In vivo transfer and microevolution of avian native IncA/C$_2$ bla$_{NDM-1}$-carrying plasmid pRH-1238 during a broiler chicken infection study

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Abstract

The emergence and spread of carbapenemase-producing Enterobacteriaceae (CPE) in wildlife and livestock animals poses an important safety concern for public health. With our in vivo broiler chicken infection study we investigated transfer and experimental microevolution of the blaNDM-1-carrying IncA/C2 plasmid (pRH-1238) introduced by avian native Salmonella (S.) Corvallis, without inducing antibiotic selection pressure. We evaluated dependency of the time point of inoculation on donor [S. Corvallis (12-SA01738)] and a plasmid-free Salmonella spp. recipient [S. Paratyphi B (dTa+), 13-SA01617] excretion by quantifying their excretion dynamics. Using S1-PFGE plasmid profiling we gained insight into the variability of native plasmid content among S. Corvallis reisolates as well plasmid acquisition in S. Paratyphi B (dTa+) and enterobacterial gut microflora. Whole genome sequencing enabled us an in-depth insight into microevolution of pRH-1238 plasmid in S. Corvallis and enterobacterial recipient isolates. Our study revealed that the fecal excretion of avian native carbapenemase-producing S. Corvallis is significantly higher and not hampered by S. Paratyphi (dTa+). Acquisition of pRH-1238 in other Enterobacteriaceae and several transfer events of pRH-1238 plasmid to different E. coli sequence types and Klebsiella pneumoniae demonstrate interspecies broad-host range. Regardless of the microevolutionary structural deletions in pRH-1238, the single carbapenem resistance marker, blaNDM-1, was maintained on pRH-1238 throughout the trial. Furthermore, we showed the importance of the gut E. coli population as vector of pRH-1238. In a potential scenario of NDM-1-producing S. Corvallis introduced into a broiler flock, the
pRH-1238 plasmid can persist and spread to a broad-host range even in absence of antibiotic pressure.
**Introduction**

*Salmonella* infections continue to play an important role for 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 last option in treating severe human infections, their use is not licensed in veterinary medicine (3-5). However, the reports revealing appearance of carbapenem resistant/non-susceptible bacteria in livestock (poultry, cattle and swine), food products, wild animals and environment have increased in recent years (6-11). The true extent of carbapenemase producing bacteria in livestock might be underestimated in Europe, due to voluntary basis for screening at EU level (12). One of the most common mechanisms leading to carbapenem resistance is the production of carbapenem-hydrolysing β-lactamases mainly encoded by bla<sub>VIM</sub>, bla<sub>IMP</sub> and bla<sub>NDM</sub> (production of class B metallo-β-lactamases), bla<sub>KPC</sub> (class A β-lactamases) and bla<sub>OXA-48</sub> (class D β-lactamases) genes (13). Worrisome is the worldwide spread of these enzymes by mobile genetic elements, like ICEs and plasmids (14). Among carbapenem resistant/non-susceptible bacteria, the 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 broad geographical reservoirs in many unrelated bacterial species, due to the location of the bla<sub>NDM-1</sub> genes on broad host-range plasmids (15). A recent study has revealed the localization of bla<sub>NDM-1</sub> gene on type 1 IncA/C<sub>2</sub> pRH-1238 [in Fischer et al. publication (11) referred to as pRH-1738] plasmid in avian native *S. Corvallis* strain (12-SA01738) isolated from a...
wild bird (Milvus migrans) 2012 in Germany (11). The discovery of this first completely sequenced \( \text{bla}_{\text{NDM-1}} \)-fosA3-IncA/C plasmid (GenBank Accession number KR091911.1) (16) is of great value due to its host and dissemination potential 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 coexistence of several resistance genes (\( \text{bla}_{\text{NDM-1}}, \text{bla}_{\text{CMY-16}}, \text{fosA3}, \text{sul1}, \text{sul2}, \text{strA}, \text{strB}, \text{aac}(6^{\prime})\text{-Ib}, \text{aadA5}, \text{aph}(\text{A}), \text{tet}(\text{A}), \text{mphA}, \text{dre17}, \text{floR} \) and \( \text{fosA} \)), facilitating resistance to carbapenems, fosfomycins, aminoglycosides, co-trimoxazole, tetracyclines and macrolides (16). The above mentioned studies and recent reports on VIM-1-producing E. 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 is a reality. With recent reports of VIM-1-producing S. Infantis 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 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 major opportunistic pathogen in chickens with a potential for zoonotic transfer to humans (21). Therefore it is of relevance to explore if and to which extent different genus and clonal lines might act as
potential recipients of $\text{bla}_{\text{NDM-1}}$-carrying pRH-1238 plasmid. This is an important concern due to previous confirmation of multidrug-resistant and NDM-1 producing S. Corvallis in a wild bird. Knowing the broad-host range of $\text{bla}_{\text{NDM-1}}$ carrying plasmids, our aim was an insight into a potential scenario of this introduction under experimental conditions, but still mimicking rearing management common to commercial broiler production. Therefore, we aimed to investigate intra- (Salmonella to Salmonella) and interspecies (Salmonella to endogenous gut microflora) transfer capacities of this $\text{bla}_{\text{NDM-1}}$ encoding plasmid without inducing antibiotic selection pressure in a broiler chicken infection study. The objective of our study was (i) to determine excretion dynamics of avian native donor [S. Corvallis (12-SA01738)] and poultry-associated recipient [S. Paratyphi B ($dTa^+$), 13-SA01617] during different inoculation time points, (ii) to analyze the in vivo broad-host range capacity of pRH-1238 plasmid, and (iii) to analyze microevolution of pRH-1238 plasmid (GenBank Accession number KR091911.1) using whole genome sequencing.
Results

Fecal excretion of challenge strains

Group 1 (simultaneous inoculation of challenge strains)

Fecal excretion of challenge strains (expressed as log CFU/g feces) over time in Group 1 is represented in Fig. 2. Highest excretion rates for S. Corvallis (log 5.09 CFU/g) and S. Paratyphi B (dTa+) (4.19 log CFU/g) were observed on 28th and 11th day of life, respectively. On the second day p.i, five animals started to shed S. Corvallis leading up to eleven animals shedding S. Corvallis on day 28. As for S. Paratyphi B (dTa+), on the first day p.i seven animals shed S. Paratyphi B (dTa+), with varying excretion on 9th (seven animals) and 16th day (three animals) of life. Statistical analysis revealed a significant difference between challenge strain excretion on 8th (P=0.018), 16th (P=0.008), 21st (P=0.008), 25th (P=0.003) and 28th (P=0.003), contrary to 9th (P=0.161), 10th (P=0.285) and 11th (P=0.169) day of life.

