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Bacterial genome sequencing tracks the housefly-associated dispersal of fluoroquinolone- and cephalosporin-resistant *Escherichia coli* from a pig farm

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Abstract

The regular use of antimicrobials in livestock production selects for antimicrobial resistance. The potential impact of this practice on human health needs to be studied in more detail, including the role of the environment for the persistence and transmission of antimicrobial-resistant bacteria. During an investigation of a pig farm and its surroundings in Brandenburg, Germany, we detected abundant cephalosporin- and fluoroquinolone-resistant *Escherichia coli* in pig faeces, sedimented dust, and house flies (*Musca domestica*). Genome sequencing of *E. coli* isolates revealed large phylogenetic diversity and plasmid-borne extended-spectrum beta lactamase (ESBL) genes CTX-M-1 in multiple strains. [Correction added on 28 February 2023, after first online publication: In the preceding sentence, 'and TEM-1' was previously included but has been deleted in this version.] Close genomic relationships indicated frequent transmission of antimicrobial-resistant *E. coli* between pigs from different herds and across buildings of the farm and suggested dust and flies as vectors for dissemination of faecal pathogens. Strikingly, we repeatedly recovered *E. coli* from flies collected up to 2 km away from the source, whose genome sequences were identical or closely related to those from pig faeces isolates, indicating the fly-associated transport of diverse ESBL-producing *E. coli* from the pig farm into urban habitation areas. The observed proximity of contaminated flies to human households poses a risk of transmission of antimicrobial-resistant enteric pathogens from livestock to man.

Wiebke Behrens, Baban Kolte, and Vera Junker contributed equally to this study.

INTRODUCTION

Antimicrobial-resistant (AMR) *Escherichia coli* was recently estimated to have directly caused 219,000 human deaths globally in 2019, which was the highest death toll among all drug-resistant bacteria (Murray et al., 2022). More than 50% of these deadly infections had been caused by *E. coli* that was resistant to either third-generation cephalosporins or fluoroquinolones (Murray et al., 2022). Bacterial resistance to these drugs is caused most commonly by production of extended-spectrum beta lactamases (ESBL) and by mutations in topoisomerase and gyrase enzymes, respectively (Poirel et al., 2018). At the same time, *E. coli* with similar resistance traits was reported to colonize livestock, including cattle, poultry and pigs (Seiffert et al., 2013). However, the extent of pathogen transmission between food animals and humans is uncertain (Lazarus et al., 2015; Muloi et al., 2018). While a recent study concluded that human-to-human transmission was the predominant route for spread of ESBL-producing *E. coli* in the United Kingdom (Day et al., 2019), identical genotypes of AMR *E. coli* among livestock, animal-derived food products and human infections have been reported repeatedly (Dahms et al., 2015; de Been et al., 2014; Overdevest et al., 2011).

A large and increasing share of globally consumed antimicrobials is used for disease prevention and growth promotion in animal agriculture (Van Boeckel et al., 2015). While it is clear that this practice selects for the emergence of antimicrobial resistance, the complex transmission routes of AMR bacteria and their impact on human health require additional study (Castillo-Ramirez et al., 2021; Robinson et al., 2016). Among the potential linkages between animal health, human health, and the environment highlighted in the 'One Health' concept, arguably, the persistence and spread of antimicrobial resistance in the environment is least well understood (Essack, 2018). Various pathways for the transmission of AMR *E. coli* from livestock reservoirs to humans are conceivable, including the consumption of contaminated meat products (Overdevest et al., 2011; Randall et al., 2021), direct contact to animals (Dahms et al., 2015; de Been et al., 2014), and environmental routes, for example, in association with aerosols or insect vectors (Friesema et al., 2012). The airborne spread of *E. coli* is limited by the pathogen's low desiccation tolerance (Siller et al., 2021). The common house fly (*Musca domestica*), however, has been identified as a vector of AMR *E. coli* and diverse other pathogens (Graczyk et al., 2001; Khamesipour et al., 2018; Onwugamba et al., 2018; Yin et al., 2022). House flies are coprophagic, that is, they feed on faeces and manure, which contain abundant faecal pathogens. At the same time, they are synanthropic, that is, they live closely associated with humans and are particularly abundant in livestock husbandry (Graczyk et al., 2001). House flies contaminated with viable ESBL-producing *E. coli* have been reported from both, human dwellings and livestock barns, and since individual flies may have flight ranges of several kilometres, they might disperse pathogens between hosts (Fukuda et al., 2020; Zurek & Ghosh, 2014). Indeed, a modelling study suggested that the fly-associated route may pose a higher risk of transmission of enteric bacterial pathogens from poultry to humans than the consumption of chicken meat (Evers et al., 2016).

Analyses of pathogen spread and transmission rely on epidemiological analyses in conjunction with molecular genotyping. Traditional methods for bacterial genotyping have been replaced by genome sequencing recently, since this technology has become more affordable and provides maximum discriminatory power (Besser et al., 2019). Genome comparisons can be performed very efficiently with the EnteroBase platform (https://enterobase.warwick.ac.uk/), which currently holds 219,226 genome sequences from *Escherichia* spp. isolates (as of October 2022) together with associated metadata, and is equipped with powerful tools for standardized data analysis and quality control (Zhou et al., 2020). Large-scale assessment of genomic relatedness is achieved with core-genome multilocus sequence typing (cgMLST) in combination with single-linkage hierarchical clustering of allelic profiles in EnteroBase (Achtman et al., 2022). For *E. coli*, hierarchical clusters at level HC5 (i.e. chains of genomes with up to five pairwise differences among cgMLST alleles) correspond to transmission chains, whereas clusters at level HC1100 are congruent to sequence type (ST) complexes, which had previously been defined on the basis of classical 7-gene MLST (Zhou et al., 2020).

