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Article

Occurrence of Mobile Colistin Resistance Genes *mcr-1–mcr-10* including Novel *mcr* Gene Variants in Different Pathotypes of Porcine *Escherichia coli* Isolates Collected in Germany from 2000 to 2021

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Abstract: In the European Union, gastrointestinal disease in pigs is the main indication for the use of colistin, but large-scale epidemiologic data concerning the frequency of mobile colistin resistance (*mcr*) genes in pig-associated pathotypes of *Escherichia coli* (*E. coli*) are lacking. Multiplex polymerase chain reactions were used to detect virulence-associated genes (VAGs) and *mcr-1–mcr-10* genes in 10,573 porcine *E. coli* isolates collected in Germany from July 2000 to December 2021. Whole genome sequencing was performed on 220 representative *mcr*-positive *E. coli* strains. The total frequency of *mcr* genes was 10.2%, the most frequent being *mcr-1* (8.4%) and *mcr-4* (1.6%). All other *mcr* genes were rarely identified (*mcr-2*, *mcr-3*, *mcr-5*) or absent (*mcr-6* to *mcr-10*). The highest frequencies of *mcr* genes were found in enterotoxigenic and shiga toxin-encoding *E. coli* (ETEC/STEC hybrid) and in edema disease *E. coli* (EDEC) strains (21.9% and 17.7%, respectively). We report three novel *mcr* variants, *mcr-1.36*, *mcr-4.8*, and *mcr-5.5*. In 39 attaching and effacing *E. coli* (AEEC) isolates analyzed in our study, the *eae* subtype β1 was the most prevalent (71.8%). Constant surveillance for the presence of *mcr* genes in various sectors should consider the different frequency of *mcr*-positive isolates in pathogenic *E. coli*.

Keywords: *Escherichia coli*; pathotype; mobile colistin resistance; *mcr-1*; *mcr-4*; *mcr-5*; plasmid; *eae*; swine

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1. Introduction

Polymyxins are considered last-resort antibiotics in human medicine against infections caused by multidrug-resistant Gram-negative bacteria [1]. The polymyxin antibiotic colistin (polymyxin E) has been widely used for treating intestinal infections in swine caused by *Escherichia coli*. Enterotoxigenic *E. coli* (ETEC) and edema disease *E. coli* (EDEC) are the causative agents of enteric diseases and edema disease in piglets, resulting in significant economic losses in the swine industry worldwide [2]. ETEC are defined by the possession of at least one of the adhesive fimbriae F4 (encoded by the *fae* genes), F5 (*fan*), F6 (*fas*), F18 (*fed*), and F41 (*fimF41*) in combination with one of the heat-labile toxins LT-Ia or LT-Ib (*eltB-lp*) or heat-stable toxins ST-Ia or ST-II (*estap* or *estb*) [3]. EDEC characteristically harbors genes for shiga toxin 2e (*stx2e*) and for adhesive fimbriae F18 (*fed*) [4]. In addition, other *E. coli* isolates that do not strictly apply to these pathotype definitions might be involved in porcine intestinal disorders that are commonly treated with antibiotics. According to a recent study from 2022 about the use of colistin in veterinary medicine in the European Union, it was stated that the main indication for the use of colistin was gastrointestinal disease in pigs [5].

In 2015, the plasmid-mediated mobile colistin resistance (*mcr*) gene *mcr-1* was identified in a porcine *E. coli* isolate from China, followed by reports of nine additional *mcr* genes (*mcr-2*–*mcr-10*) and their variants [6]. A number of studies from across the globe, including studies from Germany, reported different frequencies of *mcr* genes in fecal *E. coli* isolates from healthy pigs, as recently summarized [7]. In contrast, many fewer studies were performed to determine the frequency of *mcr* genes among clinical isolates, i.e., obtained from pigs with post-weaning diarrhea or from enteric colibacillosis [8–12]. In addition, the latter studies rarely provided a detailed molecular typing of *E. coli* isolates regarding their affiliation to distinct intestinal pathogenic pathotypes predicted by the presence of virulence-associated genes (VAGs). This would indeed be very helpful to explore, even though it would only be based on an observational approach, if certain pathotypes are more prone to acquire *mcr* genes than others.