Group 2 [time delayed inoculation of S. Paratyphi B (dTa+)]

In group 2, highest excretion rates for S. Corvallis (log 5.51 CFU/g) and S. Paratyphi B (dTa+) (3.22 log CFU/g) were on 12th and 16th day of life, respectively (Fig. 3). On the first day p.i, six animals shed S. Corvallis, all through 28th day of life, when ten animals were still excreting S. Corvallis. After S. Paratyphi B (dTa+) inoculation on 10th day of life, four (11th day of life) to nine animals (16th day of life) were excreting S.
Paratyphi B ($dTa+$). In group 1 and 2, after 16th day of life a decrease in $S$. Paratyphi B ($dTa+$) excretion was observed (Fig. 2 and 3).

Group 3 (time delayed inoculation of $S$. Corvallis)

In group 3, highest excretion rates for $S$. Paratyphi B ($dTa+$) ($4.78 \log\ CFU/g$) and $S$. Corvallis ($5.18 \log\ CFU/g$) were reached on 14th and 21st day of life (Fig. 4). In this group, the detection of $S$. Paratyphi B ($dTa+$) varied from three (8th day of life) to eleven animals on 16th day of life. Contrary to the results in groups 1 and 2, where a decrease in $S$. Paratyphi B ($dTa+$) excretion from 16th day onwards was observed, seven and four animals were still excreting $S$. Paratyphi B ($dTa+$) in this group on 21st and 28th day of life, respectively. As for $S$. Corvallis, on the first day p.i two and on 28th day of life eleven animals were shedding $S$. Corvallis.

Fecal excretion of transconjugants

In Group 1 and 3, earliest Enterobacteriaceae transconjugants were detected three days p.i., whereas in Group 2, earliest detection was after two days p.i., respectively (Table 1). In all groups, $E. coli$ transconjugants were detected, whereas Klebsiella transconjugants were detected only in Group 2. During the 21 days p.i., $S$. Paratyphi B ($dTa+$) transconjugants were not detected.
Variability of native plasmid content and plasmid acquisition in challenge strains

The S1-PFGE analysis revealed higher variability as well as losses of ~310 kb IncHI2 and >20 kb ColRNAI plasmids (Fig. S2 and S3) in the native plasmid content of S. Corvallis reisolates in Group 1 and 2 compared to Group 3 (Fig S4). In S. Corvallis reisolates from all groups slight [Group 1 (G1-28d-T10, G1-28d-T10 Post mortem), Group 2 (G2-16d-T1) and Group 3 (G3-28d-T3, G3-28d-T3 Post mortem)] to larger deviation [in Group 1 (G1-11d-T10), Group 2 (G2-8d-T1 and G2-12d-T1) and Group 3 (G3-16d-T3)] in size of ~180 kb pRH-1238 plasmid progeny were observed (Fig. S2, S3 and S4). One S. Paratyphi B (dTa+) reisolate (G1-16d-T5) acquired a ~100 kb plasmid (Fig. S1) lacking of the bla\textsubscript{NDM-1}. Furthermore, S. Corvallis reisolates hybridised with NDM-1 probe revealed that the bla\textsubscript{NDM-1} gene was encoded on 110-130 kb (G1-11d-T10, G2-8d-T1, G2-12d-T1 and G3-16d-T3) and >400 kb (G2-28d-T1) plasmids (Fig S2, S3 and S4) further to 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 and S1-PFGE, respectively. In all three experimental groups macrorestriction with XbaI endonuclease revealed identical PFGE patterns for NDM-1 producing E. coli transconjugants of phylogenetic group A. At individual - animal level, different PFGE
patterns of NDM-1 producing *E. coli* transconjugants on a particular sampling day, e.g. 21st day of life [Group 1 (G1-21d-T7 - I (*E. coli* phylogroup A) and G1-21d-T7 – II (*E. coli* phylogroup B1)], as well as different genera of Enterobacteriaceae [G2-25d-T2 - II (*E. coli* phylogroup D) and G2-25d-T2 - III (*K. pneumoniae*)] (Fig. S5) were observed. Digestion with S1 nuclease revealed that all selected NDM-1 producing Enterobacteriaceae transconjugants acquired the ~180 kb pRH-1238 plasmid, with plasmid contents differing in *E. coli* strains that are belonging to the same phylogenetic group (e.g. phylogenetic group D and A) (Fig. S6). All strains, except one *E. coli* [G3-21d-Environment (I)] isolate, encoded *bla*<sub>NDM-1</sub> gene on a ~180 kb plasmid (see Table 2 and Fig. S6).

**Whole genome sequencing analysis (WGS)**

With WGS analysis we confirmed *in vivo* transfer of pRH-1238 plasmid to different *E. coli* ST-types [ST-117, ST-156, ST-2040 and ST-2485] as well as to a *K. pneumoniae* strain (ST-1106). Furthermore, we reconfirmed the loss of ~310 kb IncHI2 plasmid in one (G2-16d-T1) and 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, ~310 kb IncHI2 plasmids were not detected in any of the NDM-1 producing Enterobacteriaceae transconjugants (Table 2). The position of *bla*<sub>NDM-1</sub> gene in all *S. Corvallis* reisolates on pRH-1238 plasmid progeny could be confirmed, whereas one strain (G1-11d-T10) did not harbor the *bla*<sub>CMY-16</sub> gene (Table 2). One *S. Corvallis* reisolate (G2-28d-T1) harbored a *bla*<sub>NDM-1</sub>.
carrying >400 kb plasmid (Fig. S3) (based on Southern blot and hybridization of S1 restricted PFGE). Mapping of this strain to reference pRH-1238 yielded consensus sequence identity of 99.53% and the >400 kb plasmid for this strain might resemble a fusion of IncA/C2 (pRH-1238) and IncHI2 (~490 kb).