In this study, we applied bacterial genome sequencing to track the spread of AMR *E. coli* within and beyond a pig farm. We found that contaminated house flies carried diverse fluoroquinolone- and cephalosporin-resistant *E. coli* from the farm into urban habitation areas, over distances of at least 2 km.

EXPERIMENTAL PROCEDURES

Sample collection at pig farm

Faeces, fly and dust samples were collected at an experimental pig fattening facility in Brandenburg, Germany. Pigs were kept in herds of 6–20 animals per pen with one to three pens located in each barn compartment. Barn compartments were closed rooms connected by small ventilation holes and doors that were usually shut. Faeces samples were collected from 11 herds from 5 compartments and from 1 herd that was kept in an open stable with free outdoor range, which was located in a separate, directly adjacent

building. Upon arrival at the farm, piglets were 28 days old and just weaned. Within the first 4 days at the farm, all piglets were administered medical food containing antiviral and anti-worm components as well as Enteroxid (OGRIS Pharma, Wels, Austria), to reduce the introduction of pathogens and parasites to the farm. Enteroxid contains colistin sulfate (25 mg/g) and zinc oxide (480 mg/g); it was administered at 5 mg of colistin sulfate/kg body weight per day. Upon bacterial diarrhoea, individual pigs were treated with enrofloxacin (Powerflox, 3 days with subcutaneous injection of 4 mg/kg*d) or marbofloxacin (Marbocyl, 3 days with subcutaneous injection of 2 mg/kg*d). In addition, ampicillin was used to treat occasional pneumonia or other infections, but not during our sampling period (November 2019 to August 2020).

Faeces were collected from several droppings within one pen with sterile spoons. Samples were collected at weekly intervals beginning within 24 h after the arrival of piglets at the farm. Deposited dust material was collected from exposed surfaces in selected stables with sterile spoons. Flies were caught individually with sterile polypropylene tubes. Outside buildings, flies were caught at 14 sampling points at increasing distances up to 5 km from the farm and in two directions (i.e. north and south). At each sampling point, flies were lured to jars filled with pig faeces and covered with netting and then collected individually in tubes. All samples were stored at 4°C and processed for cultivation of bacteria within 24 h.

Bacterial cultivation

For the enumeration of living bacteria in faeces samples, 10 g of sample material was mixed with 40 mL buffered peptone water (Roth, Karlsruhe, Germany), homogenized for 30 s with a bag mixer (Interscience) and left for 30 min at room temperature for the sedimentation of coarse particles. Similarly, 0.4 g of dust was mixed with 1.4 mL buffered peptone water. For cultivation of bacteria from sampled flies, two living flies were cold-shocked for immobilization and then crushed in a mortar and suspended in buffered peptone water (Roth). Subsequently, homogenized samples were diluted to extinction and streaked on MacConkey Agar No.3 (MC3; Oxoid, München, Germany). To determine the numbers of antibiotic-resistant enterobacteria, MC3 was supplemented with 1 mg/L cefotaxime, 2 mg/L colistin sulfate, or 0.5 mg/L ciprofloxacin, respectively (Sigma-Aldrich, Darmstadt, Germany). After incubation in ambient air at 37°C for 18 h, colony-forming units were counted and proportions of antibiotic-resistant enterobacteria were calculated in relation to the numbers of colony-forming units on MC3 without antibiotic. Out of 272 cultivations, 33 (12%) had cell counts under the limit of quantification (i.e. below 20 colonies/plate). Since the majority (25) of these were on the lowest possible dilution, these were still included in further analyses to allow the most accurate representation. Weekly time points were merged to 2-week intervals, and when data from both weeks were available, the mean was calculated. Distributions of percent-resistant CFU between time intervals were checked for statistically significant differences by non-parametric, pairwise testing with the Wilcoxon signed rank test using the R package *stats* (version 4.1.0). Bacterial colonies were picked for sub-cultivation on MC3, *E. coli* was identified by species-specific PCR (Torres et al., 2017), and 117 other bacterial isolates were species-identified by mass spectrometry on a MALDI Biotyper Smart System GP (Bruker Daltonik, Bremen, Germany) (Thiel et al., 2020).

Antibiotic susceptibility testing

Minimum inhibitory concentrations (MICs) of cefotaxime and ciprofloxacin were determined by using Etests according to the manufacturer's instructions (Biomerieux, Nürtingen, Germany). The MIC of colistin was determined by a broth microdilution test applying cation-adjusted Mueller Hinton broth as recommended by the joint CLSI-EUCAST Polymyxin Breakpoints Working Group (www.eucast.org). Colistin-susceptible *E. coli* DSM 1103 (ATCC 25922) and colistin-resistant *E. coli* DSM 105182 (NCTC 13846) were included for quality control. Isolates were considered antibiotic resistant if MICs were above breakpoints as recommended by EUCAST: ciprofloxacin (>0.5 mg/L), colistin (>2 mg/L), and cefotaxime (>2 mg/L).