To narrow this knowledge gap, we investigated the distribution of *mcr* genes *mcr-1* to *mcr-10*, which were defined at the time of writing, among a collection of more than 10,000 porcine *E. coli* isolates, according to their pathotype designation. The genomes of selected *mcr*-positive *E. coli* isolates were sequenced and analyzed for *mcr* gene variants and their location on distinct plasmids as well as for *E. coli* multi locus sequence types and phylogenetic groups. Finally, the presence of extended-spectrum β-lactamase (ESBL), AmpC, and carbapenemase genes among sequenced isolates was explored.

2. Materials and Methods

2.1. Sample Processing and Isolation of Putative E. coli Colonies

We investigated 9421 *E. coli* isolates that were obtained mainly from feces or mucosal swabs (rectum or small intestine) of piglets suffering from neonatal diarrhea, post-weaning diarrhea, or edema disease. The isolates were collected as part of routine microbiological diagnostics at the Institute for Hygiene and Infectious Diseases of Animals, Faculty of Veterinary Medicine, Justus Liebig University Giessen, Germany, from July 2000 to December 2021. Additional porcine *E. coli* isolates (n = 1152) were received through submissions of other veterinary diagnostic laboratories for further molecular typing in our institute. Some of these isolates have already been included in a recent study on the presence of *mcr-1* and *mcr-2* genes in porcine *E. coli* isolates [7]. Ethical review and approval were waived for this study due to the fact that the sample collection was not for research but for diagnostic purposes, and only the results obtained were used for scientific purposes. No additional pain, suffering, or harm was inflicted on the animals as a result of our study.

According to available metadata on the origin of samples and/or *E. coli* isolates, strains were obtained from neonatal diarrhea in piglets (i.e., isolates obtained from piglets $\leq$ 8 kg and/or $\leq$28 days with diarrhea) (n = 1473); PWD and diarrhea in elderly pigs (i.e., isolates obtained from pigs > 8 kg to $\leq$30 kg and/or >4 weeks to $\leq$12 weeks with diarrhea) (n = 3687); edema disease (i.e., isolates from pigs and/or farms, where edema disease occurred), either with (n = 702 isolates) or without diarrhea (n = 1413); diarrhea in fattening pigs (between 12 weeks and 7 months and/or >30 kg) (n = 672); diarrhea in pigs of unknown age and/or weight (n = 2605). The remaining 21 samples/isolates were provided for the typing of VAGs associated with diarrheal diseases in swine.

The maximum number of samples per farm was limited to samples from six pigs per submission. As the samples were provided for diagnostic services, they were treated immediately upon arrival at the laboratory. Fecal samples and mucosal swabs were streaked for single bacterial colonies on blood agar plates (blood agar base, Merck Chemicals, Darmstadt, Germany) containing 5% sheep blood and on Gassner agar (sifin diagnostics GmbH, Berlin, Germany). The cultures were incubated for approx. 18 h at 37 °C. Subsequently, up to six morphologically different, putative *E. coli* colonies were picked per sample and stored individually as pure bacterial suspensions in lysogeny broth (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) for further analysis. A single colony was regarded as putative *E. coli* in case the following phenotypes were observed: (i) circular, shiny, greyish diameter of 1.0–2.0 mm on blood agar or (ii) deep blue with a blue halo, diameter of 1.0–2.5 mm

on Gassner agar. If hemolytic and non-hemolytic colonies of putative *E. coli* occurred on the same blood agar plate, representative colonies of both phenotypes were picked. Species identification was performed by matrix-assisted laser desorption time-of-flight mass spectrometry MALDI-TOF MS (Bruker Daltonics, Bremen, Germany) by applying the standard MBT Compass reference library (different versions according to the study year).