Further to the resistome of pRH-1238, additional resistance genes conferring resistance to beta-lactams (blaTEM-1B) were detected in three S. Corvallis (G1-11d-T1, G1-21d-T1, G1-28d-T1) whereas one S. Paratyphi B (dTa+) reisolate (G1-16d-T5) besides the acquisition of ~100 kb IncI1 plasmid did not harbor additional resistance genes. Additional resistance genes for NDM-1 producing Enterobacteriaceae are shown in Table 2.

Based on the consensus sequence mapping of the pRH-1238 plasmids progeny to reference pRH-1238 from S. Corvallis reisolates in all groups a deletion in Tra1 region (~50 to 60 kb in size) was observed (Fig. 5) which in one strain (G1-11d-T10) led to loss of blaCMY-16. Another noteworthy result is the high percent of sequence identity among the pRH-1238 progeny from selected Enterobacteriaceae transconjugants in contrast to S. Corvallis reisolates (Table 2. and Fig 6).
Discussion

Recent publications have reported occurrence of carbapenem non-susceptible 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 co-selective pressure, since $bla_{NDM-1}$ encoding plasmids, as chosen 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 mechanisms contributing to the spread of carbapenem resistance or carbapenem non-susceptible isolates in vivo the objective of our animal trial was to explore broad-host range capacity and stability of a conjugative $bla_{NDM-1}$ carrying plasmid pRH-1238 IncA/C2, hosted by a S. Corvallis strain, in chickens, without antimicrobial selection pressure, representing non-use of carbapenems in livestock. With the help of WGS, such setup enabled us an insight into the microevolution of the plasmid in vivo.

Challenge strains excretion

During our study, we observed prolonged fecal excretion of NDM-1 carbapenemase-producing S. Corvallis (12-SA01738), contrary to S. Paratyphi B ($dTa$) (13-SA01617). The statistical analysis of data in Group 1 [simultaneous inoculation of S.
Corvallis and S. Paratyphi B (dTa+) reveals that the fecal excretion of S. Corvallis is significantly higher towards the end of trial (16th, 21st, 25th and 28th day of life) (Fig. 2) and not hampered by later inoculation of S. Paratyphi B (dTa+), as in Group 2 (statistically significant difference between S. Corvallis excretion in Group 1 and 2 only by 8th day of life) (Fig 2 and 3). Because of previous studies reporting invasiveness of S. Paratyphi B (dTa+) towards epithelial cells, macrophages and their presence in ceca, the liver, and the spleen (24), the decreased excretion of S. Paratyphi B (dTa+) observed in our in vivo trial is a noteworthy appearance. Although this serovar is reported to be broiler-associated, we have not observed competitive advantage contrary to S. Corvallis. On the other hand, prolonged excretion of NDM-1 producing S. Corvallis in the absence of antibiotic pressure is an important concern due to its resistome and broad host range of pRH-1238 plasmid.

In vivo transfer of blaNDM-1 harboring plasmid pRH-1238

In our study we demonstrate in vivo transfer of IncA/C2 blaNDM-1 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 MLST-types (ST-117, ST-156, ST-2040 and ST-2485) and a K. pneumoniae isolate (ST-1106), respectively (Table 2). At individual level, on particular sampling days, we observed not only pRH-1238 acquisition in different E. coli strains but also in different Enterobacteriaceae genera (Table 2). This, together with their rapid onset of excretion (Table 1), demonstrates broad host range and high transferability of this multidrug-resistance plasmid leading to multidrug-
resistance acquisition in one horizontal gene transfer event. The affected genera (*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*<sub>NDM-1</sub> (25). This deserves attention, especially in commercial broiler production where contamination pressure, due to continuous rearing cycles as well as short inter-service breaks could lead to continuous propagation of pRH-1238 within a mixed bacterial population. With previous detection of avian native NDM-1 carbapenemase-producing *S. Corvallis* in wild birds, such entry scenario in commercial broiler production would presumably lead to rapid and diverse *bla*<sub>NDM-1</sub> dissemination within a broiler flock even without antibiotic pressure. This might also lead to environmental contamination, as observed for ESBL/AmpC-producing *E. coli* strains (26). The broad-host range and the high transferability without antibiotic pressure should be kept in mind with implementation of preventative measures. Instead of relying only on selective and co-selective pressure as a measure to minimize carbapenemase-producing bacteria, further approaches assessing quantification of resistance genes dissemination with and without selective antibiotic pressure should be taken into account.

The detection of enterobacterial transconjugant strains until the end of the trial [Group 1 (from 10th), Group 2 (from 9th) and in Group 3 (from 13th day of life onwards)] (see Table 1) underlines 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), which is further emphasized by the observed plasmids and resistance gene acquisition from gut microflora in challenge strains (Table 2).
As we did not detect NDM-1 producing S. Paratyphi B (dTa+) transconjugants, we assume that the host’s *E. coli* population has an important influence on reception and further spread of pRH-1238 plasmid. This might be linked to their dense and diverse population and host gut adaptation, 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 (dTa+) (Table S2), their absence *in vivo* might be linked to (i) serovar colonization dynamics, (ii) abundance, diversity and interference of *E. coli* strains and (iii) the detection limit of the method used in this study (~100 CFU/g).

