench 9.5.2.

Whole-genome SNP analysis. Reisolates from chicks T1 ($n = 8$) and T5 ($n = 8$) in group 1 were selected. Three *S.* Corvallis reisolates ($n = 3$) carrying variants of pSE12-01738-2 (D2, D3, and D4) were also included (Table 1). Single-nucleotide polymorphism (SNP) analysis was performed using BioNumerics 7.6 (Applied Maths, Ghent, Belgium). Sequencing raw data were trimmed and mapped against the reference chromosome of *S.* Corvallis 12-01738 (GenBank accession number CP027677). To reconfirm SNP position in encoding genes, trimmed reads were mapped also to the annotated reference chromosome of *S.* Corvallis.

Statistical analysis. For comparison of CTFs among donors (D1 to D4) under different temperature conditions (room temperature [RT], 37°C, and 41.5°C), statistical analysis with SPSS (ver. 21.0; SPSS Inc., USA) was performed. The distribution of the CTFs is presented by box-whisker plots with outliers and extreme outliers included. For the determination of statistical significance, one-way analysis of variance (ANOVA) was

TABLE 2 Overview of plasmid content based on S1-PFGE for 90 selected *S.* Corvallis reisolates in groups 1 to 4, indicating complete plasmid loss and strains carrying pSE12-01738-2 variants[a]

Group/s	Chick ID	Days of life																								
		11d			13d			15d			17d			21d			24d			29d			*Post mortem*		
		IncHI2	IncA/C_2	ColRNAI	IncHI2	IncA/C_2	ColRNAI	IncHI2	IncA/C_2	ColRNAI	IncHI2	IncA/C_2	ColRNAI	IncHI2	IncA/C_2	ColRNAI	IncHI2	IncA/C_2	ColRNAI	IncHI2	IncA/C_2	ColRNAI	IncHI2	IncA/C_2	ColRNAI
G1	T1				■						■						■			■			■		
	T5	■						■						■											
	T9													n.a			n.a			■			■		
G2	T1	n.a															■			■					
	T4				n.a									D2											
	T7	n.a																							
G3	T3	■												■			■			■			■		
	T7																						■		
	T9																■			■			■	D3	
G4	T1							■									■			■			■		
	T5													■						■			■		
	T7								D4											n.a					

■ plasmid present
■ plasmid absent
n.a strain not available for the analysis
D2 ~450 kb IncHI2-IncA/C_2 co-integrated megaplasmid
D3 ~100 kb IncA/C_2 plasmid
D4 ~160 kb IncA/C_2 plasmid

[a]Light gray shading, plasmid present; black shading, plasmid absent; n.a, strain not available for the analysis; D2, ~450-kb IncHI2-IncA/C_2 cointegrated megaplasmid; D3, ~100-kb IncA/C_2 plasmid; D4, ~160-kb IncA/C_2 plasmid.

performed and least significant difference (LSD) was used as a *post hoc* test. The differences were considered significant if the *P* value was <0.05.

Plasmid contents of 90 *S.* Corvallis reisolates detected after selective isolation are shown in Table 2, whereas seven *S.* Corvallis reisolates detected without selective isolation are shown in Fig. S2 in the supplemental material. Variation in plasmid content was mainly seen in the loss of the IncHI2 plasmid (31 from 97 selected reisolates). Two reisolates harbored, besides the <10-kb ColE-like (ColRNAI) plasmid, an ~450-kb megaplasmid (G2-21d-T4 [further marked as D2]) and G1-13d-T9 (shown in Fig. S2 but not included in further *in vitro* analysis). In relation to the bla_{NDM-1}-carrying IncA/C_2 pSE12-01738-2 plasmid, ~70-kb (G3-29d-T9 postmortem [D3]) and ~10-kb (G4-15d-T7 [D4]) deletions were observed.

Figure 2 shows distributions of *in vitro* CTF rates for donors (D1 to D4) under different temperature conditions in relation to three *Salmonella* recipients. Among conjugative pSE12-01738-2 variants, the CTFs were highest at 41.5°C. The ~100-kb pSE12-01738-2 variant D3 was not transferable (Fig. 2). The donor with the ~450-kb IncHI2-IncA/C_2 cointegrate (D2) had a statistically significant lower CTF, in contrast to D1 with unaltered pSE12-01738-2 and D4 with an ~160-kb pSE12-01738-2 plasmid under all three temperature conditions (Fig. 2; see also Table S1).

Following *in vitro* experiments, we observed variation in colony size and prolonged growth of *Salmonella* transconjugants after conjugation with *S.* Corvallis carrying the ~450-kb cointegrated megaplasmid. Therefore, 24 of these transconjugants (four small and four large colonies per recipient) from conjugation experiments at 41.5°C were analyzed by S1-PFGE. Analysis revealed that the size of the colony is not linked to full ~450-kb cointegrate acquisition. Additionally, resolution of the cointegrate (plasmids from ~170 to ~350 kb in size) in transconjugants was observed (data not shown).

The WGS analysis revealed that pSE12-01738-2 variant D3 has a consensus sequence size of 117,289 bp and the pSE12-01738-2 variant D4 has a size of 172,146 bp, in

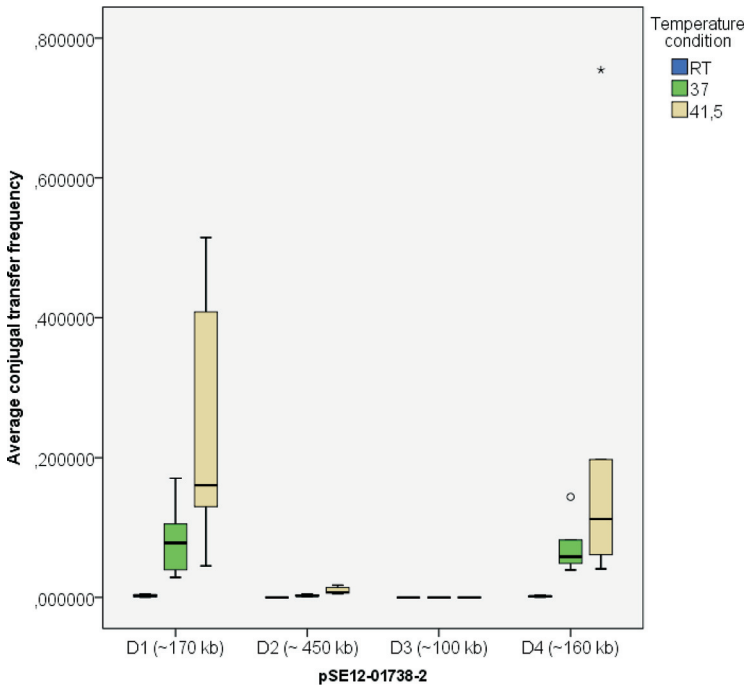

FIG 2 Distribution of the CTF rates for donors (D1 to D4) carrying pSE12-01738-2 variants in relation to three *Salmonella* recipients under different temperature conditions (room temperature [RT], 37°C, and 41.5°C) with outliers (°) and extreme outliers (*) included.

contrast to the 177,190-bp pSE12-01738-2 reference plasmid (GenBank accession number CP027679) (Fig. 3). The D2 variant of the pSE12-01739-2 plasmid is a cointegrated megaplasmid (462,435 bp) of pSE12-01738-1 IncHI2 and pSE12-01738-2 IncA/C_2 (Fig. 4). In three (G1-11d-T1 [position 978935, G→A], G1-29d-T5 [position 2226323, A→G], and G1-29d*-T5 [position 4307016, A→T]) out of 16 selected *S.* Corvallis reisolates from group 1, nonsynonymous polymorphic nucleotide exchanges (SNPs) were observed. These were attributed to genes encoding citrate lyase subunit alpha (G1-11d-T1), dihydroxy-acid dehydratase (G1-29d-T5), and galactitol-1-phosphate 5-dehydrogenase (G1-29d*-T5). In *S.* Corvallis strains harboring variants of the pSE12-01738-2 plasmid, SNPs were not detected.

Structural alterations of the bla_{NDM-1}-carrying pSE12-01738-2 plasmid were seen in ~10-kb and ~70-kb deletion and ~450-kb megaplasmid formation. The ~450-kb megaplasmid (462,435 bp) is a cointegrate of IncHI2 (pSE12-01738-1) and the multiresistance bla_{NDM-1}-carrying IncA/C_2 (pSE12-01738-2) plasmid (Fig. 4) and was detected in 2 out of 97 strains. The fusion was mediated by IS*6*-like family genetic elements. In a study of movement of IS*26*, which can be identical to IS*6,* it was observed that IS*26* can form cointegrates between DNA molecules (10). Other studies have shown plasticity of IncHI2 and fusion with IncF plasmids (11, 12). A fusion event can potentially facilitate dissemination of other genetic elements, such as heavy metal resistance in the case of tellurite (*Ter* cluster) present in pSE12-01738-1 (Fig. 4). A study by Lin et al. (13) revealed that spread of the $bla_{CTX-M-17}$ gene present on a nonconjugative plasmid was due to fusion with a conjugative ~73-kb plasmid. As our IncHI2-IncA/C_2 cointegrate was detected in only two reisolates, we assume that such an *S.* Corvallis population persists *in vivo* but in lower numbers. This could be due to instability of the cointegrate, supported by our *in vitro* conjugation experiments where resolution of the IncHI2-IncA/C_2 cointegrate was observed. Plasmid resolution was observed by Xie et al. (14) in the case of the ~190-kb cointegrated multireplicon bla_{NDM-5} plasmid, suggesting plasmid instability in new recipients or during conjugation. Besides instability and decreased CTF effect, our cointegrate acquisition caused an elongated growth time for *Salmonella* recipients and variation of the colony size *in vitro*. In

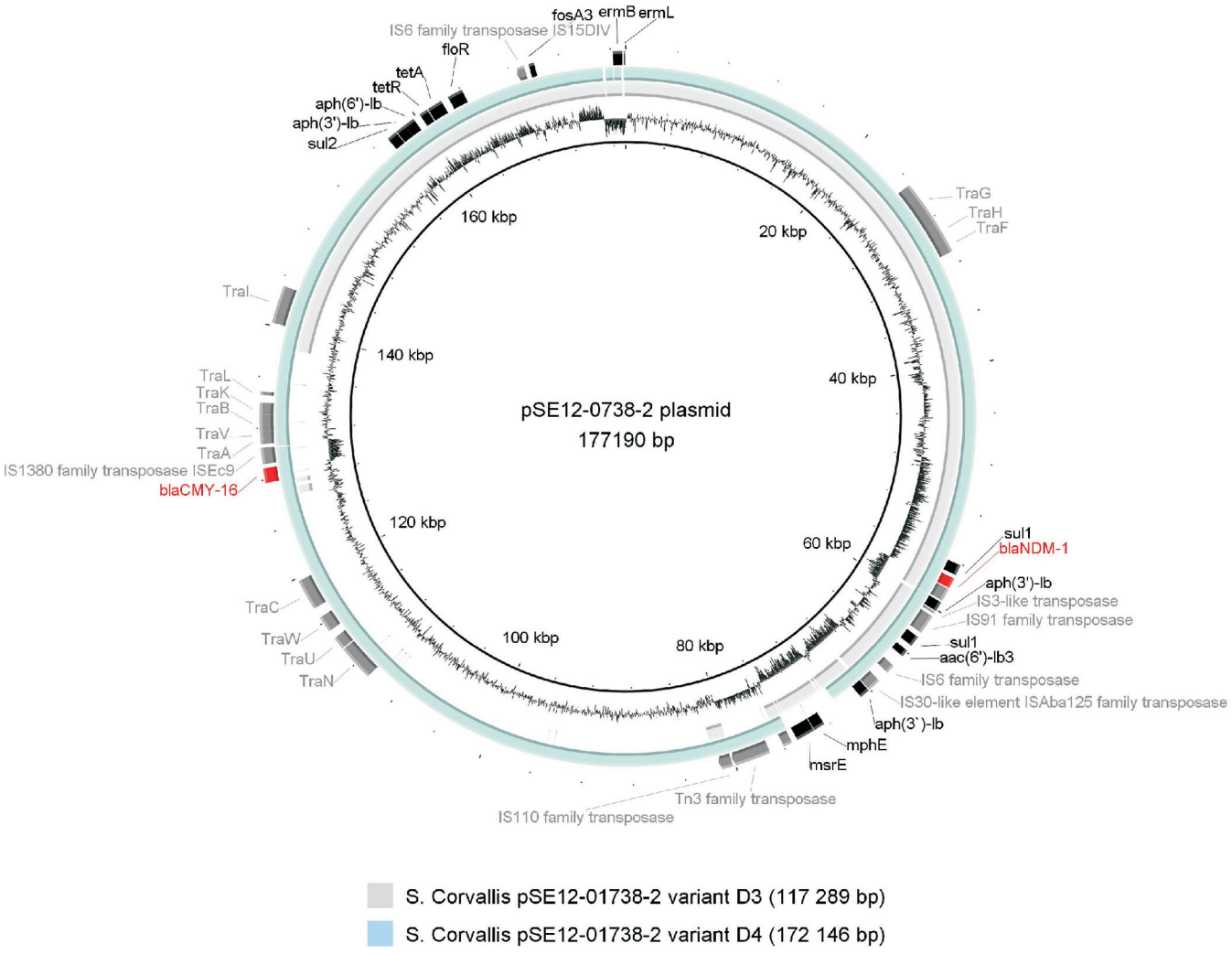

FIG 3 Visualization of the bla_{NDM-1}-carrying pSE12-01738-2 variants D3 and D4 compared to PacBio RSII reference sequence of pSE12-01738-2 plasmid (GenBank accession number CP027679) using BRIG (6) with resistance genes (red, beta-lactam genes; black, other resistance genes) as well as IS elements, transposase, and *tra* genes (all marked gray).

a study on the transmission and burden of an ~1-Mb *Pseudomonas syringae* megaplasmid, pMPP1a107, a decrease in fitness was also observed (15). In another study, it was observed that the same plasmid can have up to 2.5-fold-higher fitness costs in different *Pseudomonas* species (16).