2.2. Escherichia coli Pathotyping PCR and Prediction of Pathotypes

About 3 µL of lysogeny broth cultures (about 3×10^5 CFU) were used as template DNA in a modified multiplex PCR (MP-PCR-VAGs), in total targeting 10 virulence-associated genes (VAGs), which are associated with different pathotypes of intestinal pathogenic *E. coli* [13–15]. In detail, *E. coli* isolates were tested for the presence of genes of adhesive fimbriae F4 (encoded by the gene *faeG*), F5 (*fanA*), F6 (*fasA*), F18 (*fedA*), and F41 (*fimF41a*), the afimbrial adhesin intimin (*eae*), heat-labile *E. coli* enterotoxins LT-Ia and LT-Ib (*eltB-Ip*), heat-stable *E. coli* enterotoxins ST-Ia and ST-II (*estap/estb*), and shiga toxin 2 (*stx2*). Positive controls used were *E. coli* strains B41 (*fimF41a*, *fanA*, *estap*), 987P (*fasA*, *estap*), E57 (*fedA*, *estap*, *estb*, *stx2*), G7 (*faeG*, *eltB-Ip*), and TTP-1 (*eae*, *stx2*). An *E. coli* K-12 laboratory strain was used as a negative control. Details regarding primers and controls used in the MP-PCR-VAGs are provided in Table S1. Each study isolate that proved positive for at least one of the tested VAGs was stored in a glycerin stock at −80 °C. If isolates from the same pig showed different VAG profiles, a representative isolate of each profile was stored. Part of the pathotyping PCRs have already been conducted as part of a recent study [7].

Pathotype prediction was conducted based on the presence of VAGs determined by PCR: Adhesive fimbriae *E. coli* (in the following termed AdhF-*Ec*), positive for at least one adhesive fimbriae gene (*faeG*, *fanA*, *fasA*, *fedA*, *fimF41a*); AEEC (often also referred to as atypical EPEC), positive for *eae*; EDEC, positive for *fedA* and *stx2*; ETEC, positive for at least one adhesive fimbriae gene (*faeG*, *fanA*, *fasA*, *fedA*, *fimF41a*) and at least one enterotoxin gene (*eltB-Ip*, *estap*, *estb*); ETEC-like, positive for at least one enterotoxin gene (*eltB-Ip*, *estap*, *estb*); ETEC/STEC hybrid (in the following simply termed ETEC/STEC), positive for at least one adhesive fimbriae gene (*faeG*, *fanA*, *fasA*, *fedA*, *fimF41a*) and at least one enterotoxin gene (*eltB-Ip*, *estap*, *estb*) and *stx2*; STEC, positive for *stx2*; other, positive for a combination of VAGs not covered by the previously defined pathotypes.

2.3. PCR for the Detection of Mobile Colistin Resistance Genes mcr-1 to mcr-10

Two multiplex (MP) PCRs were applied to detect *mcr-1* to *mcr-10* genes. The first MP-PCR enabled the detection of genes *mcr-1* to *mcr-5* and was mostly based on previously published primer sequences [12,16–19]. Only one primer (MCR-5-mp-fw) was newly created in this study. The second MP-PCR protocol was based on a previous protocol [19] that we modified by including two primers to amplify the novel *mcr-10* gene in addition to genes *mcr-6* to *mcr-9*. Details regarding primers and controls used in MCR MP-PCRs I and II are provided in Table S1.

2.4. Whole Genome Sequence Analysis

Genomic DNA was extracted from *E. coli* bacteria using the Master Pure™ DNA Purification Kit (Biozym Scientific GmbH, Hessisch Oldendorf, Germany). Bacterial genomes were sequenced using an Illumina MiSeq sequencer (MiSeq Reagent Kit V.3; Illumina Inc., San Diego, CA, USA) via multiplexing of 30 samples per flow cell using 2 × 150 bp paired-end reads to obtain an average coverage of 90-fold. Quality control, including contamination removal and adapter trimming, were performed using an in-house pipeline. De novo assemblies were generated via the SPAdes Genome Assembler (v3.15.5) with the "—isolate" flag [20]. The Bakta pipeline (v1.8.2) was employed using species-specific databases for genomic annotation of the bacterial genomes [21].