Majority of *E. coli* NDM-1 producers belonged to phylogroup A (represented by ST-2040), however sequence types ST-117 and ST-156 were also detected. These ST-types are besides poultry associated also described as potential source of not only beta-lactam but also polymixin genes (29-31). In a recent publication a human acquired *mcr*-1-carrying ST-117 from avian origin was characterized highlighting the capability of this ST in resistance gene acquisition (30). Observed dominance of *E. coli* strains belonging to phylogroup A might resemble their occurrence in the gut or their capability in serving as native recipients for pRH-1238, as described for certain clonal lines being dominant in the spread of quinolone-resistance plasmid mediated *oqxAB* gene (23).

Furthermore, the *bla*<sub>CMY-16</sub> is a variant of *bla*<sub>CMY-2</sub> lineage which is described as the most common plasmid-mediated AmpC enzyme worldwide common to different Enterobacteriaceae (32). Therefore, the introduction of pRH-1238 plasmid into a broiler flock should be assessed as well in light of potential further dissemination of not only *bla*<sub>NDM-1</sub>, but also *bla*<sub>CMY-16</sub>, 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 plasmid-mediated resistance genes relevant to public health and questioning the genera or serovar dominance in this exchange. Such data could contribute to a wider picture and broaden our knowledge for carbapenem resistance risk assessment and serve as an asset for future approaches minimizing spread of antimicrobial resistance *in vivo*.

**Plasmid content variability in *S. Corvallis* reisolates**

The observed native plasmid variability (~310 kb IncHI2), 180 kb pRH-1238 (IncA/C2) and >20 kb ColRNAI] was predominant in *S. Corvallis* reisolates from Group 1 and 2 (Fig. S2 and S3). This observation leads us to the assumption that simultaneous and initial inoculation of *S. Corvallis* led to certain rearrangement mechanisms in native plasmid content, observed as complete loss (~310 kb IncHI2 and >20 kb ColRNAI plasmids) or partial region deletions in ~310 kb IncHI2 (up to ~100 kb) and in ~180 kb pRH-1238 (up to ~50-60 kb) (Fig. S2, S3 and S4). We speculate that an earlier (7th day of life) inoculation of *S. Corvallis* in experimental groups 1 and 2 led to this occurrence.

In a recent chemostat study by Card *et al.* (33), which mimicked the broiler microbiome, it seems that the bacterial community stabilised by day 6. In our case, this unstable microbial population might support mobilome restructuring as well interaction and subsequent acquisition of *bla*TEM-1B in *S. Corvallis* reisolates from Group 1 in later stages of the trial (see Table 2). Plasmid exchange and certain structural deletions might be also an important part of host adaptation regulation. Previous studies have reported that acquisition and losses of certain genetic elements in bacteria are stimulated by the
adaptation to 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 XLD and chromID® CARBA agar and we did not conduct metagenomics analysis, we presume that blaTEM-1B might have originated from *E. coli* ST-2040. Furthermore, it seems this sequence type played a significant role in plasmid/s exchanges (acquisition of pRH-1238 and ColRNAI and transfer of ColpVC plasmid) with *S. Corvallis* (see Table 2).

**Microevolution of pRH-1238 in S. Corvallis and enterobacterial transconjugants**

Besides plasmid content variability observed after S1 restriction for 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 region and downstream (~50 to 60 kb size) between two resistance islands: ARI-A (harboring sul1, blaNDM-1, aph6, mph(A) and aac6′lb) and ARI-B (harboring sul2, strA, strB, tet(A), floR, fosA3, sul1, aadA5, dfrA7) (16). These deletions did not lead to significant alteration of pRH-1238 beta-lactam resistome, as only one strain did not harbor *blaCMY-16* (see Table 2 and Fig. 5) due to its position adjacent to Tra1 region of pRH-1238. This occurrence is in line with observations indicating large-scale structural changes often observed in neighborhood areas of transposons and IS-elements, indicating that these elements contribute to plasmids genome evolution (36). In a recent *in vitro* study by Porse et al. (37) deletions in IncN plasmid (constituting also of Tra regions) in recipient *E. coli* strains were observed, contrary to native *K. pneumoniae* and recipient *Klebsiella* strains. The
authors state that this occurrence might possess potential competitive benefit for recipient *E. coli* strains. In contrast to our *in vivo* study, where the deletions in pRH-1238 progeny were dominant in *S. Corvallis* reisolates and not *E. coli* and *K. pneumoniae* strains (see Fig. 5 and 6), suggesting that these deletions might be host or incompatibility group dependent. Generally, observed losses of IncHI2 and ColRNAI plasmids as well deletions in pRH-1238 progeny might indicate an evolutionary background in *S. Corvallis* adaptation, which enabled maintenance of pRH-1238 resistome even without antibiotic pressure in wild birds.

A noteworthy observation is a >400 kb plasmid in sample G2-28d-T1 (see Table 2 and Fig. S3) which seems to be a fusion of IncHI2 and IncA/C2 pRH-1238. This mobilome restructuring might be triggered by intrinsic *S. Corvallis* mechanisms and also linked with persistence of bla*NDM-1* in *S. Corvallis*. Namely, plasmid fusion and co-integration is a frequent phenomenon in plasmid evolution and adaptation by preventing *e.g.* plasmid incompatibility and facilitating interaction with a broad range of hosts (38). For better understanding it is of interest to explore if these occurrences are triggered by certain metabolic processes in the gut, bacterial stress or possible interaction with competitive gut microflora. Interestingly, pRH-1238 progeny from two strains sampled from cecal content showed high percentage of sequence identity to the pRH-1238 backbone (Table 2 and Fig. 5). Such occurrence indicates that the *S. Corvallis* reisolates harboring native pRH-1238 are existing in the intestinal tract and continuously disseminating pRH-1238 *in vivo*. Previous findings report on higher colonisation of *Salmonella* in ceca leading to higher conjugation rates, observed for conjugative extended-spectrum cephalosporins resistance-encoding 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 region and revealed a higher degree of identity to the reference backbone of pRH-1238 (see Table 2 and Fig. 6). This indicates that the pRH-1238 acquisition or transfer process itself might not lead to 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 prolonged fecal excretion of avian native NDM-1 carbapenemase-producing *S. Corvallis* strain (12-SA01738), with microevolutionary deletions in the pRH-1238 backbone while preserving *bla*NDM-1 gene, during a broiler chicken *in vivo* study. The conjugative pRH-1238 IncA/C2 *bla*NDM-1 plasmid is transferable to different Enterobacteriaceae expanding its resistance pool among gut microflora in absence of antibiotic pressure throughout the trial. This study shows at molecular level how rapid and diverse dissemination of *bla*NDM-1 harboring IncA/C2 plasmids in commercial broiler production can occur even in the absence of selective pressure. Furthermore, it highlights the need for understanding interaction mechanisms of host microflora and *Salmonella* serovars and calls for additional efforts in future intervention approaches to avoid further spread of multidrug resistance plasmids in commercial broiler production.
Material and Methods