Whole-genome sequencing and bioinformatic analyses

DNA from *E. coli* isolates was extracted with the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). Libraries for sequencing were prepared by using a miniaturized Nextera XT-protocol (Steglich et al., 2018), and sequencing was performed on a NextSeq 550 machine with a NextSeq 500/550 mid output v2.5 kit (Illumina). Resulting sequencing data were deposited at GenBank (https://www.ncbi.nlm.nih.gov/genbank/) and are available under BioProject ID: PRJNA885145. In addition, read data were uploaded to EnteroBase (http://enterobase.warwick.ac.uk/), where they were assembled and checked for quality automatically (Zhou et al., 2020). Assembly statistics for 336 genome sequences are provided in Table S3. Phylogenetic analyses were performed on the basis of cgMLST by using GrapeTree as implemented in EnteroBase. Neighbour-joining phylogenetic trees were calculated by using the RapidNJ implementation in GrapeTree based on matrices of pairwise distances, calculated from differences among cgMLST allelelic profiles (Zhou et al., 2018). EnteroBase also predicted

fimH alleles. Genomic assemblies were downloaded from EnteroBase and analysed for antibiotic resistance genes and resistance-causing point mutations by using the Resistance Gene Identifier (Alcock et al., 2020) (https://card.mcmaster.ca/analyze/rgi). Perfect and strict hits (see https://card.mcmaster.ca/analyze/rgi) were visualized in relation to a genome-based phylogenetic tree with iTOL (Letunic & Bork, 2019) (https://itol.embl.de/). PlasmidFinder v2.0.1 was used locally for detection of plasmids in 336 *E. coli* genome assemblies, using the database for *Enterobacteriaceae* (downloaded from https://cge.cbs.dtu.dk/services/PlasmidFinder/ on 29 November 2021). Plasmid replicon typing was performed using BLAST v2.7.1+, with identity and coverage of ≥95% and ≥ 80%, respectively (Carattoli et al., 2014). In-house (Linux shell) scripts were used to determine co-occurrence of antibiotic resistance genes and plasmid signatures on the same contigs.

RESULTS

Cultivation of antimicrobial-resistant *Enterobacteriaceae*

We screened faeces of pigs for enterobacteria resistant to colistin, ciprofloxacin and cefotaxime, since related drugs had been used regularly in the fattening facility. Colistin had been applied to all piglets upon arrival at the farm, and fluoroquinolones (enrofloxacin or marbofloxacin) or β-lactams (ampicillin), respectively, had been used for treatment of diarrhoea or occasional pneumonia. We determined the proportions of enterobacteria resistant to each of these antimicrobials by quantitative cultivation on selective agar media.

We analysed a total of 68 samples of pooled faeces from 12 herds of pigs, with six to 20 individual animals each. From each herd, we had collected faeces samples upon arrival of piglets in the facility (i.e., within 24 h; week 0) and then at weekly to bi-weekly intervals until 8 weeks later (Week 1 to Week 8). Enterobacteria resistant to ciprofloxacin and colistin were found in almost all faeces samples, including those from Week 0, and enterobacteria resistant to cefotaxime were found in 40 out of 68 samples. After the piglets' arrival in the farm, the proportions of bacteria resistant to ciprofloxacin ($p < 0.01$) and to colistin ($p < 0.05$) increased over time, from less than 0.1% to more than 1% on average, with rates of resistance to ciprofloxacin and colistin between 10% and 100% in some samples (Figure 1).

Mass spectrometric analysis and species-specific PCR identified 39% of bacterial colonies on antibiotics-containing Mac Conkey agar as *E. coli*, whereas the remaining isolates were mostly other enterobacteria (*Enterobacterales*, including the genera *Salmonella*, *Klebsiella*, *Citrobacter*, *Providencia*, *Morganella*, *Proteus*) and a few glucose non-fermenters (i.e. *Acinetobacter*, *Pseudomonas*).

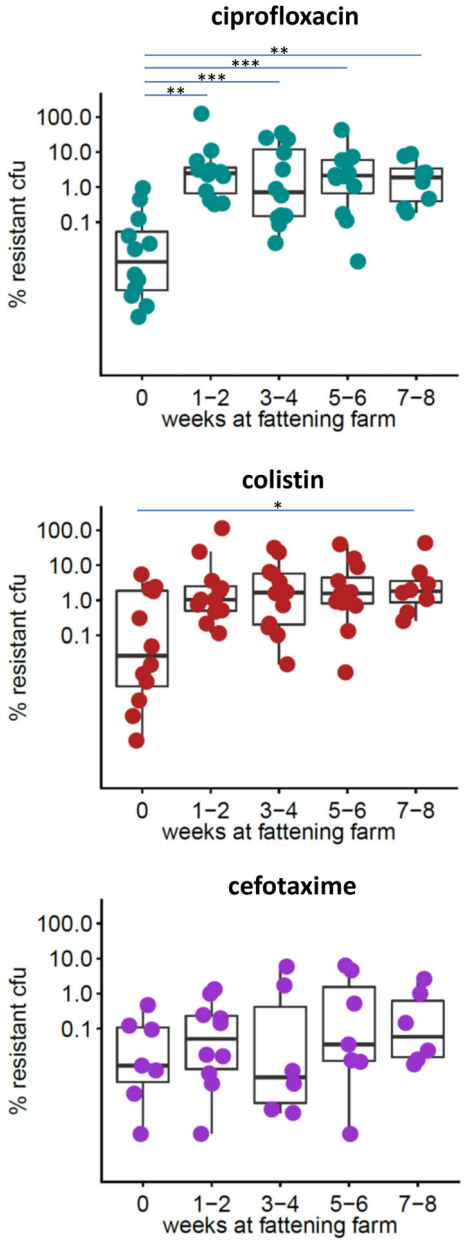

FIGURE 1 Proportions of AMR enterobacteria in pig faeces. Proportions of cultivatable enterobacteria (logarithmic scale) resistant to ciprofloxacin, colistin and cefotaxime, respectively, in 68 samples of pig faeces collected from 12 herds at several time points each, from the arrival of piglets at the farm to 8 weeks later. Boxes indicate the median (50th percentile), 25th and 75th percentiles, and whiskers reach to 1.5 times the interquartile ranges. Significant increases of resistance over time were observed as indicated (Wilcoxon signed rank test; ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$).

