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Prevalence and zoonotic transmission of colistin-resistant and carbapenemase-producing Enterobacterales on German pig farms

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ABSTRACT

The treatment of infections due to colistin-resistant (Col-E) and carbapenemase-producing (CPE) Enterobacterales challenges clinicians both in human and veterinary medicine. Preventing zoonotic transmission of these multidrug-resistant bacteria is a Public Health priority.

This study investigates the prevalence of Col-E and CPE on 81 pig farms in North-West Germany as well as among 138 directly exposed humans working on these farms. Between March 2018 and September 2020, 318 samples of porcine feces were taken using boot swabs. Farm workers provided a stool sample. Both a selective culture-based approach and a molecular detection of colistin (*mcr*-1 to *mcr*-5) and carbapenem resistance determinants (bla_{OXA-48}/bla_{VIM}/bla_{KPC}/bla_{NDM}) was used to screen all samples. Isolates from farm workers and farms were compared using core genome multilocus-sequence typing (cgMLST) and plasmid-typing.

CPE were cultured neither from porcine feces nor from human stool samples. In one stool sample, bla_{OXA-48} was detected, but no respective CPE isolate was found. Col-E were found in 18/318 porcine (5.7%) samples from 10/81 (12.3%) farms and 2/138 (1.4%) farmers, respectively. All Col-E isolates were *Escherichia coli* harboring *mcr*-1. Both farm workers colonized with Col-E worked on farms where no Col-E were detected in porcine samples.

In conclusion, CPE were absent on German pig farms. This supports findings of culture-based national monitoring systems and provides evidence that even when improving the diagnostic sensitivity by using molecular detection techniques in addition to culture, CPE are not prevalent. Col-E were prevalent in porcine feces despite a recent decrease in colistin usage among German livestock and absence of colistin treatments on the sampled farms. Farmers carried Col-E, but zoonotic transmission was not confirmed.

1. Background

The spread of antimicrobial resistance (AMR) in Gram-negative bacteria is a major threat to global healthcare. In 2017, up to 30% of all *Escherichia coli* isolates obtained from blood cultures of human patients in Southern Europe were cefotaxime-resistant, which is indicative for the production of extended-spectrum β-lactamases (ESBL) [1]. Consequently, ESBL-unaffected carbapenems were more frequently used, which facilitated the dissemination of carbapenemase-producing Enterobacterales (CPE) in healthcare settings. Although carbapenem resistance rates in Enterobacterales are still low in most European countries (e.g. Germany <1% of all *E. coli* and *Klebsiella pneumonia* [1]), CPE are an emerging problem [2] stimulating the parenteral use of colistin to treat CPE-infections. Colistin resistance can be acquired by genes located on transferable plasmids (e.g. *mcr*-1-10) [3] and recently colistin-resistant, *mcr*-positive Enterobacterales (Col-E), mainly *E. coli*, were detected globally [4].

The rise of AMR among Gram-negative bacteria in human medicine was accompanied by the occurrence of AMR in livestock. In Germany, ESBL-*E. coli* were found on 44–61% of all pig and 100% of all chicken

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farms [5,6]. In another study, up to 66% of chicken meat and 13% of pork samples at retail were contaminated with ESBL-*E. coli* [7]. Although carbapenems are not licensed in veterinary medicine, CPE (bla_{VIM-1} positive *E. coli* and *Salmonella*) were first reported in German livestock in 2012 [8,9] on a limited number of farms. Recently, it was found that *mcr*-1 positive *E. coli* were prevalent in poultry production [10], on pig farms [11], and slaughterhouses as well as fresh food items [12] in Germany. Studies in several countries, such as in the Netherlands [13], Denmark [14], and Vietnam [15], have identified direct exposure to livestock as a risk factor for the carriage of ESBL-*E. coli* as carriage rates among farmers and slaughterhouses exceeded the proportions observed in the general population. For Col-E and CPE, data about the colonization of livestock-exposed persons are rare.

This study aims to investigate the prevalence of CPE and Col-E harboring *mcr* on German pig farms and corresponding farm workers and compare their genomic background using molecular typing techniques.