Challenge strains

As donor strain, avian native *Salmonella enterica* subsp. *enterica* serovar Corvallis [S. Corvallis (12-SA01738)], sequence type ST-1541 harboring *bla*\(_{NDM-1}\)* IncA/C\(_2\) pRH-1238 plasmid (GenBank Accession number KR091911.1) was selected. As recipient, native *d*-tartrate fermenting *Salmonella enterica* subsp. *enterica* serovar Paratyphi B (13-SA01617) [S. Paratyphi B (*dTa*)] ST-28, isolated in 2013, with intrinsic resistance to nalidixic acid was selected. The pRH-1238 plasmid is the first completely sequenced *bla*\(_{NDM-1}\)-*fosA*3-IncA/C plasmid with 187,683 bp in size, 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 the *bla*\(_{NDM-1}\) located in ARI-A and *bla*\(_{CMY-16}\) in Tra1 region (16). Besides the pRH-1238, the donor strain harbors two additional plasmids of incompatibility group IncHI2 (~310 kb) and ColRNAI (>20 kb), whereas S. Paratyphi B (*dTa*) was selected as plasmid-free recipient strain. Phenotypic and genotypic properties of donor and recipient strains are listed in Table S1. The selection of S. Paratyphi B (*dTa*) was based on high prevalence in commercial poultry production in Germany (40) as well as optimal *in vitro* conjugation transfer frequency (CTF) at 42°C (corresponds to average body temperature of birds) per S. Corvallis as 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 (see Table S2) were conducted to determine average conjugation transfer frequency (CTF) for four potential recipient strains per S. Corvallis (12-SA01738) as donor. After aerobic growth with gentle shaking at 37°C to obtain optical density (OD 560 nm) value of 0.25, a mixture of a ratio of 1 to 2 between Salmonella donor and recipient was centrifuged (20,000 × g during 2 min), inoculated on 0.45 μm filter membranes (Merck Milipore, Germany) previously placed on Lysogeny Agar - LBA (Thermo Fisher Scientific, Germany) and incubated for 4 h at room temperature (RT), 37°C and 42°C. Following incubation, filter membranes were suspended in 4 ml of Lysogeny Broth - LBL (Thermo Fisher Scientific, Germany), decimally diluted and plated on transconjugant selective plates (according to Table S3). All filter mating conjugation experiments were conducted in triplicates 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 one day-old chicks, without prior chick sexing. Housing, clinical examination, individual labelling and sampling followed. Animals were randomly divided into three experimental groups (G1, G2 and G3), each containing 11 animals (T1-T11) and housed in the facilities for Animal Experiments at the Federal Institute for Risk Assessment, Berlin,
Germany. In order to evaluate the dependency of the time point of inoculation on challenge strains, excretion during 28 days of experiment, three experimental setups resembled in Group 1, 2 and 3 were designed. In Group 1, simultaneous inoculation of donor and recipient on 7th day of life, whereas in Group 2 (7th day donor and 10th day recipient) and Group 3 (7th day recipient and 10th day donor) time delayed inoculations followed. At the end of the experiment (28th day of life), all animals were handled carefully following electrical stunning before being sacrificed for Post mortem ceca extirpation. Experiment design, containing the time frame and related activities is shown in Fig. 1. During the experiment, microambiental conditions complied to hybrids management guide and animals were checked daily for health and wellbeing criteria. Animal trials were approved by the German State Authority for Health and Social Affairs (Lageso) (Nr. 0308/15).

To prevent unintentional cross-reaction with intestinal microbiota, one day-old chicks were tested for a 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 spp. following ISO 6579:2002/Amd 1:2007 (International Organization for Standardization, Switzerland). The procedure was repeated on the day of inoculation to reconfirm 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 optical density (OD 560 nm) value of 0.35, which corresponded to bacterial counts of $4 \times 10^6$ CFU per 100 µl for both strains used as inoculum. On day 7, animals were orally inoculated [Group 1, (donor and recipient), Group 2, (donor), and Group 3, (recipient strain)], followed by second inoculation [for Group 2 (recipient) and for Group 3 (donor strain)] on 10th day of life. After inoculation, a four day consecutive sampling was performed and further sampling two times per week towards the end of trial (Fig. 1). Animals were always sampled individually in a particular time frame with preweighed cotton cloacal swabs (Deltalab, Spain) in order to determine counts of excreted challenge strains, expressed as CFU/g feces.