Characterization of antimicrobial-resistant *E. coli*

Considering breakpoints for resistance recommended by EUCAST, measurements of minimum inhibitory

concentrations indicated that the majority (83% or 100%) of *E. coli* isolated from MC3 agar containing ciprofloxacin or cefotaxime, respectively, were fully resistant to either antibiotic (Table S1). There was little cross resistance to more than one of these antibiotics (Table S1). In contrast, we found no colistin-resistant *E. coli* with MIC greater than the recommended breakpoint (>2 mg/L), even among isolates recovered from agar medium containing 2 mg/L colistin sulfate, a finding that may be related to the poor diffusion of colistin through agar (Poirel et al., 2017) (Table S1).

Genome sequences from 336 *E. coli* isolates from MC3 agar containing one of three antibiotics (ciprofloxacin, 133; colistin, 121; cefotaxim, 82) revealed large phylogenetic diversity (Figure 2). Core-genome MLST-based hierarchical clustering indicated there were 27 HC1100 clusters, each of which corresponds to a specific ST complex (Zhou et al., 2020). Among these, the ST10 complex (HC1100_13) was most prevalent (210 isolates, 63%), followed by the ST4198 complex (HC1100_4505, 35 isolates, 10%), the ST23 complex (HC1100_5, 19 isolates, 6%), and the ST155 complex (HC1100_106, 18 isolates, 5%). The majority of these HC1100 clusters had been recovered mostly from livestock in the past, even though all of them had also been detected in humans occasionally (Table S2). Four isolates were affiliated to the ST131 complex (HC1100_7) (Figure 2), of which subclade *C* (also termed H30R/H30Rx) is infamous for its multidrug resistance and for causing a global epidemic of healthcare-associated infections in humans (Petty et al., 2014; Price et al., 2013; Stoesser et al., 2016). However, our four isolates had *fimH* gene variant 22 and hence were related to subclade *B* of ST131, rather than *C* (Stoesser et al., 2016). Furthermore, they did not possess a CTX β-lactamase as would be typical for epidemic subclade *C* (Figure 2).

Out of 336 genome-sequenced *E. coli* isolates, 85 carried genes encoding ESBL of the type $bla_{\text{CTX-M-1}}$ (Figure 2), and these were commonly associated with cephalosporin resistance. Among 119 isolates tested for phenotypic resistances, 22 carried the $bla_{\text{CTX-M-1}}$ gene, and 20 of these were resistant to cefotaxime according to EUCAST breakpoints (i.e. MIC >2 mg/L). Three additional isolates were phenotypically cefotaxime resistant without carrying the $bla_{\text{CTX-M-1}}$ gene; yet, it is currently unclear by which mechanism. While genomes from these strains encoded additional beta-lactamases (including bla_{ampC1} and $bla_{\text{TEM-1}}$), each of these genes occurred in many additional isolates without causing phenotypic cefotaxime resistance (Figure 2, Table S3). [Correction added on 28 February 2023, after first online publication: In the preceding sentence, "ESBL" has been changed to "beta-lactamases" in this version.] An explanation for this observation may be that some chromosomally encoded beta lactamases require enhanced expression for causing fully fledged cephalosporin resistance (Jørgensen et al., 2010).

Resistance to fluoroquinolones in our genome-sequenced *E. coli* was caused most commonly by specific point mutations in gyrase and topoisomerase genes. Among 29 ciprofloxacin-resistant isolates, 23 carried a *gyrA* mutation and 20 of these carried an additional *parC* mutation (Table S3). Furthermore, eight genome-sequenced isolates carried genes for quinolone resistance proteins (QnrS1, QnrB19) (Figure 2), but these *qnr* genes were not associated with increased fluoroquinolone MICs (Table S3).

We did not detect any colistin-resistant *E. coli* or any transferable colistin-resistance (*mcr*) genes in the genome sequences. Multiple additional presumptive resistance determinants were found, including genes and target mutations that may cause resistances against aminoglycosides, sulfonamides and several other drug classes (Figure 2, Table S3), but these were not verified by phenotypic susceptibility tests. In addition, each of the *E. coli* genomes was indicated to carry 37–46 genes potentially associated with the efflux of various antibiotics (not shown). The genome-based phylogenetic analysis showed that identical genetic resistance determinants in many cases occurred in multiple, distantly related phylogenetic clades (Figure 2), indicating that each of these resistance traits got acquired several times independently during the course of evolution.