2. Material and methods

2.1. Samples

Samples were taken at 81 pig farms in North-West Germany (North Rhine-Westphalia, Hesse & Lower Saxony), between March 2018 and September 2020. Of the 81 farms, 57 performed conventional, 13 alternative, and 11 ecological farming approaches. Farms were defined as "ecological" following the guidelines of special associations ("Bioland", Naturland" etc.) or other organizations (e.g. guidelines of the EU) and included straw bedding and/or outdoor climate in the stable and free ventilation. "Conventional" farms were characterized by slatted or partially slatted floor in the bay, forced ventilation, and closed buildings. Conventional farms that did not fulfill all criteria to be classified as "ecological" but had straw bedding and/or outdoor climate in the stable and free ventilation were defined as "alternative".

Fecal samples from the pigs were collected as bulk samples using two sets of boot swabs on each farm per visit. The use of boot swabs for sampling sedimented bacteria in farm environments is well established [16]. Therefore, beret hoods made of nonwoven viscose (Hygostar, Buchloe, Germany) were autoclaved and moistened in a sterile blender bag with 10 ml peptone water (Merck, Darmstadt, Germany) as nutrient and transport medium. For sampling, these hoods were pulled over additional overshoes (overshoes CPE, VWR, Langenfeld, Germany) to walk approximately 60 steps through all areas of the pen to collect a representative sample. All except three farms were visited twice within the same fattening period resulting in 318 fecal samples. Farmers, additional farm workers, and their family members (in the following text summarized as farm workers) were asked to voluntarily provide one self-collected stool sample per person (eSwab® with Amies medium, Hain, Nehren, Germany). In total, stool samples from 138 humans belonging to 49 different farms were provided.

2.2. Microbiological analysis targeting Col-E and CPE

The boot swabs were placed in tryptic soy broth (Merck, Darmstadt, Germany) to homogenize the fecal material attached to the swabs. Human fecal samples were homogenized within the eSwabs' amies medium. Aliquots of these emulsions were mixed 1:1 with glycerol and stored at −20 °C until further processing.

For the detection of Col-E, 100 µl of these mixtures were transferred into 9 ml buffered peptone water (BPW) (Thermo Scientific™ Oxoid™, Waltham, Massachusetts, USA) supplemented with 4 µg/ml colistin and incubated for 18–22 h at 37 °C. Up to five of these enrichments were pooled by transferring 200 µl of each into a 1.5 ml reaction tube followed by centrifugation (5 min, 13,200 rpm). DNA extraction from the pellets was conducted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the 'Purification of total DNA from Animal Tissue' protocol. DNA was stored at 4 °C for <24 h or at −20 °C for later use. The presence of *mcr* genes was tested via PCR following the protocol by Rebelo et al. [17]. If *mcr* was detected, the procedure was repeated for single samples. For *mcr*-positive samples, 10 µl of the enrichment were streaked on selective media (chromID Colistin R, bioMérieux, Marcy l' Etoile, France). In addition, the enrichment broth of all samples was used for direct inoculation of 10 µL on chromID Colistin R to enhance screening sensitivity as described before [12].

For the detection of CPE, an analogous procedure was performed. BPW vials were supplemented with 50 mg/l vancomycin and 0.25 mg/l ertapenem. Presence of bla_{NDM}, bla_{KPC}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48} was examined in a real-time PCR using the Carbaplex® IVD PCR Kit (Bruker, Billerica, Massachusetts, USA). ChromID Carba and ChromID OXA-48 agar plates (bioMérieux, Marcy l' Etoile, France) were used as selective media for CPE detection.

Bacteria growing on the selective agars were sub-cultured on blood agar and MALDI-TOF MS was performed for species identification [18]. All except intrinsically colistin- or carbapenem-resistant species (e.g. *Providencia*, *Proteus*, *Serratia, Shewanella*) were subjected to PCR of single colonies to confirm the presence of resistance genes.