Bacterial strain isolation

After suspending the fecal material (~0.2 g) in 5 ml of 0.85% (w/v) NaCl, suspension was subjected to decimal dilution and 100 µl deposition volume per plate were plated with an automatic spiral plater in duplicates on selective agar plates using the spiral colony counting technique by Whitley Automatic Spiral Plater (Don Whitley Scientific, UK). On the first day post inoculation (p.i.), dilutions of 1:10 and $1:10^3$ were plated, and later adjusted to 1:10 and $1:10^2$ based on 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/l), cefotaxime (1 mg/l) and/or nalidixic acid (50 mg/l)] depending on target strain (donor, recipient and *Salmonella* transconjugants), and chromID® CARBA (BioMérieux, France) for detection of carbapenemase-producing Enterobacteriaceae (CPE) (Table S3). *Salmonella* suspicious colonies were detected on XLD agar as red-yellow colonies with black center, and CPE (*e.g.* *E. coli, Klebsiellae*) on chromID® CARBA as purple and blue colonies, respectively. In order to further characterize [*e.g.* plasmid content variability in *S. Corvallis* and plasmid acquisition in *S. Paratyphi B* (*dTα+*)] challenge strain reisolates from particular chicks within each group [*S. Corvallis* reisolates in Group 1 (T10 chick, see below), Group 2 (T1), Group 3 (T3), and for *S. Paratyphi B* (*dTα+*) in Group 1 (T5), in Group 2 (T1), in Group 3 (T3)] were preserved, whereas when possible four subcolonies (marked as I to IV) of presumptive NDM-1 carbapenemase-producing Enterobacteriaceae transconjugants were selected for molecular characterisation. Selected strains were denoted based on the group (G1 to G3), days of life (1d to 28d), chicks (T1 to T11) and subcolony (I-IV) origin.

**Confirmation of presence of pRH-1238 in transconjugants**

Transconjugants were screened by *bla*<sub>NDM-1</sub> and *bla*<sub>CMY-16</sub> PCR-amplification using 1:10 diluted overnight culture as template according to previous publications (42, 43). PCR reactions (25 µl reaction volume) contained 17.5 µl of mastermix [4 µl primer mix (each 400 nM), 2.5 µl dNTPs mix (200 µM), 2.5 µl 10X buffer, 1.25 µl MgCl<sub>2</sub> (2.5 mM), 7.05 µl PCR grade water, 0.2 µl Taq DNA polymerase (Invitrogen, USA)] and 7.5 µl DNA template under following conditions: initialization (5 min at 94°C), denaturation,
annealing and extension [30 s at 94°C, 30 s at 54°C and 1 min at 72°C (30 cycles)],

elongation (5 min at 72°C) and final hold at 4°C.

**Genus/ species identification of transconjugants through Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS)**

Random selection of phenotypically different (purple and blue) colonies of Enterobacteriaceae transconjugants grown on chromID® CARBA were tested by MALDI-TOF-MS (Bruker Daltonik, Germany) to confirm taxonomical classification at genus and species level. After subculturing on LBA, a small amount of bacterial colony was transferred in duplicates onto target wells of MSP 96 polished steel BC plate (Bruker Daltonik, Germany) and suspended in 1 µl of HCCA matrix (Bruker Daltonik, Germany), according to the manufacturer’s instructions. After air dried at room temperature, measurement of mass spectrometry using MALDI Flexcontrol (Bruker Daltonik, Germany) and identification of microorganisms according to obtained measurement values: 2.300-3.000 (very sure species identification), 2.000-2.299 (sure genus identification, probable species identification), 1.700-1.999 (probable genus identification) and 0.000-1.699 (not reliable identification) by the use of MALDI Biotyper (Bruker Daltonik, Germany) database was conducted.
Molecular characterisation of transconjugants

Phylotyping of E. coli transconjugants

Multiplex PCR assay for phylotyping of 104 selected E. coli transconjugants [Group 1 (n=43), Group 2 (n=19) and Group 3 (n=42)] into A, B1, B2 and D phylogenetic groups was conducted (44). All PCR reactions were done in 25 µl of reaction volume, containing 18 µl of mastermix [5 µl of primer mix (each 10 pmol), 2.5 µl dNTPs mix (200 µM), 2.5 µl 10X buffer, 7.5 µl PCR grade water, 0.5 µl Taq DNA polymerase (Invitrogen, USA)] and 7 µl DNA template (1:10 diluted overnight culture) under following conditions: initialization step (5 min at 94°C), denaturation, annealing and extension [30s at 94°C, 30s at 65°C and 30s at 72°C (30 cycles)], elongation (5 min at 72°C) and final hold at 4°C.

XbaI-PFGE analysis

Macrorestriction with XbaI endonuclease (Roche Applied Sciences, Switzerland) was performed for 104 NDM-1 producing Enterobacteriaceae transconjugant strains according to PulseNet standardized protocol (www.pulsenetinternational.org) (45) using the CHEF-DRIII system (Bio-Rad Laboratories, Madrid, Spain) for separation of fragments. As molecular-sized standard, Salmonella Braenderup strain H9812 (restricted with XbaI) was used. Gel imaging in GenBox (Syngene, UK) and gel documentation by GeneSnap (Syngene, UK) software was conducted.
Molecular characterisation of pRH-1238 plasmid

S1-PFGE plasmid profiling

In order to evaluate variability in native plasmid content [IncHI2 (~310 kb), IncA/C2 pRH-1238 (187,683 bp) and ColRNAI (>20 kb)] among S. Corvallis and plasmid/s acquisition in S. Paratyphi B (dTa+), in total 20 [Group 1 (T10, n=6), Group 2 (T1, n=8) and in Group 3 (T3, n=6)] and 18 [Group 1 (T5, n=4), Group 2 (T1, n=6) and in Group 3 (T3, n=8)] reisolates were typed by S1-PFGE, respectively. 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 Blot, as size marker MidRange PFG Marker (Biolabs, USA) was used. The generated fragments were separated using the CHEF-DRIII system (Bio-Rad Laboratories, Spain) with S-1 running conditions (1s-25s, 17 h, 6 V/cm, 120 V), as previously described (46).

Southern Blot of S1-PFGE gel hybridised with blaNDM-1 probe

To map the position of a single carbapenem resistance marker - the blaNDM-1 gene on pRH-1238, Southern blot and hybridization of S1-PFGE gel with an digoxigenin-labelled NDM-1 probe of 20 selected S. Corvallis reisolates and 16 NDM-1 producing Enterobacteriaceae [Group 1 (n=5), Group 2 (n=6) and Group 3 (n=5)] belonging to different E. coli phylogroups as well one K. pneumoniae was conducted as previously described (46).
Whole genome sequencing analysis

Based on variability of plasmid content detected with S1-PFGE analysis, the genome of 25 strains was sequenced including 15 challenge strain reisolates [S. Corvallis (n=14) and S. Paratyphi B (dTa+) (n=1) and ten NDM-1 producing Enterobacteriaceae transconjugants. All strains selected for whole genome sequencing are listed in Table 2.