Recently, Paskova et al. (17) detected a bla_{NDM-1}-carrying ~300-kb multireplicon (IncA/C_2 and IncR) plasmid in an *E. coli* strain from human urine. The type I IncA/C_2 sequence part of this megaplasmid was 99% identical to pRH-1238, which is the same plasmid as pSE12-01738-2, with only a minor structural deletion in the latter (3). These findings confirmed our hypothesis of the broad host range and adaptation potential of this particular bla_{NDM-1}-carrying plasmid *in vivo*. This also suggests possible bla_{NDM-1} spillover from human clinical settings where carbapenems are an alternative to cephalosporin in cases of resistance (18).

We observed frequent loss of the IncHI2 pSE12-01738-1 plasmid, despite genes associated with the toxin-antitoxin system being present. Plasmids are undergoing selection pressure, and to control costs and maximize their spread, the host adapts strategies to cope with their presence (19). The cost of the pSE12-01738-1 plasmid might have outweighed the benefits for the host, leading to the plasmid loss (20, 21). Structural alterations were more common in IncA/C_2 pSE12-01738-2 than in the pSE12-01738-1 and pSE12-01738-3 plasmids. These were seen in two deletion events of

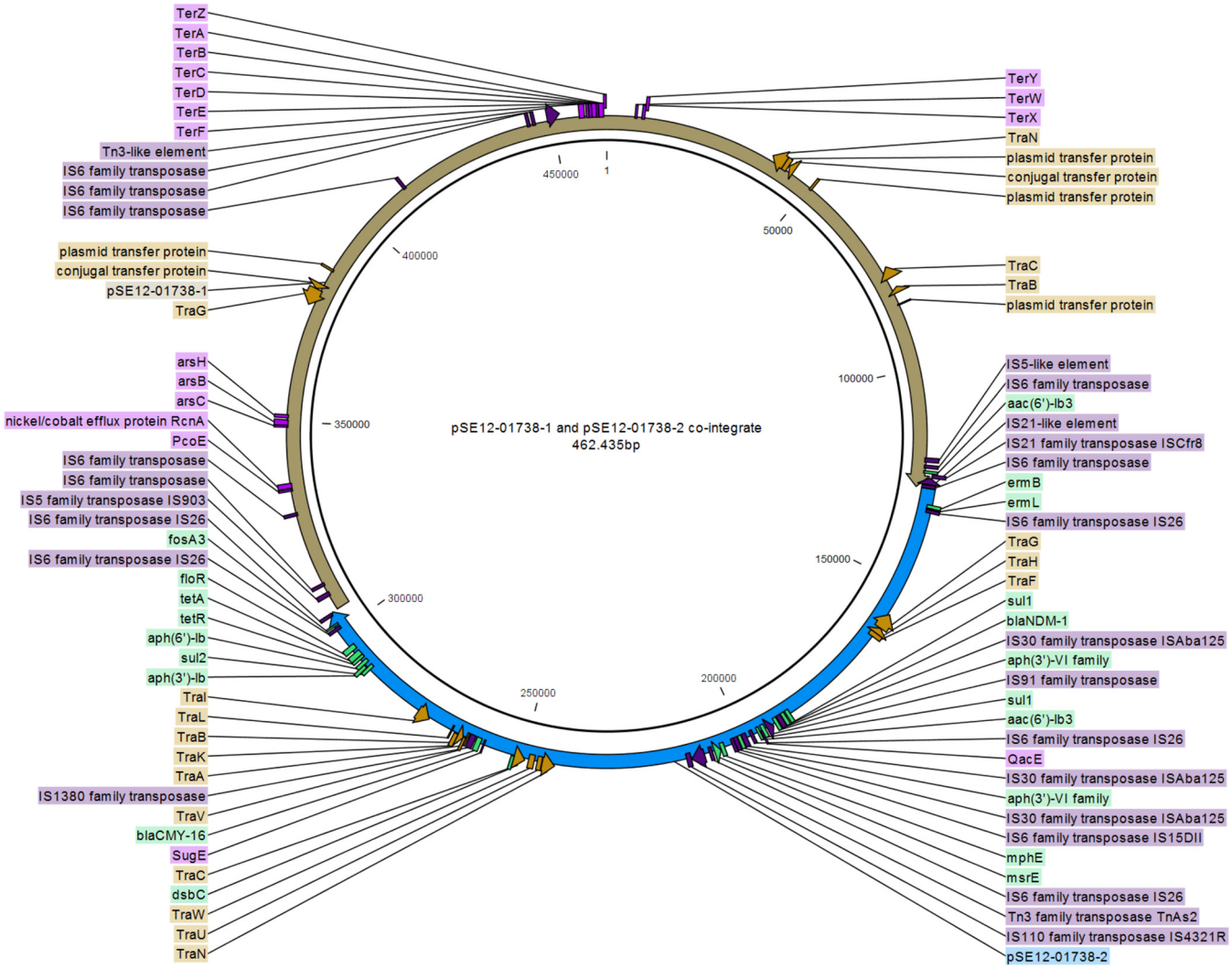

FIG 4 Structure of newly emerged IncHI2 pSE12-01738-1 and IncA/C_2 pSE12-01738-2 cointegrated megaplasmid (462,435 bp). The fusion of IncHI2 pSE12-01738-1 (brown) and IncA/C_2 pSE12-01738-2 plasmid (blue) is shown. Resistance genes are marked green, heavy metal resistance genes are pink, transposase and IS elements are purple, and transfer (*tra*) genes are brown.

the pSE12-01738-2 plasmid. The first, smaller deletion (~10 kb) covers IS*6* family transposase-flanked macrolide resistance genes (*mphE* and *msrE*), and a larger deletion (~70 kb) included two *tra* clusters (*traL-traK-traB-traV-traA* and *traC-traW-traU-traN*) (Fig. 3). As *tra* genes are required for pilus assembly (*traW*), the structure of pilus (*traC*), and mating pair stabilization (*traN*) (22–24), the absence of some *tra* genes led to loss of the conjugation machinery in this pSE12-01738-2 variant (D3) (Fig. 2 and 3). The remaining *traG*, *traH*, *traF*, and *traI* genes did not maintain conjugation ability for this plasmid derivative *in vitro*.

Antimicrobial usage is the most common trigger for the spread of antimicrobial resistance (25); however, reducing antibiotic use alone is not sufficient to reverse resistance (26). Eliminating antimicrobial selection pressure alone does not lead to plasmid loss in all plasmid-host combinations (27). This was observed in our *in vivo* study. Therefore, insights into mechanisms which trigger and enhance plasmid loss might be an effective addition to support current knowledge as future intervention measures.

Our study revealed the most common structural alterations of a public-health-relevant bla_{NDM-1}-carrying IncA/C_2 plasmid once carried with *S.* Corvallis into a broiler flock. Despite structural alterations and plasmid cointegration, the bla_{NDM-1} gene is

maintained in different IncA/C_2 variants. For the future, synergy of reduction in antimicrobial usage and alternative approaches, such as promoting plasmid loss, might be an additional contribution aiming to slow the spread of resistance.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.00380-19.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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Beatriz Guerra is currently employed with the European Food Safety Authority (EFSA) in its BIOCONTAM Unit, which provides scientific and administrative support to EFSA's scientific activities in the area of Microbial Risk Assessment. The positions and opinions presented in this article are those of the authors alone and are not intended to represent the views or scientific works of EFSA.

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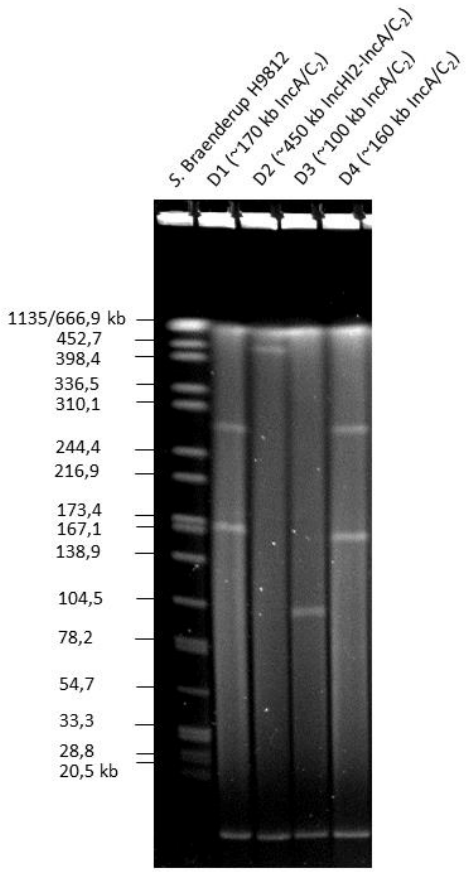

FIGURE S1. The S1-PFGE gel of *S*. Corvallis re-isolates selected as donors (D1-D4) for *in vitro* conjugation experiments. In lane 2 *S*. Corvallis (D1) with unaltered plasmid content. In lanes 3-5 variants of the IncA/C_2 pSE12-01738-2 plasmid; D2 (~450 kb IncA/C_2 pSE12-01738-2 co-integrate), D3 (~100 kb IncA/C_2 pSE12-01738-2) and D4 (~160 kb IncA/C_2 pSE12-01738-2 plasmid). As size marker in lane 1, *S*. Braenderup H9812 (restricted with XbaI).

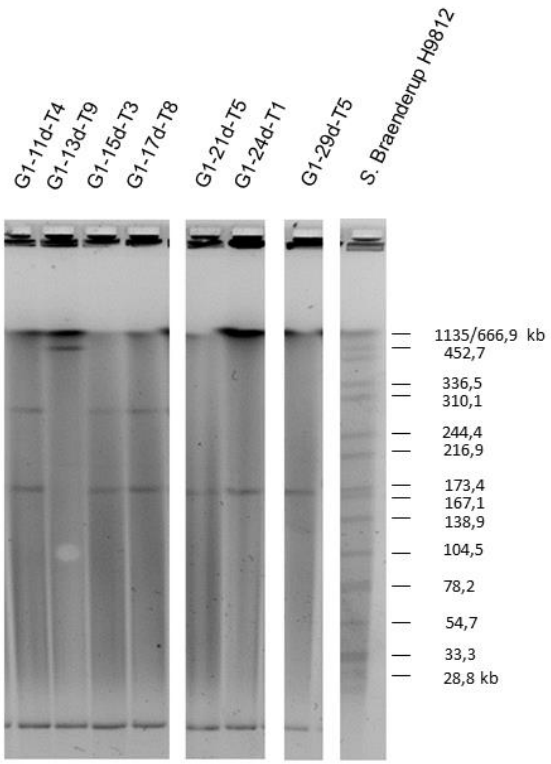

FIGURE S2. S1-PFGE gel of 7 *S*. Corvallis re-isolates from Group 1 (G1) detected on XLD. As size marker in lane 8, *S*. Braenderup H9812 (restricted with XbaI).