PlasmidFinder analysis (Carattoli et al., 2014) detected signatures of a total of 36 different plasmids in 336 *E. coli* genome assemblies (Table S4), with up to seven plasmids in individual genomes. Some of these plasmids were found in numerous genomes of the strain collection (Table S4), indicating their widespread occurrence in the *E. coli* population. Simultaneous presence of antibiotic resistance genes and plasmid signatures on the same assembly contigs suggested that those resistance traits were encoded on extra-chromosomal elements. Among these, plasmids of at least three types (IncFII, IncI1-I, IncN) each encoded resistances to three to four drug classes, including β-lactams, aminoglycosides, sulfonamides and diaminopyrimidines (Table S4). While the ESBL gene $bla_{\text{CTX-M-1}}$ was detected exclusively on IncI1-I plasmids, $bla_{\text{TEM-1}}$ was found in association with several different plasmids (Table S4).

Detection of pathogen spread within the pig farm and beyond

We found *E. coli* isolates with identical core genomes or related at the level HC5 (Figure S1A) in different barn compartments and in the open stable, suggesting that *E. coli* was able to spread across rooms and buildings in the farm (Figure S1B). Further, we detected equally closely related *E. coli* among pig herds that had been delivered from different breeding farms (Figure S1C), indicating that the pathogen got transmitted between pigs from different herds. Of note, within

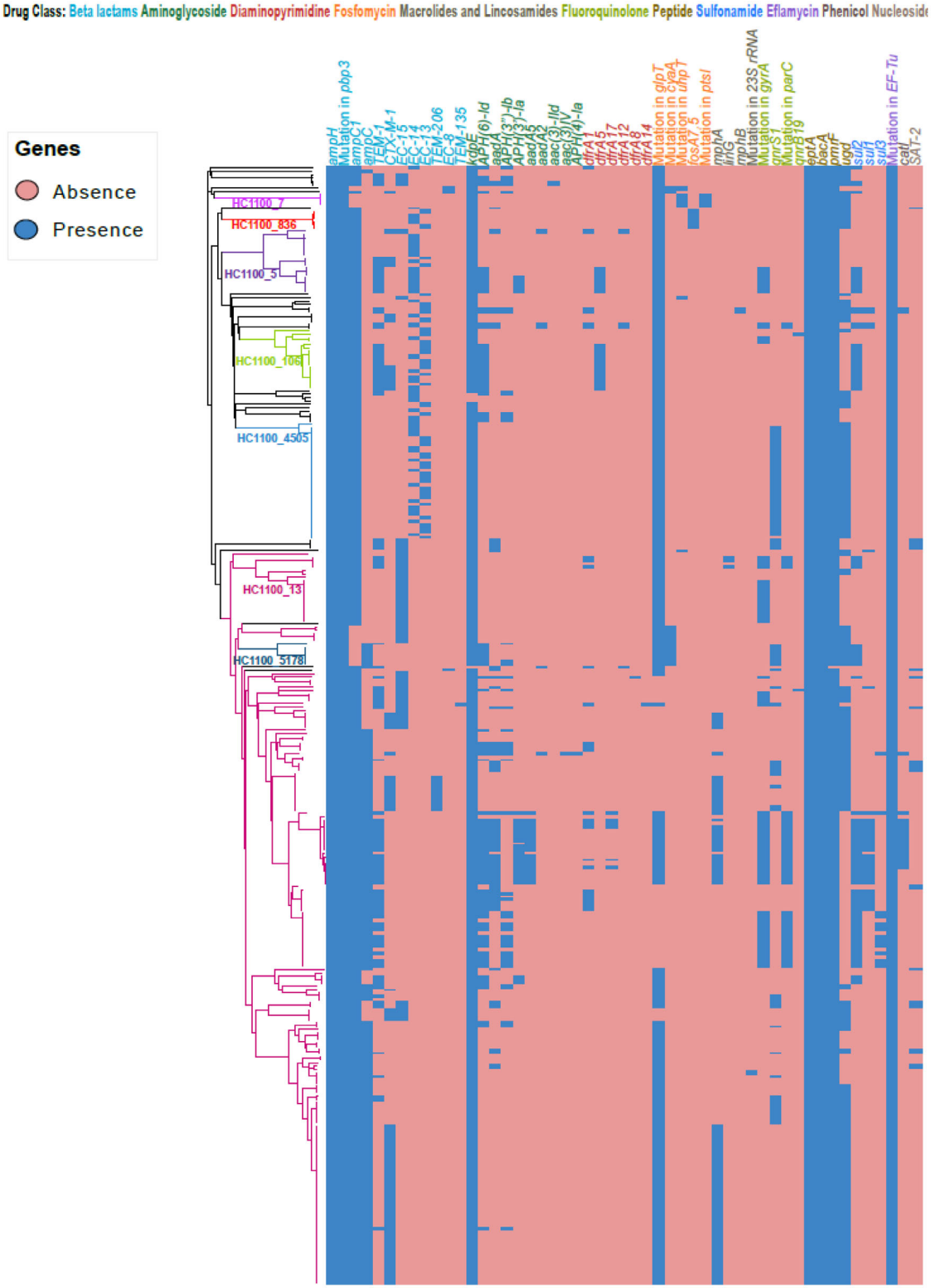

FIGURE 2 Phylogenetic distribution of genetic resistance determinants among 336 *E. coli* isolates. Neighbour-joining phylogenetic tree based on core-genome (cgMLST) variation among 336 *E. coli* isolates. Branch colours indicate HC1100 (with >4 entries), which correspond to previously named ST complexes as follows (Zhou et al., 2020): HC1100_13, ST10 complex; HC1100_4505, ST4198 complex; HC1100_106, ST155 complex; HC1100_5, ST23 complex; HC1100_7, ST131 complex.

each of those five HC5 clades that included *E. coli* from piglets of different origins (Figure S1C), piglets from only one breeder had freshly arrived in the facility (i.e. they had been sampled in week '0'), whereas closely related *E. coli* from all other piglets had been sampled later (i.e. in Weeks '1' to '8'), consistent with

the acquisition of these *E. coli* clones during the piglets' stay on the farm (not shown). Multiple *E. coli* clones (HC5) persisted in the facility during the entire study, that is, for at least 8 months (Figure S1D).