2.3. Molecular typing of isolates and plasmids

For nine *mcr*-1 positive *E. coli* isolated from human stool ($n = 2$) and porcine feces ($n = 7$) of six different farms, molecular typing using whole-genome sequencing (WGS) was performed. Genomic DNA (gDNA) was extracted using the NEB Monarch Genomic Purification Kit (New England Biolabs, Ipswich, Massachusetts, USA). Isolates were sequenced on a PacBio Sequel IIe system (Pacific Biosciences, Menlo Park, California, USA) using a 20 kb insert size library and the SMRTbell® Express Template Prep Kit 2.0. Raw sequences were de novo assembled using the hierarchical genome assembly process (HGAP) and analyzed using the SMRTLink software suite v9 with default parameters for microbial assembly. Final assembly contigs were extracted in FASTA format.

For genotyping of isolates, allelic profiles were created using a task template based on 2325 cgMLST targets of *E. coli* Sakai [19] in Ridom SeqSphere$^+$ v7.0.1 (Ridom GmbH, Münster, Germany). A minimum spanning tree was created from these profiles using the 'missing values are own category' option in SeqSphere$^+$. The contigs containing the *mcr* genes, presumably reflecting plasmids, were determined using ResFinder v3.2 [20]. Subsequently, the respective contigs were checked for complete circularization and uploaded to PlasmidFinder v2.1 [21] to predict the plasmid replicon type.

3. Results

Overall, 318 bulk samples of porcine feces from 57 conventional ($n = 224$), 13 alternative ($n = 50$) and 11 ecological farms ($n = 44$) were analyzed. In addition, stool samples of 138 humans associated with 49 farms were obtained.

In both porcine and human samples, no CPE were detected. One human stool sample was positive for bla_{OXA-48}. However, we were not able to culture an isolate harboring this gene. We found *mcr*-1 in 18 of 318 porcine samples (5.7%) from 10/81 farms (12.3%; 14 samples from 8/57 conventional (14%) and 4 samples from 2/13 (15.4%) alternative farms). On two farms, *mcr* was detected in samples from both visits, while positive samples were only found at visit 1 on the other eight farms. We were able to culture *mcr*-positive *E. coli* isolates from seven of the 18 porcine samples. For the other samples, this was unsuccessful due to overgrowth mainly by *Proteus* spp. and *Pseudomonas* spp. We also detected *E. coli* carrying *mcr*-1 in stool samples from two (1.4%) farm workers of two different conventional farms although no *mcr* genes were detected in the fecal samples of the respective farms.

3.1. Genotypic characterization of mcr-1 positive E. coli from farm workers and porcine feces

Fig. 1 shows a minimum spanning tree based on cgMLST allelic profiles of *mcr*-1 positive isolates from farmers ($n = 2$) and porcine feces ($n = 7$) on six different farms. Except for two pairs of presumably clonal CPE isolates from the same two farms (S91/S92, S112/S113), no genotypic clusters were detected. The closest isolates between farms, a human (F025) and an unrelated porcine isolate (S292) differed in 1176 alleles.

In addition to genotypic characterization of the chromosomal backbone, the plasmids carrying the *mcr* gene as well as additional resistance genes were analyzed (Table 1).

All nine *mcr*-1 plasmids of both human and porcine origin belonged to one of two plasmid types. One, with a size of ~33–35 kb, harbored the IncX4 replicon (n = 7) and the other harbored the IncI2 replicon and ranged around a size of ~60 kb ($n = 2$). All isolates harbored genes conferring resistances against colistin, aminoglycosides, and beta-lactams. Tetracycline resistance genes were present in all except one isolate. In addition, trimethoprim and sulfamethoxazole resistance was common in porcine isolates. One porcine isolate carried a chloramphenicol resistance gene.

Table 1
Overview of plasmids containing the *mcr*-1 gene and resistance profiles of seven Col-E isolates. Plasmid lengths in base pairs (bp) and detected plasmid replicon types as well as resistance profiles as determined by ResFinder v3.2 are given for each sequenced isolate. Origin: pig = porcine feces, hum = human stool.