TABLE S1. Calculated P-values of CTFs among pSE12-01738-2 variants (D1-D4) based on one-way analysis of variance (ANOVA) and LSD as Post-hoc-Test

	Room temperature (RT)				37°C				41,5°C			
	D1	D2	D3	D4	D1	D2	D3	D4	D1	D2	D3	D4
D1		0,001	0,000	0,158		0,000	0,000	0,537		0,026	0,021	0,806
D2	0,001		0,955	0,015	0,000		0,886	0,001	0,026		0,921	0,044
D3	0,000	0,955		0,013	0,000	0,886		0,001	0,021	0,921		0,036
D4	0,158	0,015	0,013		0,537	0,001	0,001		0,806	0,044	0,036	

2.4. Unpublished material

As unpublished material of the doctoral thesis, *in vivo* acquisition of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ (from experiment 2) and *bla*$_{VIM-1}$-carrying IncHI2 (from experiment 3) plasmid in *S.* Paratyphi B (*d*T+) and *S.* Infantis recipient strains was investigated. Additionally, structural alterations of the *bla*$_{VIM-1}$-carrying IncHI2 plasmid (from experiment 3) *in vivo* were presented.

2.4.1. Acquisition of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ and *bla*$_{VIM-1}$-carrying IncHI2 plasmids in *S*. Paratyphi B (*d*T+) and *S*. Infantis

Another aim of the conducted broiler chicken infection experiments was to investigate potential acquisition of the MDR-encoding *bla*$_{NDM-1}$-carrying IncA/C$_2$ and *bla*$_{VIM-1}$-carrying IncHI2 plasmids in *Salmonella* recipients. The inoculation of *Salmonella* recipients followed orally on 7th day of life, followed by NDM-1-producing *S*. Corvallis and VIM-1-producing *S*. Infantis donor strains inoculation of 10th day of life (experiment 2 and 3). Due to inconclusive results in the experimental group where *S*. Enteritidis was used as recipient, only transfer of these two plasmids to *S*. Paratyphi B (*d*T+) and *S*. Infantis recipients was analysed further. For the detection of carbapenemase-producing *S*. Paratyphi B (*d*T+) and *S*. Infantis transconjugants, fresh fecal droppings of each chick were suspended in 4.5 ml of 0.85% (w/v) NaCl. From this suspension, 500 µl was further processed for detection of *Salmonella* transconjugants. To prevent potential *in vitro* conjugation this volume was kept on ice and to concentrate potentially low abundant transconjugants, centrifugation (16,000 x g for 5 min) followed. After centrifugation, 300 µl was discarded and pellet was resuspended in remaining 200 µl and plated on two XLD plates (100 µl each) supplemented with 1 mg/L cefotaxime (CTX), 0.125 mg/L meropenem (MEM) and 50 mg/L nalidixic acid (NAL). The XLD plates were incubated for 24 hours at 37°C. The *Salmonella* strains which grew on XLD plates supplemented with CTX, MEM and NAL were serotyped and preserved in cryoprotective LB (Lysogeny Broth) supplemented with 1 mg/L CTX at -80°C for further in-depth molecular analysis. Further one, the *Salmonella* transconjugants were submitted to S1-PFGE plasmid profiling for the confirmation of plasmid acquisition and insight into structural alterations of acquired plasmids as described in **publication 3** (241).

In broiler chicken experiment 3, where VIM-1-producing *S.* Infantis was used as donor strain, acquisition of the MDR-encoding *bla*$_{VIM-1}$-carrying IncHI2 plasmid in selected *Salmonella* recipients and native enterobacteria was not detected. However, in animal experiment 2, where NDM-1-producing *S.* Corvallis was used as donor strain, in three animals *S.* Paratyphi B (*d*T+) strains and in ten animals *S.* Infantis with *bla*$_{NDM-1}$-carrying

IncA/C_2 plasmid were detected. All of these 13 *Salmonella* transconjugants were selected for the S1-PFGE plasmid profiling analysis (Table 3). Based on the S1-PFGE, in one *S*. Paratyphi B (*d*T+) transconjugant strain (G2-24d-T4) a smaller (~10 kb) deletion and in one *S*. Infantis transconjugant strain (G4-29d-T5) a larger (~50 kb) structural deletion of the bla_{NDM-1}-carrying IncA/C_2 plasmid was observed (Table 3).

Table 3. Plasmid content and size of plasmids for the NDM-1-producing *S*. Paratyphi B (*d*T+) and *S*. Infantis transconjugants.

Strain designation	**Serovar (sequence type, STs)**	**Plasmid size (in kb)**	**bla_{NDM-1} and bla_{CMY-16} genes present**	**Additional plasmids**
G2-13d-T7	Paratyphi B (*d*T+) (ST28)	~170	yes	no
G2-15d-T1	Paratyphi B (*d*T+) (ST28)	~170	yes	no
G2-24d-T4	Paratyphi B (*d*T+) (ST28)	~160	yes	no
G4-13d-T8	Infantis (ST32)	~170	yes	no
G4-13d-T9	Infantis (ST32)	~170	yes	no
G4-15d-T9	Infantis (ST32)	~170	yes	no
G4-17d-T5	Infantis (ST32)	~170	yes	no
G4-17d-T10	Infantis (ST32)	~170	yes	no
G4-21d-T5	Infantis (ST32)	~170	yes	no
G4-24d-T4	Infantis (ST32)	~170	yes	no
G4-24d-T8	Infantis (ST32)	~170	yes	no
G4-29d-T5	Infantis (ST32)	~120	yes	no
G4-29d-T9	Infantis (ST32)	~170	yes	no

The *S*. Paratyphi B (*d*T+) (G2-24d-T4) and *S*. Infantis (G4-29d-T5) transconjugant strains with structural alterations of the bla_{NDM-1}-carrying IncA/C_2 plasmid (Table 3) were submitted to Illumina WGS analysis for an in-depth insight into these structural deletions as described in **publication 3** (241). The smaller structural deletion of the bla_{NDM-1}-carrying IncA/C_2 variant from the *S*. Paratyphi B (*d*T+) transconjugant strain (G2-24d-T4) did not lead to the loss of AMR genes. On the other hand, the larger structural deletion of the bla_{NDM-1}-carrying IncA/C_2 plasmid in *S*. Infantis transconjugant strain (G4-29d-T5) led to the loss of macrolide resistance encoding genes *mph*E and *msr*E and *traC-traW-traU-traN* cluster (Figure 5).

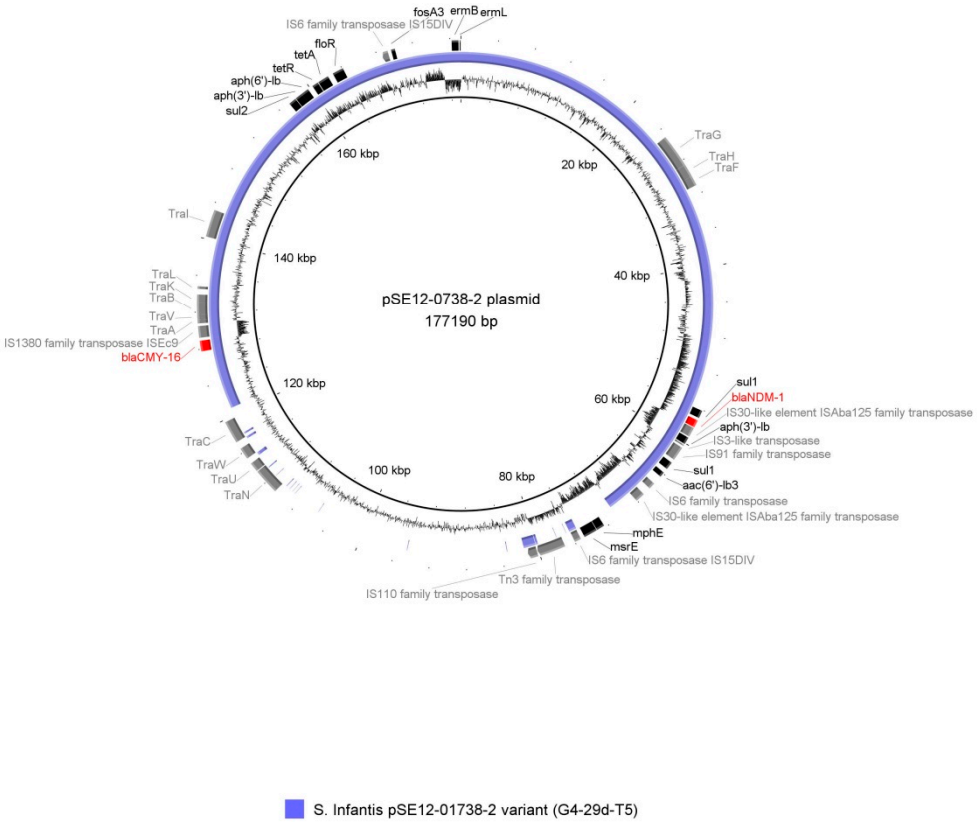

Figure 5. Visualization of the *bla*$_{NDM-1}$-carrying pSE12-01738-2 variant *S*. Infantis (G4-29d-T5) compared to PacBio RSII reference sequence of pSE12-01738-2 plasmid (GenBank Accession number CP027679) using BRIG (166) with resistance genes (red, beta-lactam genes and black other AMR genes) as well as IS elements, transposase, and *tra* genes (all marked with gray color).

2.4.2. Structural alterations of *bla*$_{VIM-1}$-carrying IncHI2 plasmid *in vivo*

Beside the *in vivo* structural alterations of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid which was presented in **publication 3** (241), a closer look into structural alterations occurring in a *bla*$_{VIM-1}$-carrying IncHI2 plasmid after an *in vivo* passage was provided. The VIM-1-producing *S.* Infantis reisolates were selected for further analysis as described for NDM-1-producing

S. Corvallis strains in **publication 3** (241). Aim was to analyze VIM-1-producing *S*. Infantis strains from at least three animals within each experimental group which have continuously excreted this strain during the *in vivo* trial. For this purpose, in total 80 strains [Group 1 (n=24), Group 2 (n=18), Group 3 (n=21) and Group 4 (n=17)] of VIM-1-producing *S.* Infantis from different timepoints were selected. In experimental group 4 it was not possible to select three animals, in which VIM-1-producing *S.* Infantis reisolates were continuously excreted. Therefore, *S.* Infantis reisolates from 8 animals were selected (Figure 6.). The S1-PFGE plasmid profiling and subsequent Illumina WGS analyses were conducted as described for NDM-1-producing *S*. Corvallis in **publication 3** (241).

From 80 selected VIM-1-producing *S*. Infantis reisolates, in 67 reisolates size of the ~310 kb IncHI2 plasmid was not changed based on S1-PFGE plasmid profiling analysis. In 13 strains structural alterations of the ~310 kb IncHI2 plasmid were observed. Among these 13 strains, in 11 strains, smaller deletions (~10 kb) and in 2 strains, the IncHI2 plasmid was slightly larger (~320 kb). Larger structural deletions of the IncHI2 plasmid were not observed. As for acquisition of other plasmids, in one strain (G1-24d-T5) beside the ~310 kb IncHI2 plasmid an additional ~30 kb plasmid was observed. In two strains (G4-13d-T7 and G4-21d-T7) beside the ~310 kb IncHI2 plasmid, additional ~100 kb plasmids were detected (Figure 7). The S1-PFGE gels of these three strains were submitted to Southern blotting and *bla*$_{\text{VIM-1}}$ hybridization. Transposition of the *bla*$_{\text{VIM-1}}$ gene from IncHI2 plasmid onto other plasmids was not observed (Figure 7).