To investigate potential transport vectors that may have facilitated the spread of bacteria within the facility, we collected flies (*Musca domestica*, $n = 22$) and samples of deposited dust ($n = 11$) inside stables and found that these samples carried abundant viable, drug-resistant enterobacteria (Figure 3). Moreover, genome sequencing revealed numerous indistinguishable *E. coli* isolates among pig faeces, flies, and dust, respectively, and additional isolates that were closely related at the HC5 level across these sources (Figure 4), indicating their frequent exchange.

We then collected flies ($n = 64$) outside buildings, at increasing distances from the farm and in two opposite directions. As a result, we repeatedly recovered *E. coli* from flies caught up to 2 km away from the facility, whose genome sequences were identical to those from pig faeces isolates collected in the stables (Figure 4). A total of eight different *E. coli* HC5 genotypes were detected in both faeces and flies from outside the stable buildings (Figure 4, Figure S1A).

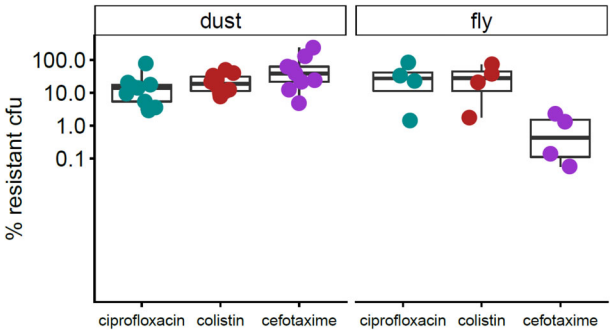

FIGURE 3 Proportions of AMR enterobacteria in houseflies and in deposited dust. Proportions of cultivatable enterobacteria (logarithmic scale) resistant to ciprofloxacin, colistin and cefotaxime, respectively, are shown. Boxes indicate the median (50th percentile), 25th and 75th percentiles, and whiskers reach to 1.5 times the interquartile ranges.

Relatedness to previously published *E. coli* genome sequences

The *Escherichia* database on the EnteroBase platform currently (as of October 2022) holds 219,226 assembled genome sequences from public databases and their associated metadata, including information about the sources of bacterial isolates (https://enterobase.warwick.ac.uk/). The 336 *E. coli* genome sequences from our dataset were classified into 109 clusters at level HC5 (i.e. with pairwise differences of maximally five cgMLST alleles; Figure S1A). All these HC5 clusters were unique to our dataset, that is, no genome sequences in the *Escherichia* database were related at this level to genomes from our study. At level HC10, in contrast, a single cluster (HC10_118309) included 35 previously published genome sequences from *E. coli*, all of which had been collected from pigs in

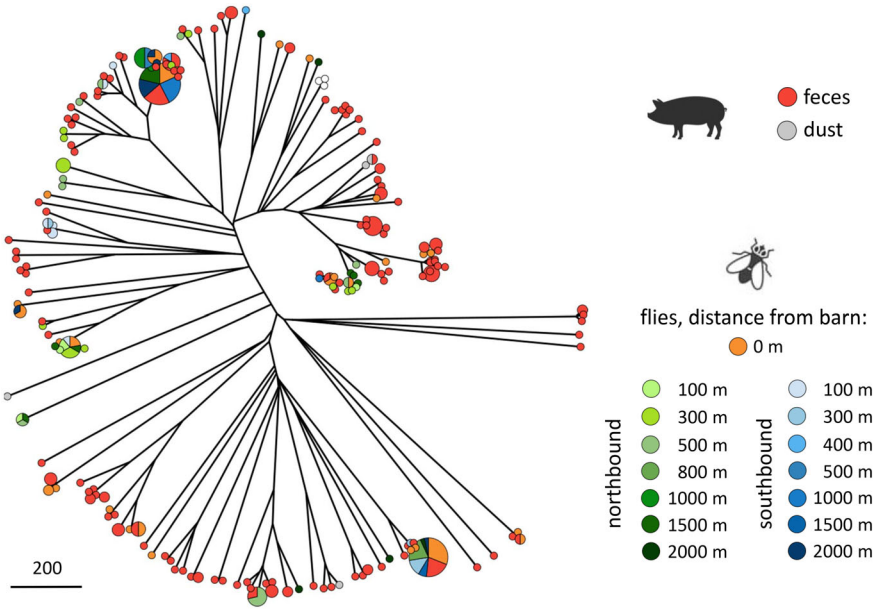

FIGURE 4 Tracing the spread of *E. coli* based on bacterial genome sequences. Neighbour-joining phylogenetic tree based on variation among core-genomes (cgMLST) from 336 *E. coli* isolates. The scale bar indicates 200 differences among cgMLST alleles. Node colours indicate origins of *E. coli* isolates from pig faeces, dust and flies, respectively. Flies had been collected within the pig fattening facility (distance, 0 m) and outside at different distances and in two directions from the source, as indicated.