Genomic DNA was extracted using the PureLink® Genomic DNA Mini Kit (Invitrogen, USA), followed by fluorometric DNA concentration (ng/µl) measurement by Qubit Fluorometric Quantitation (Invitrogen). Sequencing libraries were prepared using NEXTERA XT DNA Sample Preparation Kit (Illumina, San Diego, USA) according to the manufacturer’s protocol. Paired-end sequencing was performed on the Illumina Miseq benchtop (Miseq Reagent v3 600 cycle Kit, 2 x 300 cycles). Raw reads were assembled de novo using CLC Genomics Workbench 9.5 (Qiagen, Denmark) and ST-types, plasmid content and resistance genes were detected using the services BatchUploader (47), PlasmidFinder (48) and ResFinder (49), available at Center for Genomic Epidemiology - CGE (http://www.genomicepidemiology.org).

In order to evaluate microevolutionary changes in blaNDM-1-carrying pRH-1238 plasmid among S. Corvallis reisolates and to assess possible structural deletions in its 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 9.5. Percent of sequence identity was calculated based on the consensus sequence size in
reference to sequence of pRH-1238 whereas their visualisation was done using BLAST Ring Image Generator 0.95 – BRIG (50).

**Statistical analysis of challenge strains excretion**

For statistical analysis of challenge strains faecal excretion, SPSS21 version 2.0 (SPSS Inc., USA) was used. Due to non-normal distribution of bacterial counts excretion data, a log-transformation was conducted. In experimental group 1 (simultaneous inoculation) excretion of challenge strains was compared using Wilcoxon Matched Pairs Test, based on the related data assumption, and between groups by Mann-Whitney U-Test. Differences were considered significant if the \( P \) value was less than or equal 0.05. Challenge strains 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). Outliers, numerically distant from the rest of the data, were included for determination of the statistical significance.
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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 analysed whole genome sequencing data. B.M, B.G, A.K and B.G.Z. revised the draft version of the manuscript.

Disclaimer

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

| Days of life | Number of positive animals | Group 1<sup>a</sup> | Group 2<sup>a</sup> | Group 3<sup>b</sup> |
|-------------|----------------------------|---------------------|---------------------|---------------------|
| 8th         |                            | 0                   | 0                   | -                   |
| 9th         |                            | 0                   | 1                   | -                   |
| 10th        |                            | 1                   | 3                   | -                   |
| 11th        |                            | 3                   | 3                   | 0                   |
| 12th        |                            | n.d.<sup>c</sup>    | 4                   | 0                   |
| 13th        |                            | n.d.                | 3                   | 2                   |
| 14th        |                            | n.d.                | 1                   | 2                   |
| 16th        |                            | 1                   | 3                   | 1                   |
| 21st        |                            | 9                   | 4                   | 6                   |
| 25th        |                            | 4                   | 4                   | 6                   |
| 28th        |                            | 5                   | 2                   | 5                   |

<sup>a</sup> inoculation of S. Corvallis on 7th day of life  
<sup>b</sup> inoculation of S. Corvallis on 10th day of life  
<sup>c</sup> not determined  
<sup>d</sup> days prior to S. Corvallis inoculation
TABLE 2. Molecular characteristics of selected challenge strain reisolates and transconjugants