Group/s	ID	Sampling days							
		11d	13d	15d	17d	21d	24d	29d	*Pm*
Group 1	T4								
	T5						*		
	T8								
Group 2	T4	n.a							n.a
	T5	n.a							n.a
	T6	n.a							n.a
Group 3	T1								n.a
	T4								n.a
	T7								n.a
Group 4	T1	n.a		n.a			n.a	n.a	n.a
	T2	n.a		n.a			n.a		n.a
	T3	n.a	n.a	n.a		n.a	n.a	n.a	n.a
	T4	n.a		n.a			n.a	n.a	n.a
	T6	n.a		n.a	n.a		n.a	n.a	n.a
	T7	n.a	*	n.a	n.a	*	n.a	n.a	n.a
	T10	n.a		n.a	n.a		n.a	n.a	n.a
	T11	n.a		n.a	n.a	n.a	n.a	n.a	n.a

	Strains available for an in-depth molecular analysis
	Strains not available (n.a)
Pm	*Post mortem* cecal content isolates
*	Acquisition of additional plasmids

Figure 6. Selection of VIM-1-producing *S*. Infantis reisolates for an in-depth molecular analysis of the plasmid content, experiment 3.

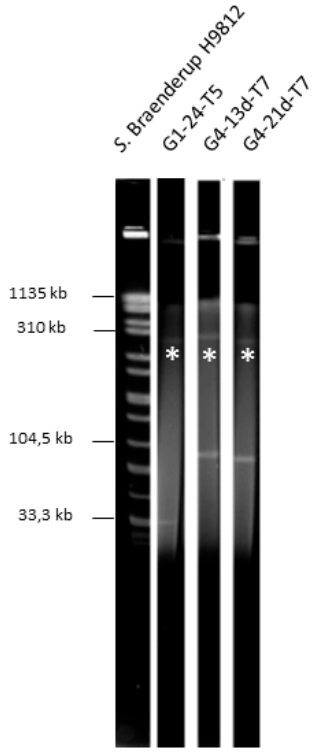

Figure 7. Three VIM-1-producing *S*. Infantis reisolates with acquisition of additional plasmids. With asterisk marked is the position of the *bla*$_{VIM-1}$ gene based on Southern blotting and *bla*$_{VIM-1}$ hybridization. In lane 1 as size marker *S*. Braenderup strain H9812, experiment 3.

3. Discussion

Antimicrobial resistance remains an important threat to the public and animal health globally (2). Although is the emergence of AMR in bacteria a natural occurrence, main driver of AMR as an emerging public health concern nowadays is the increased AMU in recent years (38). Ironically, already Alexander Fleming at his Nobel Price speech in 1945 warned that the ignorant use of antibiotics could lead to the emergence of resistance (242). A common observation is that AMR in commensal and pathogenic bacteria emerges soon after new antimicrobial compounds are brought to the market (60, 243). This is also reflected in the emergence of penicillinase, cephalosporinases, ESBLs and carbapenemases which coincides with the introduction of these beta-lactam classes into the market (80). Another challenge for the global fight against AMR is diversity of regulations and management options of antibiotic stewardship in different countries. The increasing demands for animal proteins in a rapidly growing world population are intertwined with high production pressure and increased AMU in livestock production in developing countries. The globalization, international trade and travel favored the spread of different AMR genes, such as genes encoding resistance to CIA (92, 213).

Another challenge for the global aim of slowing down the spread of AMR is the association of AMR-encoding genes to mobile genetic elements, such as plasmids. Plasmids enable bacteria of a particular environment acquisition of genes which encode new functions. Such new functions might be useful for the host under certain conditions, such as exposure to heavy metals, biocides and antimicrobials (7, 130, 133). The diversity of plasmids and their incompatibility groups, their plasticity and continuously adapting mechanisms, constrain the control of plasmid-mediated AMR. As plasmids are broad host range genetic elements, they can spread between genetically distant bacteria leading to the spread of AMR in different ecological environments.

Particular concerns for the public health were first studies which reported detection of bacteria resistant to carbapenems (9, 84, 87, 174, 180). This observation was concerning as carbapenems are perceived as last option antimicrobials for the treatment of severe life-threatening infections in humans. However, as a consequence of their use in humans, first reports of CPB in humans and human clinical settings started to emerge. Another concern was that the “big five” (KPC, OXA, IMP, NDM and VIM) carbapenemases were not only related to certain bacterial clones, but also to broad host range plasmids, which led to their spread in different bacteria and new geographical boundaries (87). An illustrating example is the dissemination of $bla_{\text{NDM-1}}$ gene, which is nowadays found in different plasmids and bacteria. From its initial source in Indian subcontinent, this NDM-1 carbapenemase-encoding gene has spread globally, being detected in Europe and North America (4).

Despite occasional reports, CPE are still sporadic in livestock. An explanation is the non-use of carbapenems in livestock and lack of the antibiotic pressure which would favor persistence of CPE (174). The non-use of carbapenems in livestock, suggests that the behavior and spread of CPB in livestock might differ from their counterpart of human origin. This shows that there is a gap in a comprehensive picture of the plasmid-mediated carbapenem resistance in food-producing animals (57). As for Germany, sporadic reports suggest that CPE have found a way into livestock production as an ecological niche. Namely, in recent years, sporadic cases of CPE in animals and animal products were reported in Germany (12, 14, 15, 17, 169). For *Salmonella* strains, a NDM-1-producing *S.* Corvallis was isolated from a wild bird (black kite) and VIM-1-producing *S*. Infantis in pig, pork and poultry production. Moreover, a recent publication by Roschanski et al. (16) reported detection of a VIM-1-producing *S*. Goldcoast strain in a pig breeding farm in Germany.

The ongoing trends of AMR in livestock call not only for a need to develop new and effective antibiotics, but primarily their prudent use through strictly regulated and monitored antibiotic stewardship. A comprehensive knowledge on the *in vivo* transfer and stability of particular public health relevant AMR-encoding plasmids is also needed. As this knowledge is still very limited, in this doctoral thesis scenarios where avian native NDM-1-producing *S*. Corvallis and VIM-1-producing *S*. Infantis entered a broiler flock were investigated, with the focus on transfer and stability of their $bla_{\text{NDM-1}}$ and $bla_{\text{VIM-1}}$-carrying plasmids *in vivo*.

3.1. *In vivo* transfer of the $bla_{\text{NDM-1}}$-carrying IncA/C_2 plasmid to avian gut enterobacteria

The interest in plasmids increased substantially in last few decades due to their role in the spread of AMR (121). By evolutionary acquisition of genetic elements, such as AMR genes, plasmids provide specific benefits when bacteria are exposed to unfavorable conditions, such as exposure to antimicrobials (7). By incorporating not one but several AMR genes, persistence of AMR plasmids can be enhanced by selection pressure due to only one antimicrobial agent (57). Variety of plasmids, their genetic plasticity and broad host range have made also the prevention of plasmid-mediated spread of carbapenem resistance difficult.

In **publication 1** of the doctoral thesis, *in vivo* persistence of a NDM-1-producing *S*. Corvallis donor strain (12-01738) once entered into an experimental broiler flock in the absence of antibiotic pressure was investigated (240). This particular NDM-1-producing *S*. Corvallis strain was initially isolated from a wild bird (black kite) in Germany and was first CPB detected in a wild animal (12). As black kite (*Milvus migrans*) is a migratory bird which spends summers in Europe and winters in North Africa, the authors hypothesized that this

strain originated from North Africa where *S.* Corvallis is frequently isolated in different animal species and environment. Migratory route, which includes Balkan region, were NDM-1-carbapenemases are prevalent, might have led to acquisition of NDM-1-carbapenemases in this strain due to intake of polluted water along the birds migratory route. Actually, beside Indian subcontinent, Balkan region is another geographic region where NDM-1-carbapenemases are prevalent (98). Polluted water as a source of MDR bacteria was investigated in several studies (76, 244-246). In a study by Walsh et al. (93) where environmental origin of the NDM-1-carbapenemases in India was investigated, the *bla*$_{\text{NDM-1}}$ gene was detected in 4% of drinking water samples, 30% of seepage samples and in eleven bacterial species. In a study by Guenther et al. (247) it was concluded that fecal polluted water is an important vector for wild birds to acquire different AMR genes. Recently, Dolejska et al. (65) reviewed wildlife and their role in the epidemiology of medically important AMR bacteria. The authors concluded that wildlife animals are important sentinel species which reflect AMR of the environment, but also act as reservoirs and vectors of MDR bacteria. The changes in feeding behaviors (feeding from landfills and human refuse) as well migration patterns of birds have led to the changes in the epidemiology of AMR and facilitated spread of important AMR genes to different geographic areas (65, 67). Ahlstrom et al. (226) recently reported first detection of a carbapenemase-producing *E. coli* strain in Alaska and in wildlife from United States. The above-mentioned studies have shown that wastewaters and wild bird reservoirs play an important role in the epidemiology of MDR CPB and could act as an important vector for their spread into other ecological niches.

The results of the *in vivo* study showed that the avian-native NDM-1-producing *S*. Corvallis can persist in a broiler flock in the absence of antibiotic pressure. Namely, in all three experimental groups excretion of this strain was detected until the end of trial (240). This is an important observation reflecting a natural condition since carbapenems are not licensed for the use in food-producing animals. Furthermore, this *S*. Corvallis strain harbors a broad host range MDR-encoding *bla*$_{\text{NDM-1}}$-carrying IncA/C$_2$ plasmid (33) which, as shown in conducted *in vivo* studies, would disseminate in mixed and dense bacterial population, such as chicken gut microbiota. Namely, gut is defined as a “hot spot” for transfer of antibiotic resistance genes from exogenous to indigenous bacteria (248). The hypothesis of the intestinal tract serving as a “melting pot” for HGT is due to the microbial load and diversity of bacteria, resistance genes and hostile environment which can exert stress to bacterial population. This stress can subsequently promote different *in situ* HGT events (249). The *in vivo s*tudies of this thesis revealed acquisition of the *bla*$_{\text{NDM-1}}$-carrying IncA/C$_2$ plasmid in different gut enterobacteria without administering antibiotic pressure (240). As this plasmid carries not only genes which encode carbapenem resistance, but also resistance to fosfomycins, aminoglycosides, tetracyclines and macrolides, the persistence of such MDR-

encoding *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid could be promoted by mechanism of co-resistance by usage of one of these antimicrobial substances. Namely, an *in vivo* murine study conducted by Ye et al. (212) revealed that use of other antibiotics might contribute to the spread of *bla*$_{NDM-1}$-carrying IncX3 plasmid. The authors showed that use of antibiotics which are a common choice for treatment of infections with gram-negative bacteria, such as ampicillin and amoxycillin, facilitated persistence of carbapenem-resistant enterobacteria and *in vivo* transfer of the *bla*$_{NDM-1}$-carrying IncX3 plasmid in a murine infection model.

The broad host range of the IncA/C$_2$ plasmids (133, 142) was also observed in the *in vivo* study. Namely, acquisition of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid in different *E. coli* STs (ST117, ST156, ST2040, ST2485) and a *K. pneumoniae* strain (ST1106) in the absence of antibiotic pressure was detected. Moreover, acquisition of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid in different strains from one individual animal on a particular sampling day was observed (240). In a study conducted by Card et al. (250) transfer of a MDR-encoding *bla*$_{CTX-M-1}$-carrying plasmid from *Salmonella* to commensal *E. coli* was investigated. The authors used an *in vitro* chemostat system, which mimicked cecal microbiome of chickens, to investigate transfer of this plasmid. High transfer rates of the MDR-encoding *bla*$_{CTX-M-1}$-carrying plasmid to seven different *E. coli* STs without administration of cefotaxime were observed. In a study by Smet et al. (251), an *in situ* system, which resembles human cecum and ascending colon was used, to study behavior of an avian native ESBL-producing *E. coli* strain. The authors observed persistence of this ESBL-producing strain under different conditions and transfer of the *bla*$_{TEM-52}$-carrying plasmid to limited number of commensal bacteria. In a study by Anjum et al. (248), the fate of a *bla*$_{CMY-2}$-carrying IncI1 plasmid in an *in vitro* gut model was investigated. By using a lower inoculum dose, which resembles a real-life infection dosis, the authors observed that even such inoculum dose is sufficient for a *bla*$_{CMY-2}$-carrying IncI1 plasmid to be transferred to other enterobacteria. Regarding 3R (Replacement, Reduction and Refinement) guiding principles for animal experimentation (252), overall aim is to reduce number of animals used in animal experiments. Promising models, which can be used for investigating the HGT of MDR-encoding plasmids in mixed bacterial population, are *in vitro* gut models. Although gut models do not completely resemble physiological conditions and microbiological content of the gut, they can serve as an indication of dissemination potential of certain AMR-encoding plasmids. With the rise of metagenomic analysis of the avian gut microbiome, a more reliable resemblance of the gut microbiome *in vitro* might be achieved in the near future. The advantage of gut models is that they enable insight into the effect of different conditions, such as exposure to different substances on the extent of plasmid transfer, without the need to include additional animals in animal experiments.