2.5. Phylogroups, Sequence Types, Clonotypes, Antimicrobial Resistance Genes, Virulence-Associated Genes

Bacterial genome sequence data were analyzed in silico to classify isolates into one of the eight *E. coli* phylogenetic groups (A, B1, B2, C, D, E, F, and G) or into a cryptic clade using the refined ClermonTyping method, based on the in vitro PCR assay, targeting *chuA*, *yjaA*, *TspE4.C2*, *arpA*, and *trpA* (http://clermontyping.iame-research.center/, accessed on 20 October 2023). MLST 2.0 (https://cge.food.dtu.dk/services/MLST/, accessed on 20 October 2023) was used to determine sequence types (STs) according to the Achtman scheme, employing seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*). The clonotyping was based on the internal 469- and 489-nucleotide sequences of the *fumC* and *fimH* genes, respectively [22]. Allele assignments for *fumC* and *fimH* and their combinations (=clonotypes) were determined using CHTyper 1.0 (https://cge.food.dtu.dk/services/CHTyper/, accessed on 28 November 2023) [23]. Sero(geno)types were determined by applying SerotypeFinder 2.0 (https://cge.food.dtu.dk/services/SerotypeFinder/, accessed on 13 November 2023). The O25b serogroup was investigated in selected isolates by comparing the *papB* sequence with the reference sequence of the O25b:H4-ST131 uropathogenic strain EC958 (accession number HG941718; ENA; http://www.ebi.ac.uk/ena, accessed on 13 November 2023). AMR genes and chromosomal point mutations related to antimicrobial resistance were determined using ResFinder 4.1 (https://cge.food.dtu.dk/services/ResFinder/, accessed on 12 September 2023). ST131 isolates were additionally investigated for VAGs related with extraintestinal pathogenic *E. coli* (ExPEC) using VirulenceFinder 2.0 (https://cge.food.dtu.dk/services/VirulenceFinder/, accessed on 28 November 2023).

2.6. Statistical Analysis

Pathotype frequencies were reported as descriptive data. Fisher's exact tests (https://www.graphpad.com/quickcalcs/contingency1/, accessed on 19 November 2023) were used to characterize the association of specific pathotypes with the occurrence of *mcr* genes. In particular, we performed pairwise comparisons of the pathotype associated with the highest *mcr* prevalence with the *mcr* abundance of the other pathotypes characterized within this study. We considered p-values below 0.05 to be statistically significant. All reported p-values are two-tailed.

3. Results

3.1. E. coli Pathotypes

More than half (60.5%) of the 10,573 porcine pathogenic *E. coli* isolates could be clearly delineated to an intestinal *E. coli* pathotype following the definition provided in Material and Methods (Section 2.2) and in Table 1. They were determined as ETEC (31.9%), EDEC (12.8%), AEEC (12.4%), and STEC (3.5%). The remaining isolates were assigned to the groups of ETEC-like (25.1%), i.e., harboring at least one enterotoxin but lacking adhesive fimbriae genes, AdhF-*Ec* (8.1%; positive for at least one adhesive fimbriae gene), and to hybrid groups termed ETEC/STEC (5.3%) or AEEC/STEC (0.22%), fulfilling the predictive criteria for both pathotypes simultaneously. Several other VAG profiles were observed, leading to further delineation of a small proportion of the isolates (0.78%) into additional hybrid pathotypes (Table 1).

E. coli isolates obtained from pigs with clinical signs or suspected of having edema disease on the farms were predominantly defined as EDEC (25.5%), ETEC (21.9%), and ETEC-like (12.0%). Isolates obtained from piglets with neonatal diarrhea were mostly assigned as ETEC (47.6%), ETEC-like (24.0%), and AEEC (17.0%), while isolates collected from cases of PWD were predominantly allocated to the pathotypes ETEC (31.6%), ETEC-like (30.2%), AEEC (11.1%), and EDEC (8.3%). Also, among the isolates obtained from diarrheic fattening pigs and from diarrheic pigs of unknown ages, pathotypes ETEC and ETEC-like *E. coli* were predominant (35.2%, 26.1% and 29.2%, 24.0%, respectively).

Table 1. Pathotype distribution and occurrence of *mcr* genes among 10,573 porcine *E. coli* isolates collected from 2000 to 2021 in Germany.