isolate ID	origin	*mcr* plasmid length (bp)	*mcr* plasmid replicon type	resistance profile
S081	pig	33,310	IncX4	*aadA1, aadA2b, bla*$_{\text{TEM-1B}}$, *mcr-1.1, cmlA1, sul3, tet(B)*
S112	pig	33,298	IncX4	*aph(3″)-Ib, aph(6)-Id, bla*$_{\text{TEM-1B}}$, *mcr-1.1, sul2, tet (A)*
S113	pig	33,298	IncX4	*aph(3″)-Ib, aph(6)-Id, bla*$_{\text{TEM-1B}}$, *mcr-1.1, sul2, tet (A)*
S239	pig	60,970	IncI2	*aph(3′)-Ia, bla*$_{\text{TEM-1B}}$, *mcr-1.1, dfrA5*
S240	pig	60,959	IncI2	*aadA1, bla*$_{\text{TEM-1A}}$, *mcr-1.1, dfrA1, sul2, tet(B)*
S291	pig	35,138	IncX4	*aadA1, aph(3″)-Ib, aph(6)-Id, bla*$_{\text{TEM-1B}}$, *mcr-1.1, dfrA1, sul2, tet(B)*
S292	pig	35,138	IncX4	*aadA1, aph(3″)-Ib, aph(6)-Id, bla*$_{\text{TEM-1B}}$, *mcr-1.1, dfrA1, sul2, tet(B)*
F025	hum	34,504	IncX4	*aac(3)-IV, aph(4)-Ia, bla*$_{\text{TEM-1B}}$, *bla*$_{\text{TEM-1C}}$, *mcr-1.1, mph(A), tet(A)*
F105	hum	33,310	IncX4	*aadA22, bla*$_{\text{TEM-1C}}$, *mcr-1.1, erm(B), tet(A)*

4. Discussion

In this study, we investigated the prevalence of CPE and Col-E on German pig farms and among farm workers. To date, CPE were described on only a few pig and broiler farms in Germany [8,9,22–24] We are not aware of their emergence in livestock in neighboring countries. All German isolates harbored the *bla*$_{\text{VIM-1}}$ gene and were associated with the species *E. coli*, *Enterobacter cloacae,* and *Salmonella* spp. None of the farms enrolled in this study were located in geographical areas where CPE has been described so far [22]. In our study, we did not detect CPE in farm samples confirming the assumption that the prevalence of CPE in livestock is currently still low in Germany. However, this finding adds important value to the current knowledge since we performed a selective approach to screen for CPE while in most previous investigations on German farms, CPE were found in samples that were initially tested for the presence of ESBL-E using cefotaxime-agars rather than explicitly screening for CPE. This has a low sensitivity as demonstrated by Fischer et al. who re-tested 249 stored cultures from three pig farms where CPE had been detected before [22]. Moreover, we used both a culture-based and a genotypic approach to screen the samples for bacteria and resistance genes. This is important as many studies describing CPE among animals have demonstrated that isolates (especially those associated with carbapenemase genes *bla*$_{\text{OXA-48}}$ and *bla*$_{\text{VIM-1}}$)