As in other studies, the *E. coli* transconjugants detected in conducted *in vivo* trials were shown to be associated with the acquisition of additional plasmids and resistance genes. In a study by Macesic et al. (253) *E. coli* ST117 strain of human origin carrying an IncX4 *mcr-1*-harboring plasmid was detected, suggesting that this particular avian pathogenic *E. coli* ST117 can acquire also other resistance genes, such as *mcr-1*. In a study by Tang et al. (214), a NDM-5-producing *E. coli* ST156 was detected in a poultry farm in China. The *bla*$_{NDM-5}$ gene was associated to a transferable IncX3 plasmid. In a study by Oikarainen et al. (204) it was observed that the ST2040 and ST429 are predominant *E. coli* STs found in ESBL/AmpC-producing *E. coli* isolates in poultry production pyramid. Interestingly, Paskova et al. (135) recently detected a *bla*$_{NDM-1}$-carrying multireplicon (IncA/C$_2$ and IncR) plasmid in *E. coli* ST58 from human urine. The type I IncA/C$_2$ sequence part of this ~300 kb megaplasmid was 99% identical to pRH-1238 of *S*. Corvallis. This suggests that not only enterobacteria from different ecological environments can acquire this *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid, but also adaptation of this plasmid to other bacteria. The *E. coli* transconjugants which acquired the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid from the *in vivo* study were detected until the end of the trial (240). Other studies have shown that *E. coli* strains can act as carriers of the *bla*$_{NDM-1}$ gene, either in humans (254-256), animals (103) or food products (257). As *E. coli* strains are ubiquitously present in livestock animals, acquisition of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid in these strains could lead to their continuous propagation in a livestock setting, environmental contamination and potentially downstream the food production chain. This is particularly important in commercial broiler production where high production pressure and short inter-service breaks could lead to continuous persistence of such MDR-encoding bacteria. This is of notice as it is also shown that *E. coli* strains can act as vehicles of the carbapenemases from one environmental source to another (76). Stable *in vivo* persistence of transconjugant population which acquired an AMR-encoding plasmid, in the absence of antibiotic pressure, was observed also in a study by Gumpert et al. (258). The authors explored persistence and transfer of a MDR-encoding plasmid in infant microbiota in the absence of antibiotic pressure and observed that the newly emerged transconjugant population remained stable for months, without antibiotic pressure.

Different non-*E. coli* enterobacteria are known to acquire *bla*$_{NDM}$ genes. In the *in vivo* study one *K. pneumoniae* ST1106 strain which acquired *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid was detected (240). In a study by Zhang et al. (259) poultry-associated NDM-producing *K. pneumoniae, E. cloacae* and a *S. enterica* strains were detected. Due to broad host range of plasmids, it is not a rare observation during an epidemiological investigation that a common MDR-encoding plasmid is detected in different bacteria. In a study by Hammerum et al. (260) carbapenemase-producing *C. freundii, E. coli, K. pneumoniae* and *K. oxytoca* strains of human origin were detected. The authors hypothesized multiple *in vivo* transfer

events of NDM-1-encoding IncA/C_2 plasmid from *C. freundii* to other strains. In a study by Tijet et al. (261) the authors also hypothesized an *in vivo* transfer of a *bla*$_{NDM-1}$-carrying IncA/C_2 plasmid and independent acquisition of the *bla*$_{NDM-1}$ in *E. coli, E. cloacae* and *P. stuartii* strains. The occurrence of highly similar MDR-encoding plasmids in different bacteria reveals a gap in the understanding of plasmid dissemination routes as it is difficult to identify which bacteria have a pivotal role in the spread of particular plasmids. For the future, a profounder understanding of such interaction would be a contribution to better understand the spread of plasmid-mediated resistance.

The observations of conducted *in vivo* studies suggest that future prevention approaches could, beside reduction of AMU, also be directed towards preventing the transfer of a MDR-encoding plasmid. As bacterial conjugation is the main mechanism contributing to the spread of AMR and main mechanism by which bacteria acquire resistance, an additional approach might be the use of specific conjugation inhibitors (COINs), such as unsaturated fatty acid derivatives (122, 262). These substances target components of the bacterial cell involved in bacterial conjugation, such as conjugative relaxase proteins and conjugative pili (122). An ideal COIN should not reduce bacterial fitness as this might activate mechanisms of selection and resistance, should inhibit conjugation of plasmids belonging to main Inc groups and have an effect in different bacteria (262).

From the *in vivo* studies enterobacterial transconjugants, which were isolated on selective mediums, such as XLD and Carba chromID agar were characterized. Therefore, with targeted cultivation of enterobacteria in conducted studies it was not possible to investigate complete extent of the *bla*$_{NDM-1}$-carrying IncA/C_2 plasmid transfer in mixed and dense bacterial population, such as broiler chicken gut. Actually, there is no consensus for best methodology which could describe the extent of plasmid transfer (124). Another challenge is the quantification of plasmid transfer in mixed bacterial population, which would ideally be expressed as number of transfer events per donor or recipient. For *in vitro* conjugation experiments where one donor and one recipient strain are conjugated, this might be possible. However, with conducted *in vivo* experiments and cultivation approach, the role of other gut enterobacteria which acquired the *bla*$_{NDM-1}$-carrying IncA/C_2 plasmid as new donors of this plasmid remained unknown. Therefore, the estimation of the plasmid transfer in relation to only one donor (such as NDM-1-producing *S.* Corvallis) in mixed bacterial population such as gut microbiota based on cultivation, might lead to underestimation or overestimation of the extent of plasmid transfer. For a more accurate estimation of the plasmid transfer in mixed bacterial populations, different approaches and technologies are needed. In near future, a more precise insight into plasmid transfer might be achieved by single-cell detection (124). For the profounder understanding of the dissemination of MDR-encoding plasmids in mixed bacterial population, it is also of interest to explore how and to

which extent other bacteria, participate in HGT, giving insight into host-plasmid association. This is important as it is also known that less than 1% of bacteria are cultivable and that some bacteria can undergo a viable but non-cultivable status (VBNC), therefore underestimating the extent of HGT (124). However, this issue might be overcome by a technology which emerged recently and relies on sequencing data and physical linkage of plasmid DNA with chromosomal DNA sequences in close physical proximity within a cell based on Hi-C technology (263). Such approach, although not broadly used, would identify with higher resolution if certain bacteria have a more dominant role in the ecology of particular plasmids and the dissemination potential of particular plasmids in mixed bacterial populations, resembling more accurately a potential real-life scenario.

All of this shows that we are a long way from completely understanding and tracing HGT as that there is a significant knowledge gap on the HGT *in situ* and the factors influencing these events in an environment, such as intestine (249). The use of WGS in microbiological investigations also reveals that many plasmid-associated genes are of unknown function and their function needs to be experimentally confirmed. So far, little is known about the role of other factors, such as antibiotic or heavy metals exposure and gut conditions on the plasmid transfer *in vivo*. A study by Stecher et al. (264) has shown that even inflammation of the mouse gut can enhance HGT between pathogenic and commensal enterobacteria, contributing to dissemination of particular plasmids.

Nevertheless, the conducted *in vivo* study revealed stable persistence of a MDR-encoding *bla*$_{\text{NDM-1}}$-carrying IncA/C$_2$ plasmid in *S*. Corvallis and transfer of this plasmid to different gut enterobacteria in the absence of selective pressure. This is a worrying observation, as persistence of MDR-encoding plasmids without selective pressure might contribute to further HGT and compromise effective antimicrobial stewardship which aims to reduce spread of AMR (114).

3.2. Acquisition of the *bla*$_{\text{NDM-1}}$-carrying IncA/C$_2$ plasmid in *S*. Paratyphi B (*d*T+) and *S.* Infantis

Carbapenemase-producing *Salmonella* strains are dominantly detected in human (99, 101, 265) and rarely in samples of animal origin. However, several studies revealed detection of carbapenemase-producing *Salmonella* strains in animals. In a study by Wang et al. (102) plasmid-associated *bla*$_{\text{NDM-1}}$ gene was detected in a MDR *S*. Indiana isolate from a chicken carcass in China. Abraham et al. (266) reported detection of a plasmid-associated *bla*$_{\text{IMP-4}}$ gene in a feline *S.* Typhimurium strain. In Germany, carbapenemase-producing *S*. Corvallis and *S*. Infantis were associated to wild birds, livestock animals and minced pork

meat (12-14). Recently, Roschanski et al. (16) revealed detection of a VIM-1-producing *S*. Goldcoast strain in a pig breeding farm in Germany.

In **unpublished material** of the doctoral thesis acquisition of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ and *bla*$_{VIM-1}$-carrying IncHI2 plasmid in two broiler-associated serovars *S*. Paratyphi B (*d*T+) and *S.* Infantis was investigated. The acquisition of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid was observed in three *S*. Paratyphi B (*d*T+) and ten *S*. Infantis strains (Table 3). This reflects observation of the *in vitro* filter mating experiments, which were performed prior to the *in vivo* trials, where *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid was transferable to these *Salmonella* recipients *in vitro* (240). The transfer of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid to *S*. Paratyphi B (*d*T+) and *S*. Infantis is an important observation as these two serovars are frequently associated with commercial broiler production in Germany and *S*. Infantis is among top five public health relevant serovars in the EU (18). On the other hand, serovar *S.* Paratyphi B (*d*T+) has been associated with resistance to another last resort antimicrobial–colistin, where plasmid and chromosomally-associated *mcr-5* gene was detected in a selection of *S.* Paratyphi B (*d*T+) isolates in Germany (152). The acquisition of *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid in three *S*. Paratyphi B (*d*T+) and ten *S*. Infantis strains apparently did not led to significant alteration of the plasmid structure as only one smaller (~10 kb) in *S*. Paratyphi B (*d*T+) (G2-24d-T4) and one larger (~50 kb) structural deletion in *S.* Infantis strain (G4-29d-T5) was observed. The larger structural deletions in *S.* Infantis strain (G4-29d-T5) led to the loss of *traC-traW-traU-traN* cluster and macrolide resistance genes *mph*E and *msr*E (Figure 5). If and to which extent this structural deletion might influence further dissemination of this plasmid, from *S*. Infantis as a donor of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid, remains to be investigated *in vitro*. During *in vivo* studies, neither acquisition of the *bla*$_{VIM-1}$-carrying IncHI2 plasmid in *S*. Paratyphi B (*d*T+) nor in *S.* Infantis recipient strains was detected. This is in line with the *in vitro* experiments where *bla*$_{VIM-1}$-carrying IncHI2 plasmid was not conjugative *in vitro*.

For a real-life scenario of a *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid transfer to another *Salmonella* serovar in a broiler flock, several circumstances need to be fulfilled. Firstly, entry and colonization of the chicken gut with an infrequent avian native NDM-1 *S*. Corvallis needs to occur. Generally, serovar Corvallis is not frequently detected in German poultry production (personal communication Dr. Istvan Szabo) and so far, the detection of NDM-1-producing enterobacteria in German livestock production hasn't been reported. Secondly, for plasmid transfer to occur *in vivo*, colonization of the broiler gut with another potential *Salmonella* recipient strain is needed. The national control programs of *Salmonella* in the EU in recent years have led to gradual decrease of *Salmonella* prevalence in the *Gallus gallus* (18). Therefore, chance for such scenario occurring seems not be high, yet possible. However, one was to be aware that a carbapenemase-producing *Salmonella* dissemination along the

food chain has already taken place in the case of VIM-1-producing *S*. Infantis. Namely, this strain which carries a MDR-encoding *bla*$_{VIM-1}$-carrying InHI2 plasmid was detected at the primary production level–swine farm (13) and final product–pork in Germany (14).