Germany in 2014 (sequence accession number PRJNA552271; Johanns et al., 2019). Hence, currently available genome sequence data did not suggest the spread of these *E. coli* clones to humans or to other host species, or over long distances. Rather, strains recovered from humans were more distantly related. Accordingly, three clusters at the level HC20 (HC20_3848, HC20_4988, HC20_23529) included 63 genomes, again originating mostly from pig faeces (in the United Kingdom), but also from environmental samples (Chile and UK), from airplane sewage, and from humans in Germany, Denmark and Spain. For the latter database entries, information on any associated clinical symptoms was not available.

DISCUSSION

Pig-associated antimicrobial-resistant *E. coli*

We detected abundant cephalosporin- and fluoroquinolone-resistant *E. coli* in pig faeces, sedimented dust, and flies collected from a pig farm and its surroundings in Brandenburg (East Germany). We did not find any colistin-resistant *E. coli* (i.e. with a colistin MIC above the EUCAST breakpoint for resistance), even though colistin had been administered prophylactically to all newly arrived piglets. Cephalosporin resistance was associated with plasmid-borne extended-spectrum beta lactamase CTX-M-1. Genes encoding CTX-M-1 and TEM-1 also had been the most abundant beta-lactamase genes in pig faeces previously sampled on seven other farms in East Germany (von Salviati et al., 2015). $bla_{\text{CTX-M-1}}$ was reported to be the most prevalent ESBL gene in *E. coli* from swine in Europe, and at the same time, it was frequently found in *E. coli* causing clinical infections in humans (Seiffert et al., 2013). Even though short-read sequencing data rarely allow complete reconstruction of plasmid sequences (Arredondo-Alonso et al., 2017), co-occurrence of plasmid signatures and resistance genes on assembled, contiguous sequence stretches in our dataset allowed the identification of plasmids encoding antibiotic resistance. The β-lactamase gene $bla_{\text{CTX-M-1}}$ was exclusively found on an IncI-1 plasmid. This tight association of $bla_{\text{CTX-M-1}}$ with IncI plasmids had been observed previously (Orlek et al., 2017), and IncI plasmids carrying $bla_{\text{CTX-M-1}}$ had been reported in diverse *E. coli* strains collected from pigs, poultry, cattle, dogs and humans, mostly in Europe (Rozwandowicz et al., 2018). In contrast, we found $bla_{\text{TEM-1}}$ on more diverse plasmids (IncI-1, IncN, IncFII, IncX1), and again, similar associations were reported before (Orlek et al., 2017). Each of the ESBL genes and fluoroquinolone resistance mutations (in *gyrA*, *parC*) occurred in multiple phylogenetic clades of *E. coli*, indicating that these traits had been acquired multiple times independently, likely reflecting the selective pressure caused by widespread use of antibiotics. Our genome analyses indicated the presence of multiple additional antibiotic resistance genes and mutations. We did not identify any carbapenemase genes by scanning genome sequences from *E. coli* isolates, but their sensitive detection in faeces may require targeted, selective enrichment procedures (Irrgang et al., 2019).

Piglets freshly delivered from breeders were already colonized with resistant gut bacteria, likely due to administration of fluoroquinolones and cephalosporins to suckling pigs (Raasch et al., 2020). During pig fattening, however, proportions of *Enterobacteriaceae* in pig faeces resistant to both ciprofloxacin and colistin grew significantly with increasing duration of the animals' stay in the facility. The increase of ciprofloxacin resistance was most pronounced, even though fluoroquinolone drugs (i.e. enrofloxacin) had been administered much more rarely than colistin. However, since fluoroquinolones are particularly stable chemical compounds, large proportions of these drugs get excreted unaltered from treated animals and then are recalcitrant to microbial degradation (Rusch et al., 2019). Hence, residues in the environment may get ingested or inhaled by other animals and select for fluoroquinolone resistance in their commensal flora (Scherz et al., 2014). The observed increase of colistin resistance was not due to *E. coli* but to related *Enterobacterales* species, which frequently are intrinsically resistant to polymyxins (Torres et al., 2021), and which we did not characterize any further. In contrast, cefotaxime resistance did not increase over the first 8 weeks of the fattening period, presumably due to rare application of β-lactam antibiotics and their comparatively lower environmental persistence (Braschi et al., 2013).

Dissemination of antimicrobial-resistant *E. coli*

Our analyses of bacterial genomes indicated frequent transmission of AMR *E. coli* between pigs from different herds and suggested dust and flies as vectors for dissemination of faecal pathogens. Barn dust may carry faecal particles including bacteria (Luiken et al., 2020), which may get ingested or inhaled (and then swallowed), and subsequently become established in the pigs' gut microbiome. Airborne transmission of ESBL-producing *E. coli* in the pig barn environment had been suspected previously to cause an occupational risk for farmers and their employees (Dohmen et al., 2017; von Salviati et al., 2015).

Coprophagic flies are commonly contaminated with gut bacteria, since both adult and larval flies feed on animal faeces (Zurek & Ghosh, 2014). Due to their widespread occurrence, flies have recently been

proposed as useful targets for sentinel surveys to monitor antimicrobial resistance (Yin et al., 2022). Flying distances of several kilometres per day have been documented for individual *M. domestica* by releasing and recapturing dye-labelled flies (Quarterman et al., 1954; Schoof et al., 1952). The detection of *E. coli* or *Salmonella enterica*, respectively, with indistinguishable pulsotypes (i.e. DNA macrorestriction patterns resolved by pulsed-field gel electrophoresis) on different livestock farms had been attributed to fly-associated spread in the past (Solà-Ginés et al., 2015; Wang et al., 2011). However, other common sources of the bacteria (e.g. supplies of stocking animals from the same breeding farms) could have led to similar results and were not excluded in those previous studies (Solà-Ginés et al., 2015, Wang et al., 2011).