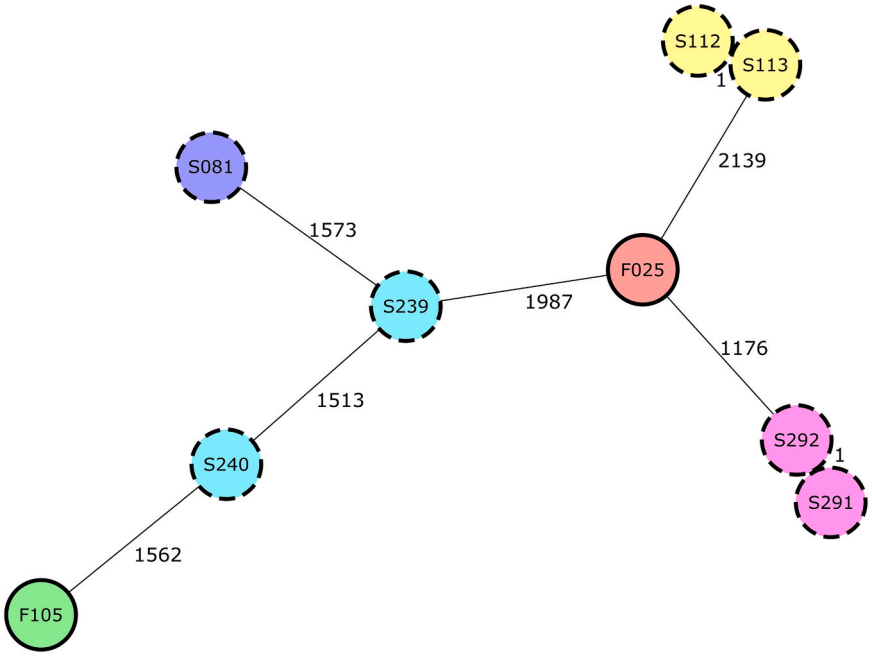

Fig. 1. Minimum spanning tree of *mcr*-1 positive *E. coli* isolates from seven porcine and two human samples. Each node represents a genotype based on allelic profiles of up to 2325 cgMLST target genes. Missing values were treated as their own category. Different node colors represent different farms and node border type marks the sample type (dashed = porcine feces, solid = human stool). Numbers on connecting lines show the number of differing alleles between genotypes.

had very low minimum inhibitory concentrations (MICs) for carbapenems, which prevents reliable identification by culture (reviewed in [25]). Furthermore, Ceccarelli et al. who analyzed samples from various food items and water samples in the Netherlands [26] demonstrated that the $bla_{\text{OXA-like}}$ genes they detected in 0.16% of their samples were chromosomally encoded and associated with *Shewanella* spp., a marine microorganism and presumably the natural reservoir of $bla_{\text{OXA-like}}$ genes [27] rather than with CPE. In this study, we detected $bla_{\text{OXA-48}}$ in one human stool sample. However, we were not able to culture a corresponding isolate, thus the respective species and origin remain unclear.

While the prevalence of CPE was expected to be low, we assumed a higher rate for the presence of Col-E in porcine feces. We found that 5.7% of the samples from pig farms were positive for *mcr*-1. Overall 12.3% of the farms were affected. These numbers are in the range of previous reports. In a retrospective study on samples taken in 2011/2012 on German pig farms, *mcr*-1 was found in 10% of the analyzed samples and 26% of the investigated farms [28]. An analysis of >10,000 non-selectively collected *E. coli* isolates from German national veterinary monitoring programs covering the years 2010–2015 revealed that 3.8% of all isolates harbored the *mcr*-1 gene. The prevalence varied between different animals and was highest on turkey farms (up to 17.9%) but lower on pig farms (1.5–2.1%) [10]. In a more recent study on samples taken in 2018/2019 for a food monitoring program in North-West Germany, *mcr*-1 was detected in 9–26% of the samples from different levels of the food production chain [12].

In Germany, the total antibiotic use in livestock was reduced by 60.7% between 2011 and 2019 [29]. This success was mainly due to a decrease in the use of tetracyclines, penicillins, macrolides, and sulfonamides. In 2019, 66 t of colistin were used (i.e. 10% of 670 t total antibiotic use), which corresponds to a 61% reduction of colistin use compared to 2011 [29]. It has been shown that stopping the use of colistin can reduce the prevalence of Col-E in livestock in the long term [30]. None of the farms investigated in this study reported colistin usage in the sampled fattening period; however, we cannot rule out colistin usage by the providing piglet breeders as these data were not available. Given the supposed absence of selective pressure, a lower prevalence rate would be expected. The evolutionary forces leading to a continuing presence of resistance genes despite the lack of antibiotic application are currently poorly understood and need further research. However, co-selective effects in cases of multi-resistance are a likely explanation [31].

Reports about Col-E among occupationally livestock-exposed humans are rare and to our knowledge, this is the first investigation in Germany regarding this issue. We found that 1.4% of the farm workers included in this study carried *mcr*-1 positive *E. coli*. In the international literature, we only identified few studies, where farm workers were tested. Bui et al. found that 33% of chicken farmers in Vietnam were colonized with *mcr*-positive Col-E [32] and Wang et al. [33] detected *mcr*-1 in a sample from one of six poultry farmers in China (without isolating the bacteria). In the general population, the prevalence of *mcr-E. coli* carriage seems to be low in Western European countries. Saly et al. detected no patient colonized with *mcr-E. coli* at admission to a French hospital [34] and Zurfluh et al. found no *mcr-E. coli* when screening >1000 healthy individuals in Switzerland [35]. To our knowledge, there are no population-based studies assessing the prevalence of Col-E among humans in the community in Europe. However, one recent study found that 11.4% of German and Dutch travelers, who were initially tested negative for *mcr* carriage, acquired fecal carriage of *mcr-E. coli* during traveling, mostly in Asia, Africa, and South America [36].