From the perspective of prevention, worrying is the rapid and broad dissemination of *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid to other gut enterobacteria in the absence of antibiotic pressure (240). This is concerning as it makes eradication of bacterial population which acquired such a MDR-encoding plasmid difficult. As enterobacteria are normal part of the gut microbiota and livestock environment, the acquisition *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid in enterobacteria, would lead to continuous propagation of such MDR-encoding plasmid in a livestock setting and environment. Therefore, persistence of such enterobacterial population could function as a reservoir for carbapenemase-encoding plasmids and would increase the probability of *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid being transferred to any other bacteria, including *Salmonella* recipients. The acquisition of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid in different enterobacteria reveals also many difficulties in the sanitation of a livestock setting where CPB carrying broad host range plasmids are detected. Practically, this means that the sanitation of such livestock setting should not only be directed towards eradication of particular carbapenemase-producing donor strain, but towards all possible recipient strains, which is a technically, timely demanding sanitation measure, with a questionable outcome. This calls not only for new sanitation strategies, but also for a need that traditional standardized and time-consuming microbiological methods are supported by faster and eventually portable on-site diagnostic tools, such as MinION for the detection of such bacterial strains. The use of onsite molecular technologies would yield more precise time needed for the successful sanitation of such livestock setting, with the aim of preventing spread of a particular MDR bacteria.

Nevertheless, the *in vivo* broiler chicken infection study revealed acquisition of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid in *S*. Paratyphi B (*d*T+) and *S*. Infantis, contrary to *bla*$_{VIM-1}$-carrying IncHI2 plasmid. Particularly is the acquisition of *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid in these public health relevant *Salmonella* serovars concerning, as an acquisition of this plasmid would significantly jeopardize effective antibiotic therapy in humans.

3.3. *In vivo* stability of MDR-encoding *bla*$_{NDM-1}$-carrying IncA/C$_2$ and *bla*$_{VIM-1}$-carrying IncHI2 plasmids

The use of high-throughput next-generation WGS technology transformed the view on molecular epidemiology of AMR. A major advantage of WGS is higher resolution for source tracking in outbreak investigations and a deeper insight into evolutionary changes of bacterial genomes. The insight into evolution of plasmids was especially made possible by long-read

sequencing technology, which made complete assembly of plasmids possible (160). In **publication 2** of the doctoral thesis (267), first publicly available genome of a *S*. Corvallis strain harboring three plasmids was presented . This carbapenemase-producing *S*. Corvallis strain was used as donor strain for the *in vivo* studies and was submitted to single-molecule real time sequencing using the Pacbio RSII instrument, leading to complete genome sequence (GenBank Accession number CP027677) and complete sequences of three plasmids (CP027678, CP027679, CP027680). Nowadays, different WGS based studies reveal mosaic structure of MDR-encoding plasmids, resembled in their plasticity, integration of other genetic elements as well fusion with other plasmids (139, 268-270). The evolutionary studies of particular MDR-encoding plasmids are mainly based on *in vitro* conditions and investigations of outbreak strains and changes in their plasmids at different timepoints (269, 271). Therefore, an objective of this doctoral thesis was to investigate if certain structural changes of the MDR-encoding *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid occur after an *in vivo* passage. This would be an indication on the stability of this MDR-encoding plasmid in a scenario of entry with *S*. Corvallis into a broiler flock in the absence of selective pressure.

In **publication 3** of the doctoral thesis (241) evolutionary changes of a MDR-encoding *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid occurring *in vivo* in a scenario of entry with *S*. Corvallis into a broiler flock were investigated. Avian native NDM-1-producing *S*. Corvallis strain, 12-01738 (GenBank Accession number CP027677) which carries three plasmids [IncHI2; pSE12-01738-1 (CP027678), IncA/C$_2$; pSE12-01738-2 (CP027679) and ColE-like–ColRNAI; pSE12-01738-3 (CP027680)] was selected due to the avian origin and carbapenem resistance. Beside NDM-1-encoding IncA/C$_2$ plasmid, insight into stability and structural alterations of IncHI2 and ColE-like–ColRNAI plasmids, as well interaction between these three plasmids was obtained. The *in vivo* structural alterations of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid were observed in four cases as three types of structural alterations. First, a smaller (~10 kb) deletion which covered macrolide resistance genes *mph*E and *msr*E and did not had a statistically significant effect on the *in vitro* conjugation rate of this *bla*$_{NDM-1}$-carrying IncA/C$_2$ variant. Second, a larger (~70 kb) deletion, led to the loss of two *tra* gene clusters (*traL-traK-traB-traV-traA* and *traC-traW-traU-traN)* and the *bla*$_{CMY-16}$ gene due to its position in Tra1 region of this plasmid. By subsequent *in vitro* filter mating conjugation experiments, it was confirmed that these two *tra* clusters play an active role in the transfer of this plasmid, as this plasmid variant was not transferable to selected *Salmonella* recipients *in vitro* (241). Both structural deletions were neighbored by IS elements, which is in line with observations by Huelter et al. (272) that large-scale structural changes are often observed in neighboring areas of transposons and insertion sequences, revealing the role of ISs in the evolution of plasmids. Similar occurrence was observed by Porse et al. (271), where structural deletions

were mediated by an IS*26* element in a conjugative MDR-encoding *bla*$_{CTX-M-15}$-carrying IncN plasmid, leading to the loss of conjugation ability in this plasmid.

The third structural alteration was a fusion event of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ and IncHI2 plasmids and was observed in two *S*. Corvallis strains. By using Nanopore long-read WGS in combination with Illumina short-read sequencing complete structure of this *bla*$_{NDM-1}$-carrying IncHI2-IncA/C$_2$ megaplasmid was obtained (241). In subsequent *in vitro* conjugation experiments, it was observed that this co-integrate formation influenced conjugation transfer of this plasmid variant, resembled in significantly lower conjugation rate of this megaplasmid compared to the native IncA/C$_2$ plasmid or the IncA/C$_2$ plasmid with a ~10 kb deletion (241). This fusion event was probably mediated by an IS*6*-like genetic element. In a study which investigated mobility of IS*26*, which is a member of IS*6* family, it was observed that IS*26* mediates formation of plasmid co-integrates (273, 274). Formation of co-integrated multireplicon plasmids is a frequent observation in plasmids of different Inc groups (135, 268, 270, 275). The clinical importance of plasmids co-integrating results from the potential spread of genes encoding resistance to carbapenems (139, 270, 276). In several studies which investigated carbapenemase-producing *K. pneumoniae* strains it was observed that these carbapenemase-encoding genes were associated to co-integrated plasmids (277, 278).

Plasmids belonging to IncHI2 group are prone to co-integrate with plasmids of other Inc groups (178, 268, 279). In studies conducted by Fang et al. (178, 268) it was observed that the IncHI2 plasmids co-integrated with IncFII and IncN plasmids leading to their high genetic plasticity. In a study by Doublet et al. (279) which investigated cephalosporin resistance in poultry and human-associated *S*. Paratyphi B (*d*T+) strains, the *bla*$_{CTX-M-2}$ gene was frequently associated to multireplicon IncHI2/P plasmid. On the other hand, the IncA/C$_2$ plasmids also form multireplicon plasmids. In a study by Drieux et al. (280) a multireplicon *bla*$_{VIM-1}$-carrying plasmid was detected in a multiresistant *P. stuartii* strain. Subsequent sequencing analysis revealed fusion of an IncA/C$_2$ and IncR plasmid. In a study by Desmet et al. (270) it was shown that a *bla*$_{OXA-427}$-carrying IncA/C$_2$ plasmid co-integrated with an IncFIb plasmid, leading to a ~300 kb megaplasmid with multiple AMR genes. The authors suggested that plasmid co-integration might be an important pathway for the interspecies transfer of this plasmid. As for *in vivo* studies conducted here, acquisition of the IncHI2-IncA/C$_2$ megaplasmid was not observed in other enterobacteria. This might be due to the size and instability this megaplasmid. Namely, although *in vitro* filter mating experiments do not resemble *in vivo* conditions, resolution of this particular megaplasmid into smaller plasmids in recipient strains after *in vitro* conjugation was observed. This suggests instability of this megaplasmid in new recipients or during conjugation itself. Similar occurrence was reported by Xie et al. (281), where resolution of a multireplicon *bla*$_{NDM-5}$-carrying plasmid was

observed once transferred to new enterobacterial hosts. Another reason for IncA/C_2-IncHI2 megaplasmid absence in other enterobacteria might be the lower transfer capacity of this megaplasmid, as observed *in vitro*, where significantly lower transfer rate in contrast to native and *bla*$_{NDM-1}$-carrying IncA/C_2 plasmid with a ~10 kb deletion were observed (241). The formation of the IncHI2-IncA/C_2 megaplasmid from the *in vivo* study was detected only in two from 97 *S*. Corvallis strains. This is in line with the assumption that the co-integration of plasmids occurs at low frequency in donor strains (138).

Other studies have shown that co-integration of plasmids might facilitate dissemination of resistance genes which are associated to non-conjugative plasmids. In a study conducted by Lin et al. (282) it was found that a non-conjugative *bla*$_{CTX-M-17}$-carrying ColE1-type plasmid integrated into a conjugative plasmid, facilitating the spread of *bla*$_{CTX-M-17}$ gene. In another study by Chavda et al. (139) which investigated *bla*$_{KPC}$-carrying plasmids, it was shown that fusion event of non-conjugative and conjugative plasmid into an IncFIA conjugative co-integrated plasmid facilitated the spread of *bla*$_{KPC}$ gene. The study of Paskova et al. (135) showed also potential of this particular *bla*$_{NDM-1}$-carrying IncA/C_2 plasmid to persist under structural rearrangement in other bacterial hosts. The authors namely detected a *bla*$_{NDM-1}$-carrying ~300 kb multireplicon (R and A/C) plasmid in an *E. coli* strain from human urine. This particular multireplicon plasmid was 99% identical to pRH-1238 of *S*. Corvallis. This confirms also an observation of CPE occurring frequently in human clinical samples and hospital settings, which is triggered by antibitiotic pressure and the use of carbapenems to treat severe human infections (174). This, along with the observations of conducted *in vivo* studies shows that despite structural alterations of a *bla*$_{NDM-1}$-carrying IncA/C_2 plasmid, the *bla*$_{NDM-1}$ gene is maintained in different variants of the *bla*$_{NDM-1}$-carrying IncA/C_2 plasmids and different ecological environments. Detection of this *bla*$_{NDM-1}$-carrying IncA/C_2 variant in different environments and under different structural arrangements is in line with the hypothesis of epidemic plasmids. Namely, Carattoli et al. (7) suggests that plasmids associated to CTX-M-1, CTX-M-15, VIM-1, OXA-48 and NDM-1 are epidemic plasmids due to their detection in bacteria without any epidemiological link.

Another observation of the *in vivo* study was frequent loss of the IncHI2 pSE12-01738-1 plasmid (GenBank Accession number CP027677) in selection of *S*. Corvallis reisolates. Namely, loss of this plasmid in 31 out of 97 *S*. Corvallis reisolates was observed. An explanation could be the carriage burden of a relatively large (~280 kb) IncHI2 plasmid. Namely, plasmid carriage represents a burden for the host and to control these fitness costs, the host cell adopts strategies to cope with their presence (283). The burden of carriage might have outweighed selective benefits for the host, leading to frequent loss of IncHI2 plasmid. However, for the profounder knowledge of the ecology of particular plasmids it is of interest to explore which exact factors contributed to the frequent loss of IncHI2 plasmid in

S. Corvallis, contrary to the IncA/C_2 and ColE-like plasmids which were stably maintained in characterized *S*. Corvallis strains. Recently, Carroll et al. (284) reported detection of a novel inducible and mobilized *mcr-9* gene detected on an IncHI2 plasmid in MDR *S.* Typhimurium strain isolated from a human patient in Washington. Although neither transfer nor ecology of different *mcr*-variants was subject of the *in vivo* studies, re-screening of the sequencing data of the NRL-*Salmonella* in Germany which were performed after findings of Carroll et al. (284) revealed detection of the *mcr-9* gene on the IncHI2 pSE12-01738-1 plasmid (GenBank Accession number CP027677) of carbapenemase-producing *S*. Corvallis (personal communication Maria Borowiak). This confirmed benefits of the WGS technology as a powerful tool for re-screening of existing sequences, yielding insight into dissemination of newly emerged resistance genes. On the other hand, it also shows an inevitable time gap from the time point of emergence to the time point of detection of a resistance gene and how difficult it is to control spread of particular plasmid-mediated resistance genes.