| Species or serovar | ID* | Sequence type | Inc designations                      | Percent sequence identity to pRH-1238 and size of pRH-1238 progeny in kb | Presence of bla<sub>NDM</sub> and Additional resistance genes |
|-------------------|-----|---------------|---------------------------------------|-----------------------------------------------------------------------|-------------------------------------------------------------|
| S. Corvallis      | G1-11d-T10 | ST-1541 | IncHE1,IncAC1,ColI,ColIV,ColV,ColIVA,ColIVA | 86.23 (~100 kb)                                                      | Only bla<sub>NDM</sub>-1                                    |
| S. Corvallis      | G1-16d-T10 | ST-1541 | IncHE1,IncAC1,ColI,ColIV,ColIVA       | 99.43 (~180 kb)                                                      |                                             |
| S. Corvallis      | G1-21d-T10 | ST-1541 | IncHE1,IncAC1,ColI,ColIV,ColIVA       | 99.66 (~180 kb)                                                      |                                             |
| S. Corvallis      | G1-28d-T10 | ST-1541 | IncHE1,IncAC1,ColI,ColIV,ColIVA       | 95.55 (~170 kb)                                                      |                                             |
| S. Corvallis      | G1-28d-T10<sup>II</sup> | ST-1541 | IncHE1,IncAC1,ColI,ColIV,ColIVA       | 98.69 (~180 kb)                                                      |                                             |
| S. Corvallis      | G2-6d-T11 | ST-1541 | IncHE1,IncAC1,ColI,ColIV,ColIVA       | 79.17 (~140 kb)                                                      |                                             |
| S. Corvallis      | G2-10d-T11 | ST-1541 | IncHE1,IncAC1,ColI,ColIV,ColIVA       | 99.54 (~180 kb)                                                      |                                             |
| S. Corvallis      | G2-12d-T11 | ST-1541 | IncHE1,IncAC1,ColI,ColIV,ColIVA       | 73.46 (~130 kb)                                                      |                                             |
| S. Corvallis      | G2-16d-T11 | ST-1541 | IncAC2,ColIV,ColIVA                   | 99.66 (~190 kb)                                                      |                                             |
| S. Corvallis      | G2-25d-T11 | ST-1541 | IncAC2,ColIV,ColIVA                   | 99.66 (~190 kb)                                                      |                                             |
| S. Corvallis      | G2-98d-T11 | ST-1541 | IncAC2,ColIV,ColIVA                   | 95.54 (~400 kb)                                                      |                                             |
| S. Corvallis      | G3-16d-T11 | ST-1541 | IncHE1,IncAC1,ColI,ColIV,ColIVA       | 75.69 (~140 kb)                                                      |                                             |
| S. Corvallis      | G3-28d-T11 | ST-1541 | IncHE1,IncAC1,ColI,ColIV,ColIVA       | 95.69 (~180 kb)                                                      |                                             |
| S. Corvallis      | G3-28d-T11<sup>II</sup> | ST-1541 | IncHE1,IncAC1,ColI,ColIV,ColIVA       | 99.66 (~180 kb)                                                      |                                             |
| E. coli           | G1-21d-T10<sup>II</sup> | ST-2040 | IncK1,IncAC1,ColI,ColIV,ColIVA,ColIV | 95.76 (~180 kb)                                                      |                                             |
| E. coli           | G1-21d-T10<sup>III</sup> | ST-156 | IncAC2                                 | 99.83 (~180 kb)                                                      |                                             |
| E. coli           | G1-21d-T10<sup>IV</sup> | ST-2040 | IncK1,IncAC1,ColI,ColIV,ColIVA,ColIV | 99.76 (~180 kb)                                                      |                                             |
| E. coli           | G1-21d-T10<sup>IV</sup> | ST-156 | IncAC2                                 | 99.83 (~180 kb)                                                      |                                             |
| E. coli           | G1-21d-T10<sup>IV</sup> | ST-2040 | IncAC2                                 | 99.76 (~180 kb)                                                      |                                             |
| E. coli           | G1-21d-T10<sup>IV</sup> | ST-156 | IncAC2                                 | 99.83 (~180 kb)                                                      |                                             |
| E. coli           | G1-21d-T10<sup>IV</sup> | ST-2040 | IncAC2                                 | 99.76 (~180 kb)                                                      |                                             |
| E. coli           | G2-21d-T10<sup>IV</sup> | ST-117 | IncK1,IncAC1,ColI,ColIV               | 99.68 (~180 kb)                                                      |                                             |
| E. coli           | G2-21d-T10<sup>IV</sup> | ST-2485 | IncAC2                                 | 99.85 (~180 kb)                                                      |                                             |
| E. coli           | G2-21d-T10<sup>IV</sup> | ST-2485 | IncAC2                                 | 99.85 (~180 kb)                                                      |                                             |
| K. pneumoniae     | G2-21d-T10<sup>IV</sup> | ST-1106 | ColI,MG260,ColAC1,ColIV               | 99.53 (~180 kb)                                                      |                                             |
| E. coli           | G3-21d-T10<sup>IV</sup> | ST-2040 | IncK1,IncAC1,ColI,ColIV               | 99.62 (~180 kb)                                                      |                                             |
| E. coli           | G3-21d-T10<sup>IV</sup> | ST-2040 | IncK1,IncAC1,ColI,ColIV               | 95.74 (~180 kb)                                                      |                                             |
| E. coli           | G3-21d-T10<sup>IV</sup> | ST-2040 | IncK1,IncAC1,ColI,ColIV               | 95.31 (~180 kb)                                                      |                                             |

* Designations according to group (G1 to G3), days of life (1d to 28d), animal (T1 to T11) and subcolonies (I to IV) origin
b. Based on consensus sequence length (CLC Genomics Workbench 9.5)
c. Designations based on S1-PFGE restriction
d. Post mortem (cecal content isolates)
TABLE AND FIGURE LEGENDS

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

TABLE 2. Molecular characteristics of selected challenge strain reisolates and transconjugants

FIGURE 1. Experiment design of the test groups (Group 1 to 3) with inoculation (red marked) and sampling days (blue marked) as well end of the experiment (blue-black marked)

FIGURE 2. Fecal excretion of donor (S. Corvallis) and recipient [S. Paratyphi B (dTa+)] in Group 1 (simultaneous inoculation of donor and recipient at day 7) expressed as log CFU per g feces with outliers (°) and extreme outliers (*) included

FIGURE 3. Fecal excretion of donor (S. Corvallis) and recipient [S. Paratyphi B (dTa+)] in Group 2 (delayed recipient inoculation on day 10) expressed as log CFU per g feces in Group 2 with outliers (°) and extreme outliers (*) included

FIGURE 4. Fecal excretion of donor (S. Corvallis) and recipient [S. Paratyphi B (dTa+)] in Group 3 (delayed donor inoculation on day 10) expressed as log CFU per g feces in Group 3 with outliers (°) and extreme outliers (*) included

FIGURE 5. Visualization of assemblies of 14 pRH-1238 consensus sequences from S. Corvallis reisolates mapped to reference pRH-1238 plasmid (GenBank Accession number KR091911.1). The innermost circle - pRH-1238 coordinates, second - GC content (both black), Group 1 (grey), Group 2 (green) and Group 3 (yellow). Outermost annotations of resistome (black, and red – bla genes) and deletions (dark...
grey) in Tra1 and adjacent region using BRIG (50). Note in strain G1-11d-T10 pRH-1238 progeny loss of \( \text{bla}_{\text{CMY-16}} \) gene.

FIGURE 6. Visualization of assemblies of ten pRH-1238 consensus sequences from carbapenemase-producing \( E. \ coli \) (ST-117, ST-156, ST-2040 and ST-2485) and \( K. \ pneumonias \) (ST-1106) transconjugants mapped to reference pRH-1238 plasmid (GenBank Accession number KR091911.1). The innermost circle - pRH-1238 coordinates, second - GC content (both black), ST-117 (grey), ST-156 (yellow), ST-2040 (green), ST-2485 (blue) and ST-1106 (purple). Outermost are annotations of resistome (black and red – \( \text{bla} \) genes) using BRIG (50). Note in strain G3-21d-Environment (I) pRH-1238 progeny loss of \( \text{bla}_{\text{NDM-1}} \) gene.