In this study, we found that the carriage of *mcr-E. coli* among workers on pig farms was rare. The absence of *mcr* on the corresponding farms does not exclude a zoonotic origin as we tested only a limited number of porcine samples per farm, which does not rule out the possibility of *mcr-E. coli* presence among pigs. However, other sources, such as travel, are also probable. Molecular comparison of the *mcr-E. coli* isolates from the different farms revealed a high diversity, which is in line with other studies on *mcr-E. coli* prevalence among livestock reporting a wide range of STs and serotypes [37]. No clonal relationships of isolates from different farms were detected. However, given the high diversity of *mcr* backgrounds, it is likely that different genotypes are present within individual farms, which have not been captured due to choosing only one or two isolates per farm from a single fattening period for sequencing. Plasmid-typing revealed that *mcr* was harbored by two different replicon family types, IncX4 and IncI2. Both belong to the most frequently identified plasmids in the context of *mcr* carriage in Germany [28,38] and various European countries such as Spain [37], the Netherlands [39], the UK [40], Denmark [41], Switzerland [42] and France [43]. They are also among the predominant *mcr*-plasmids in Asia indicating that there is no country-specific distribution. The plasmids described here contained, in addition to *mcr*-1, a number of genes conferring resistance to other antibiotic classes, which has been described for *mcr*-containing plasmids in Enterobacterales globally [44]. Of interest, the *cml*A1 gene conferring resistance to fenicols including florfenicol, which is frequently used in veterinary medicine, was detected. Its association with an IncX4 plasmid harbored by *Salmonella* spp. has recently been reported [45].

This study had a few limitations. First, samples were only tested for *mcr*-1 to *mcr*-5, neglecting *mcr*-6-10 [3], which were first described after the study protocols had been implemented. Second, although two different time points were sampled on each farm, the study only reflects a snapshot of the prevalence in a single batch of fattening pigs. Third, recent travel history of farm workers, in particular to Asia, which is another risk factor for the acquisition of Col-E, was not recorded. Furthermore, the genotypic diversity could not be depicted due to sequencing of only single isolates per sample. For future research, we suggest a longitudinal study with higher numbers of isolates to assess the temporal dynamics and genotypic backgrounds of Col-E and other multidrug-resistant bacteria on German pig farms.

5. Conclusions

In summary, we confirmed that CPE are rare in German pig farming even when using sensitive diagnostic techniques. We found that despite a reduction in the total antimicrobial use in livestock, Col-E harboring *mcr*-1 are still prevalent on pig farms, which supports the need for further research on the driving forces that lead to AMR preservation. We did not find evidence for zoonotic transmission between pigs and farm workers but reported that 1.4% of the farmers carried *mcr*-1 positive *E. coli*.

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Authors' contributions

Conceptualization and funding acquisition: RK, AM, MB, CC. Data curation, formal analysis and methodology: NE, IK, SL, FH, HS, CC. Investigation: NE, IK, CC. Project administration: RK, MB, CC. Writing – original draft: NE, RK. Writing – review & editing: NE, IK, SL, FH, HS, AM, MB, RK, CC.

Ethics approval and consent to participate

This study was approved by the Ethical Committee of the University of Münster (No. 2018–008-f-S). All participants obtained an information describing the aim of the study and the study protocol. Prerequisite for inclusion was the participants' agreement, documented by return of a declaration of consent. All participants were informed individually

about the results of sampling with a laboratory report. No animal ethics approval was required as the pigs were not kept for scientific purposes and sampling was conducted in a non-invasive way and did not exceed routine animal care practices.

Author statement

All authors of manuscript ONE-HLT-D-21-00327 have contributed to the revision and agree with the revised manuscript as submitted now.

Declaration of Competing Interest

None of the authors had any financial and personal relationships with other people or organizations that could inappropriately influence (bias) our work.

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All authors declare that they do not have any competing interests with respect to this article.

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