The structural alterations of the *bla*$_{VIM-1}$-carrying IncHI2 plasmid, from VIM-1-producing *S.* Infantis used as donor strain in third animal experiment which were shown in **unpublished material**, revealed smaller structural deletions (~10 kb) and in two cases the IncHI2 plasmid was slightly larger (~320 kb). Larger structural deletions in *bla*$_{VIM-1}$-carrying IncHI2 plasmid were not detected. In three VIM-1-producing *S*. Infantis strains acquisition of additional plasmids (~30 kb and ~100 kb) was detected (Figure 7) revealing HGT of other plasmids *in vivo.* Although transfer of *bla*$_{VIM-1}$-carrying IncHI2 plasmid was not detected in characterized strains during conducted *in vivo* broiler infection studies, other publications have shown detection of this plasmid in other bacteria. Namely, Roschanski et al. (16) reported detection of this plasmid in *S*. Infantis, *S*. Goldcoast and *E. cloacae* strains, from a single pig breeding farm suggesting that transfer events of this plasmid might be rare, yet possible *in vivo.*

In conclusion, common structural alterations of the *bla*$_{NDM-1}$-carrying IncA/C_2 and *bla*$_{VIM-1}$-carrying IncHI2 plasmids which might occur *in vivo* were detected. As for the prevention of carbapenemase-producing *Salmonella* strains spreading in a broiler flock, a prevention approach directed towards preventing such strain entering a broiler flock would be a contribution to minimize the *in vivo* dissemination of MDR-encoding plasmids.

4. Final conclusions

I. Stable persistence of *bla*$_{NDM-1}$-carrying IncA/C$_2$ and *bla*$_{VIM-1}$-carrying IncHI2 plasmids in their native hosts once entered into a broiler flock in the absence of antibiotic pressure was observed.

II. Transfer of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid to gut native *E. coli* strains (ST117, ST156, ST2040, ST2485) and a *K. pneumoniae* strain (ST1106) in the absence of antibiotic pressure was detected.

III. The detection of transconjugant population until the end of trial suggests potential of *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid to persist in different bacterial hosts, also in the absence of selective pressure.

IV. Although less frequent in contrast to gut *E. coli* strains, acquisition of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid in *S*. Paratyphi B (*d*T+) and *S*. Infantis was observed. The acquisition of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid in public health relevant *Salmonella* serovars is particularly worrying as such scenario would jeopardize effective antibiotic treatment in humans.

V. The acquisition of the *bla*$_{VIM-1}$-carrying IncHI2 plasmid was not detected in gut native *E. coli* strains and selected *Salmonella* recipients, despite observations from other studies that this highly similar plasmid was detected in other bacteria.

VI. The observations of the conducted *in vivo* broiler chicken infection experiments suggest that an important preventive measure is to avoid entrance of a carbapenemase-producing bacteria into a broiler flock, as such entry scenario could lead to stable persistence of a particular strain and dissemination of the MDR-encoding *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmids, even in the absence of antibiotic pressure.

5. Summary

The antimicrobial resistance remains an important threat to the public and animal health. The current trends of antimicrobial resistance reveal an alarming and ongoing dissemination of associated genes to different bacteria. With the aim of preserving important antimicrobials for human treatment, certain antimicrobial classes, such as carbapenems are categorized as critically important antimicrobials and are not licensed for use in food-producing animals. However, in recent years, reports of carbapenemase-producing bacteria in livestock animals started to emerge. In Germany, several studies reported detection of carbapenemase-producing enterobacteria in wild birds, livestock, meat and seafood products. An important observation was association of New Delhi Metallo (NDM-1) and Verona Integron Metallo (VIM-1) carbapenemases to different *Salmonella* serovars, suggesting their role as reservoirs of these carbapenemases.

Motivated by the detection of carbapenemase-producing *Salmonella* serovars in a wild bird and poultry production, in this doctoral thesis scenarios of avian native NDM-1-producing *Salmonella enterica* subsp. *enterica* serovar (*S.*) Corvallis and VIM-1-producing *S.* Infantis entering a broiler flock were investigated. The aim of the *in vivo* studies was to understand behavior of these strains and their plasmids in the absence of antibiotic pressure. Particular focus was on the *in vivo* spread and stability of carbapenemase-encoding *bla*$_{\text{NDM-1}}$-carrying plasmids belonging to the incompatibility group A/C$_2$ (IncA/C$_2$) and *bla*$_{\text{VIM-1}}$-carrying plasmids belonging to group HI2 (IncHI2). The insight into transfer and structural alteration of plasmids was obtained by an in-depth molecular analysis with S1-pulsed-field gel electrophoresis plasmid profiling and subsequent short and long-read whole genome sequencing analysis.

The *in vivo* studies showed that in a scenario of NDM-1-producing *S.* Corvallis entering a broiler flock, the *bla*$_{\text{NDM-1}}$-carrying IncA/C$_2$ plasmid disseminates to different gut enterobacteria, such as *Escherichia* (*E.*) *coli* and *Klebsiella* (*K.*) *pneumoniae,* in the absence of antibiotic pressure. The acquisition of this plasmid was not restricted to a particular *E. coli* clone, as this plasmid was detected in different *E. coli* ST-types and a *K. pneumoniae* strain. The *bla*$_{\text{NDM-1}}$-carrying IncA/C$_2$ plasmid remained detected in donor and *E. coli* strains until the end of trial, revealing stable persistence of this plasmid also in new hosts. Although less frequent in contrast to gut enterobacteria, acquisition of the *bla*$_{\text{NDM-1}}$-carrying IncA/C$_2$ plasmid in broiler-associated *S*. Paratyphi B (*d*Tartrate-positive) and *S*. Infantis strains was detected. Acquisition of the *bla*$_{\text{VIM-1}}$-carrying IncHI2 plasmid was not detected in other bacteria during the course of *in vivo* study.

As for the *in vivo* stability of *bla*$_{\text{NDM-1}}$-carrying IncA/C$_2$ and *bla*$_{\text{VIM-1}}$-carrying IncHI2 plasmid, stable persistence of these plasmids in their native hosts was observed. In the case

of *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid a smaller (~10 kb) and larger (~70 kb) structural deletion was identified, along with formation of a newly emerged *bla*$_{NDM-1}$-carrying IncA/C$_2$-IncHI2 multireplicon megaplasmid. Despite structural alterations, the *bla*$_{NDM-1}$ gene was maintained in these variants of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid. In the selection of *bla*$_{VIM-1}$-carrying IncHI2 plasmids, from donor reisolates, smaller (~10 kb) structural deletions were observed.

In conclusion, the *in vivo* broiler chicken infection studies revealed stable persistence of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ and *bla*$_{VIM-1}$-carrying IncHI2 plasmids, despite sporadic structural alterations, in their hosts in a scenario of entry into a broiler flock. As for *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid, a worrying observation is the rapid and broad dissemination of the *bla*$_{NDM-1}$-carrying IncA/C$_2$ plasmid to gut enterobacteria and *Salmonella* recipients in the absence of selective pressure. Although here primarily investigating the behavior of NDM-1-producing *S*. Corvallis and VIM-1-producing *S*. Infantis strains, the results can serve as a model for the potential dissemination of other MDR-encoding broad host range plasmids in a broiler flock. Therefore, important preventive measure should be achieved to avoid such bacteria entering a broiler flock, as their entrance could lead to the spread of MDR-encoding plasmids even in the absence of antibiotic pressure.

6. Zusammenfassung

Die Übertragung und strukturelle Veränderung der Carbapenemasegen-tragende Plasmide im Tiermodell Huhn

Die Antibiotikaresistenzen stellen nach wie vor eine große Bedrohung für die Gesundheit von Menschen und Tieren dar. Die derzeitigen Entwicklungen der Antibiotikaresistenzen zeigen eine alarmierende und anhaltende Verbreitung der Antibiotikaresistenzgene bei verschiedenen Bakterien. Mit dem Ziel, die wichtigsten Antibiotika für die Behandlung von Menschen zu bewahren, werden bestimmten Gruppen von Antibiotika, wie z.B. Carbapenemen, als Reserveantibiotika eingestuft und dürfen bei Nutzieren nicht verwendet werden. In den letzten Jahren wurden dennoch Carbapenemase-produzierende Bakterien bei Nutztieren isoliert. In Deutschland haben mehrere Studien über den Nachweis von Carbapenemase-produzierende Enterobakterien in Wildvögeln, Nutztieren, Fleisch und Meeresfrüchten berichtet. Eine wichtige Beobachtung dieser Studien war der Zusammenhang der New Delhi Metallo (NDM-1) und Verona Integron Metallo (VIM-1) Carbapenemasen mit unterschiedlichen Salmonellen, was darauf hindeutet, dass die Salmonellen mögliche Reservoire dieser Carbapenemasen sind.

Motiviert durch den Nachweis von Carbapenemase-produzierenden Salmonellen bei einem Wildvogel und in der Geflügelproduktion wurden in dieser Dissertation Eintrittsszenarien einer aviären NDM-1-produzierenden *Salmonella enterica* subsp. *enterica* serovar (*S*.) Corvallis und einer VIM-1-produzierenden *S*. Infantis in einen Masthähnchenbestand untersucht. In den *in vivo* Studien wurde das Verhalten dieser Salmonellen und der dazugehörigen Plasmide ohne Antibiotika-Einsatz untersucht. Insbesondere wurden hier die *in vivo* Übertragung und die Stabilität der Carbapenemase-kodierenden bla_{NDM-1}-tragenden Plasmide der Inkompatibilitätsgruppe A/C_2 (IncA/C2) und bla_{VIM-1}-tragenden Plasmide der Inkompatibilitätsgruppe HI2 (IncHI2) untersucht. Der Einblick in der Verbreitung und die Stabilität der Plasmide wurde durch die Verwendung von molekularbiologischen Methoden S1-Puls-Feld-Gelelektrophorese und die Gesamtgenomsequenzierung ermöglicht.

Die *in vivo* Studien haben gezeigt, dass in einem Szenario in dem der NDM-1-produzierende *S.* Corvallis in einen Masthähnchenbestand eintritt, das bla_{NDM-1}-tragende $IncA/C_2$ Plasmid sich in unterschiedlichen Darmenterobakterien wie *Escherichia* (*E*.) *coli* und *Klebsiella* (*K*.) *pneumoniae* auch ohne Antibiotika-Einsatz verbreiten kann. Die Verbreitung dieses Plasmids war nicht auf einen bestimmten *E. coli*-Klon beschränkt. Das Plasmid wurde

in verschiedenen *E. coli* ST-Typen und einem *K. pneumoniae*-Stamm nachgewiesen. Das *bla*$_{NDM-1}$-tragende IncA/C$_2$ Plasmid konnte in den Donorzellen und *E. coli*-Stämmen bis Ende des Versuchs nachgewiesen werden, was für eine stabile Persistenz des Plasmids in anderen Bakterien spricht. Im Gegensatz zu Darmenterobakterien war die Aufnahme des *bla*$_{NDM-1}$-tragenden IncA/C$_2$-Plasmids in Broiler-assoziierten *S*. Paratyphi B (*d*Tartrat-positiv) und *S*. Infantis weniger häufig. Die Aufnahme des *bla*$_{VIM-1}$-tragenden IncHI2 Plasmids in anderen Bakterien konnte im Verlauf der *in vivo* Studie nicht nachgewiesen werden.

Bezüglich der *in vivo* Stabilität der *bla*$_{NDM-1}$-tragenden IncA/C$_2$ und *bla*$_{VIM-1}$-tragenden IncHI2 Plasmide wurde eine stabile Persistenz dieser Plasmide in ihren Donorzellen beobachtet. Bei den *bla*$_{NDM-1}$-tragenden IncA/C$_2$ Plasmiden wurde eine kleinere (~10 kb) und eine größere (~70 kb) Deletion in dem Plasmid sowie die Entsehung eines *bla*$_{NDM-1}$-tragenden IncA/C$_2$-IncHI2-Multireplikon-Megaplasmids beobachtet. Trotz strukturellen Veränderungen des Plasmids konnte das *bla*$_{NDM-1}$ Gen in diesen Varianten des *bla*$_{NDM-1}$-tragenden IncA/C$_2$ Plasmids nachgewiesen werden. Bei den untersuchten *bla*$_{VIM-1}$-tragenden IncHI2 Plasmiden wurden kleinere (~10 kb) Deletionen beobachtet.