In contrast, our bacterial genome sequencing approach tracked the fly-associated transport of diverse ESBL-producing *E. coli* from a pig-farm into urban habitation areas, over distances of at least 2 km. Houseflies have been shown to transfer *E. coli* and other faecal bacteria to human food through contact, which can lead to colonization and infection in humans after the food is consumed (De Jesús et al., 2004; Fukuda et al., 2020; Lindeberg et al., 2018). Hence, the observed proximity to human households poses a risk of transmission of antimicrobial-resistant enteric pathogens from livestock to man (Khamesipour et al., 2018; Onwugamba et al., 2018; Zurek & Ghosh, 2014). Interestingly, 13% of flies recently collected in central Berlin (located approximately 25 km from the pig farm investigated here) were contaminated with ESBL-producing *E. coli*, and the majority of those *E. coli* carried the $bla_{\text{CTX-M-1}}$ gene (Wetzker et al., 2019). While the endowment of bacteria with this resistance gene would be consistent with their livestock origins, genome data from those strains were not available for analysis of strain relatedness.

Comparison of our genome sequences from 336 *E. coli* isolates to >200,000 genomic assemblies publicly available from EnteroBase did not identify any closely related matches. This might suggest that transmission of *E. coli* from the pig farm to humans has not occurred frequently, or at least has not caused clinical disease in humans frequently (as most genome-sequenced *E. coli* originate from infections rather than symptom-free colonization). This result is in contrast to our previous study, which used EnteroBase to identify near-identical genomes among *Clostridioides difficile* isolates originating from chicken manure, retail chicken meat, and human infections from independent previous investigations, even though the database for *Clostridioides* at the time was 10 times smaller than the current *Escherichia* database (Frentrup et al., 2021). A more thorough assessment of pathogen spread from the farm to human residents would require simultaneous, extensive sampling of *E. coli* genomes from livestock and humans in the same area. It is quite possible, however, that *E. coli* transmits less easily between host species than *C. difficile*, since a recent study applying bacterial genome sequencing also detected little overlap between ESBL *E. coli* derived from livestock and from human bacteremia in the United Kingdom, concluding that the spread of AMR *E. coli* among hospital patients was much more relevant (Day et al., 2019). Even if *E. coli* strains from animals do not frequently cause disease in humans; however, they constitute a large reservoir of resistance genes, which may get transferred horizontally to human-adapted strains (Chang et al., 2015). In any case, measures to reduce antimicrobial resistance and its spread within and beyond animal husbandry are warranted in a 'One Health' framework (Robinson et al., 2016). Aside from a reduction of antimicrobial usage, these may include an improved hygiene management and pest control (Raasch et al., 2020). Pathogen transmission by flies can be prevented most effectively by reducing breeding sites (i.e. manure) (Meerburg et al., 2007) and by using fly screens (Hald et al., 2007). Possibly, the emission of contaminated flies from the specific farm studied here was facilitated by free-range external access of pigs from the open stable (Meerburg et al., 2007).

To our knowledge, this is the first study applying bacterial genome sequencing for tracing the insect-associated, environmental spread of any pathogens from their source. We note that this approach at the same time also tracked the dissemination of the specific insect population. Genomic analyses of microbiome constituents may be adapted and more widely applied for investigations into movements of other insect species, similar to mark-recapture methods, but without the need for any labelling.

AUTHOR CONTRIBUTIONS

Wiebke Behrens: Data curation (equal); formal analysis (lead); investigation (equal); methodology (supporting); visualization (supporting); writing – review and editing (supporting). **Baban Kolte:** Data curation (equal); formal analysis (lead); investigation (equal); methodology (supporting); visualization (supporting); writing – review and editing (supporting). **Vera Junker:** Data curation (equal); formal analysis (lead); investigation (supporting); methodology (supporting); visualization (equal); writing – review and editing (supporting). **Martinique Frentrup:** Data curation (supporting); formal analysis (supporting); investigation (supporting); methodology (supporting); writing – review and editing (equal). **Claudia Dolsdorf:** Methodology (supporting); resources (supporting); supervision (supporting); writing – review and editing (supporting). **Maria Börger:** Formal analysis (supporting); writing – review and editing (supporting). **Megarsa Jaleta:** Investigation (supporting); writing – review and editing (supporting). **Tina**

Kabelitz: Conceptualization (equal); investigation (supporting); supervision (supporting); writing – review and editing (supporting). **Thomas Amon:** Conceptualization (equal); funding acquisition (lead); investigation (supporting); supervision (supporting); writing – review and editing (supporting). **Doreen Werner:** Conceptualization (equal); funding acquisition (supporting); investigation (equal); methodology (supporting); writing – review and editing (supporting). **Ulrich Nubel:** Conceptualization (lead); funding acquisition (lead); investigation (equal); project administration (equal); supervision (lead); supervision (lead); validation (lead); visualization (supporting); writing – original draft (supporting).

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CONFLICT OF INTEREST STATEMENT

The authors declare that they do not have any competing interests.

DATA AVAILABILITY STATEMENT

The sequence data that support the findings of this study are openly available from GenBank (https://www.ncbi.nlm.nih.gov/genbank/) under BioProject ID: PRJNA885145.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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