Die im Tiermodell Huhn durchgeführten *in vivo* Studien haben eine stabile Persistenz der *bla*$_{NDM-1}$-tragenden IncA/C$_2$ und *bla*$_{VIM-1}$-tragenden IncHI2-Plasmide in Donorzellen bei einem Eintrittsszenario in einen Masthähnchenbestand gezeigt. Besorgniserregend ist die Verbreitung des *bla*$_{NDM-1}$-tragenden IncA/C$_2$ Plasmids zu den Darmenterobakterien und Salmonellen ohne Antibiotika-Einsatz. Obwohl in den dargestellten Studien das Verhalten der NDM-1-produzierenden *S*. Corvallis und VIM-1-produzierenden *S*. Infantis untersucht wurde, können die Ergebnisse dennoch als ein Modell für die Verbreitung von Multiresistenz-kodierenden Plasmiden in einem Masthänchenbestand dienen. Somit sollten Präventivmaßnahmen ergriffen werden um den Eintritt solcher Bakterien in einen Masthänchenbestand zu verhindern, insbesondere weil ihr Eintritt zur Verbreitung von Multiresistenz-kodierenden Plasmiden auch ohne Antibiotika-Einsatz führen könnte.

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III List of publications

First author in publications:

Publication 1.

Hadziabdic, Sead; Fischer, Jennie; Malorny, Burkhard; Borowiak, Maria; Guerra, Beatriz; Kaesbohrer, Annemarie; Gonzalez-Zorn, Bruno; Szabo, I (2018). **In vivo transfer and microevolution of avian native IncA/C$_2$ *bla*$_{NDM-1}$-carrying plasmid pRH-1238 during a broiler chicken infection study.** In: Antimicrobial Agents and Chemotherapy (AAC) 62(4) https://doi.org/10.1128/AAC.02128-17.

Publication 2.

Hadziabdic, Sead; Borowiak, Maria; Malorny, Burkhard; Szabo, Istvan; Guerra, Beatriz; Kaesbohrer, Annemarie; Fischer, Jennie (2018). **Complete genome sequence of an avian native NDM-1-producing *Salmonella enterica* subsp. *enterica* serovar Corvallis strain.** In: Genome Announcement. 6(26) https://doi.org/10.1128/genomeA.00593-18.

Publication 3.

Hadziabdic, Sead; Fischer, Jennie; Borowiak, Maria; Malorny, Burkhard; Juraschek, Katharina; Kaesbohrer, Annemarie; Guerra, Beatriz; Deneke, Carlus; Gonzalez-Zorn, Bruno; Szabo, I (2019). **The *bla*$_{NDM-1}$ carrying IncA/C$_2$ plasmid underlies structural alterations and co-integrate formation in vivo.** In: Antimicrobial Agents and Chemotherapy (AAC) 63(8) https://doi.org/10.1128/AAC.00380-19.

Co-author in publications:

Publication 1.

Roschanski, Nicole; **Hadziabdic, Sead;** Borowiak, Maria; Malorny, Burkhard; Tenhagen, Bernd-Alois; Projahn, Michaela; Kaesbohrer, Annemarie; Guenther, Sebastian; Szabo, Istvan; Roesler, Uwe; Fischer, Jennie (2019). **Detection of VIM-1-Producing *Enterobacter cloacae* and *Salmonella enterica* serovar Infantis and Goldcoast at a breeding pig farm in Germany in 2017 and their molecular relationship to former VIM-1-Producing *S.* Infantis isolates in German livestock production.**
In: mSphere 4(3) https://doi.org/10.1128/mSphere.00089-19.

Publication 2.

Schielke, Anika; Simon, Sandra; Banerji, Sangeeta; Szabo, Istvan; Malorny, Burkhard; Borowiak, Maria; **Hadziabdic, Sead;** Becker, Natalie; Luber, Petra; Lohr, Dorothee; Harms, Carolin; Plenge-Bönig, Anita; Mellou, Kassiani; Mandilara, Georgia; Mossong, Joël; Ragimbeau, Catherine; Weicherding, Pierre; Hau, Patrick; Dědičová, Daniela; Šafaříková, Lucie; Nair, Satheesh; J. Dallman, Timothy; Larkin, Lesley; McCormick, Jacquelyn; De Pinna, Elizabeth; Severi, Ettore; Kotila, Saara; Niskanen, Taina; Rizzi, Valentina; Deserio, Domenico; Flieger, Antje; Stark, Klaus. 2019. **Salmonellosis outbreak with novel *Salmonella enterica* subspecies *enterica* serotype (11:z41:e,n,z15) attributable to sesame products in five European countries, 2016 to 2017.**

In: Eurosurveillance 24(36) https://doi.org/10.2807/1560-7917.ES.2019.24.36.1800543.

Oral presentations:

1. **Hadziabdic, Sead;** Malorny, Burkhard; Szabo, Istvan; Fischer, Jennie; Kaesbohrer, Annemarie "Current status and ongoing activities of the animal pretrial and *in vitro* experiments (BfR - WP3)" EFFORT Project Annual Meeting. 2017. Sofia, Bulgaria.
2. **Hadziabdic Sead** "Current status of the *in vivo* experiments at BfR (WP3)" EFFORT Project Annual Meeting. 2018. Brussels, Belgium.
3. **Hadziabdic, Sead;** Fischer, Jennie; Malorny, Burkhard; Szabo, Istvan. "Nachweis der Übertragung und Veränderung eines Carbapenemase-kodierenden Plasmids im Tiermodell Huhn", AVID-Tagung/ Bakteriologie. 2019. Bad Staffelstein, Germany.
4. **Hadziabdic, Sead;** Fischer, Jennie; Malorny, Burkhard; Szabo, Istvan. "*In vivo* transfer and adaptation of a carbapenemase-encoding IncA/C_2 plasmid" Symposium für Doktorandinnen und Doktoranden. 2019. Berlin, Germany.
5. **Hadziabdic Sead** "Nachweis der Übertragung und Veränderung eines Carbapenemase-kodierenden Plasmids im Tiermodell Huhn" BfR-Symposium „Zoonosen und Lebensmittelsicherheit". 2019. Berlin, Germany.

Poster presentations:

First author in poster presentations

1. **Hadziabdic, Sead;** Baumann, Bea; Guerra, Beatriz; Szabo, Istvan; Kaesbohrer, Annemarie; Malorny, Burkhard; Fischer, Jennie. In vitro conjugal transfer frequency of multidrug resistant NDM-1 carbapenemase harboring plasmid to different *Salmonella* serovars mainly implicated in human salmonellosis. 5th ASM Conference on *Salmonella*. 2016. Potsdam, Germany.
2. **Hadziabdic, Sead;** Fischer, Jennie; Malorny, Burkhard; Borowiak, Maria; Guerra, Beatriz; Kaesbohrer, Annemarie; Gonzalez-Zorn, Bruno; Szabo, Istvan. In vivo transfer of IncA/C_2 *bla*$_{NDM-1}$-carrying plasmid pRH-1238 to commensal Enterobacteriaceae in a broiler chicken infection model. ECCMID Congress. 2018. Madrid, Spain.
3. **Hadziabdic, Sead;** Fischer, Jennie; Juraschek, Katharina; Irmer, Sophie; Bloch, Angelina; Borowiak, Maria; Malorny, Burkhard; Kaesbohrer, Annemarie, Guerra, Beatriz; Szabo, Istvan. Acquisition of an IncA/C_2 *bla*$_{NDM-1}$-carrying plasmid pRH-1238 in *Salmonella enterica* subsp. *enterica* serovar Infantis during a broiler chicken infection study - a potential threat for human exposure? 26th International ICFMH Conference - FoodMicro 2018. 2018. Berlin, Germany**.**

4. **Hadziabdic, Sead;** Fischer, Jennie; Borowiak, Maria; Malorny, Burkhard; Bloch, Angelina; Juraschek, Katharina; Kaesbohrer, Annemarie; Guerra, Beatriz; Szabo, Istvan. Frequency of *bla*$_{NDM-1}$-carrying IncA/C_2 plasmid structural alterations and co-integrate formation *in vivo*. DRS Doktorandensymposium. 2018, Berlin, Germany.
5. **Hadziabdic, Sead;** Fischer, Jennie; Juraschek, Katharina; Irmer, Sophie; Bloch, Angelina; Borowiak, Maria; Malorny, Burkhard; Szabo, Istvan. *In vivo* acquisition of an IncA/C_2 *bla*$_{NDM-1}$-carrying plasmid pRH-1238 in *Salmonella* Infantis in a broiler chicken infection model. International Symposium *Salmonella* and Salmonellosis. 2018. St. Malo, France.
6. **Hadziabdic, Sead;** Fischer, Jennie; Salatowsky, Denise; Borowiak, Maria; Malorny, Burkhard; Kaesbohrer, Annemarie; Guerra, Beatriz; Szabo, Istvan. *In vivo* persistence and stability of animal *S*. Infantis native *bla*$_{VIM-1}$-carrying IncHI2 plasmid in a broiler infection study. 71. Jahrestagung der Deutschen Gesellschaft für Hygiene und Mikrobiologie e. V. 2019. Göttingen, Germany.

Co-author in poster presentations

1. Fischer, Jennie; **Hadziabdic, Sead;** Malorny, Burkhard; Borowiak, Maria; Guerra, Beatriz; Kaesbohrer, Annemarie; Gonzalez-Zorn, Bruno; Szabo Istvan. Microevolution of *bla*$_{NDM-1}$-carrying plasmid pRH-1238 harboured by avian native *Salmonella* Corvallis during a broiler chicken infection model. ECCMID Congress. 2018. Madrid, Spain.
2. Borowiak, Maria; Hammerl, Jens Andre; Fischer, Jennie; **Hadziabdic, Sead;** Malorny, Burkhard. Diversity of mcr-5 harbouring plasmids in *German Escherichia coli* and *Salmonella enterica* isolates. ECCMID Congress. 2018. Madrid, Spain.
3. Schielke, Anika; Simon, Sandra; Prager, Rita; Szabo, Istvan; Malorny, Burkhard; Borowiak, Maria; **Hadziabdic, Sead**; Becker, Natalie; Luber, Petra; Lohr, Dorothee; Harms, Carolin; Plenge-Bönig, Anita; Mellou, Kassiani; Mandilara, Georgia; Mossong, Joël; Ragimbeau, Catherine; Weicherding, Pierre; Hau, Patrick; Dědičová, Daniela; Šafaříková, Lucie; Nair, Satheesh; Dallman, Tim; Larkin, Lesley; McCormick, Jacquelyn; De Pinna, Elizabeth; Severi, Ettore; Kotila, Saara; Niskanen, Taina; Rizzi, Valentina; Deserio, Domenico; Stark, Klaus. European-wide salmonellosis outbreak with a novel serovar (11:z41:e,n,z15) attributable to sesame products. International Symposium *Salmonella* and Salmonellosis I3S. 2018. Saint-Malo, France.
4. Deneke, Carlus; Grützke, Josephine; **Hadziabdic, Sead;** Malorny, Burkhard. Application of joined shotgun metagenomics and DNA cross-linking technology allows tracking of resistance plasmid transfer *in vivo*. ASM Conference on Rapid Applied

Microbial Next-Generation Sequencing and Bioinformatic Pipelines. 2018. Washington DC area, USA.

5. Fischer, Jennie; **Hadziabdic, Sead**; Borowiak, Maria; Malorny, Burkhard; Bloch, Angelina; Juraschek, Katharina; Käsbohrer, Annemarie; Guerra, Beatriz; Szabo, Istvan. In vivo structural alterations of a *bla*$_{NDM-1}$ carrying IncA/C_2 plasmid and complete sequence of its IncHI2-IncA/C2 co-integrate megaplasmid derivative generated during gastrointestinal passage in a broiler infection study. 12th International Meeting on Microbial Epidemiological Markers (IMMEM XII), 2019, Dubrovnik, Croatia.

Certificates

1. FELASA C Certificate, 2017
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V Conflict of interest

No conflict of interest to declare.

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VII Selbständigkeitserklärung

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 05.08.2020

Sead Hadziabdic

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