Pathotype (no./% of Isolates)	*mcr-1*	*mcr-2* *	*mcr-3*	*mcr-4*	*mcr-5*	*mcr-1* and *-4*	*mcr-1* and *-5*	*mcr-4* and *-5*
ETEC (3369/31.9)	215 (6.4)	0	0	37 (1.1)	2 (0.1)	1 (0.03)	0	0
ETEC-like (2650/25.1)	230 (8.7)	2 (0.1)	0	40 (1.5)	2 (0.1)	0	0	0
EDEC (1348/12.8)	178 (13.2)	0	3 (0.2)	39 (2.9)	7 (0.5)	8 (0.6)	0	3 (0.2)
AEEC (1310/12.4)	45 (3.4)	0	0	1 (0.1)	3 (0.2)	1 (0.1)	1 (0.1)	0
AdhF-*Ec* (862/8.1)	82 (9.5)	0	0	6 (0.7)	6 (0.7)	0	0	0
ETEC/STEC (563/5.3)	95 (16.9)	0	0	22 (3.9)	6 (1.1)	0	0	0
STEC (367/3.5)	28 (7.6)	0	0	8 (2.2)	1 (0.3)	1 (0.3)	0	0
ETEC-like/STEC (75/0.7)	2 (2.7)	0	0	0	0	0	0	0
AEEC/STEC (23/0.2)	0	0	0	0	0	0	0	0
ETEC-like/AEEC (3/0.03)	0	0	0	0	0	0	0	0
AdhF-*Ec*/EDEC (2/0.02)	0	0	0	0	0	0	0	0
AdhF-*Ec*/AEEC (1/0.01)	0	0	0	0	0	0	0	0
Total (n = 10,573)	875 (8.3)	2 (0.02)	3 (0.03)	153 (1.5)	27 (0.3)	11 (0.1)	1 (0.01)	3 (0.03)

Data on the *mcr* gene distribution are given as numbers and percentages (in brackets). Percentages refer to the number of isolates among a given pathotype or group. Numbers for *mcr-6* to *mcr-10* genes are not presented, as none of the isolates were positive for any of these genes. Pathotype prediction was conducted based on the presence of VAGs, as described in Section 2.2. * *mcr-2*-positive isolates were previously published and will not be mentioned hereinafter [7].

ETEC isolates revealed 25 different VAG combinations. The most frequent combination was *estb*, *eltB-Ip*, *faeG* (n = 1451/3369; 43.1%), and, in decreasing frequency: *estb*, *estap*, *fedA* (n = 513, 15.2%); *estb*, *estap*, *eltB-Ip*, *faeG* (n = 508; 15.1%), *estb*, *estap*, *faeG* (n = 291, 8.6%), and *estb*, *eltB-Ip*, *fedA* (n = 205, 6.1%). The remaining 20 VAG patterns were each present in $\leq$2.0% (one to 67 isolates) of the 3369 ETEC isolates (Table S2). ETEC-like isolates, which lacked all fimbrial genes investigated by PCR, predominantly harbored *estb* as the sole enterotoxin gene (n = 1708/2650, 64.5%). Other frequent VAG patterns among ETEC-like isolates were *estb*, *estap* (n = 766, 28.9%), and *estb*, *eltB-Ip* (n = 142, 5.4%), whereas three other patterns occurred only rarely (*estap*, 1.1%; *eltB-Ip*, 0.04%; *estb*, *estap*, *eltB-Ip* 0.2%). Isolates of the group of AdhF-*Ec* predominantly carried the F18 fimbrial gene *fedA* (n = 790/862, 91.6%) and less often the F4 fimbrial gene *faeG* (n = 44, 5.1%), *fim41a* (n = 23, 2.7%), or other fimbrial genes. As *per definitionem*, all 1348 EDEC isolates harbored *fedA* and *stx2*, all AAEC isolates carried intimin gene *eae*, and all STEC isolates carried shiga toxin gene *stx2*.

3.2. Distribution of mcr Genes, Novel mcr Gene Alleles

Out of 10,573 *E. coli* isolates, 10.2% (n = 1075) carried one or two *mcr* genes (Table 1). With regard to different pathotypes, ETEC/STEC hybrid strains and EDEC strains revealed the highest proportion of *mcr*-positive isolates, respectively (21.9% and 17.7%) (Figure 1). Lower percentages were identified among the groups of AdhF-*Ec* (10.9%) and ETEC-like (10.3%), as well as among pathotypes STEC (10.4%) and ETEC (7.6%). The prevalence of *mcr*-positive isolates was significantly lower in non-ETEC/STEC isolates compared to the other pathotypes (p = 0.04 compared to EDEC; $p < 0.0001$ for all other pathotypes, Fisher's exact test).

With respect to the different *mcr* genes, the group of ETEC/STEC hybrid isolates showed the highest percentages, i.e., 16.9% for *mcr-1*, 3.9% for *mcr-4*, and 1.1% for *mcr-5* (Table 1), while only two ETEC-like and three EDEC isolates were positive for *mcr-2* and *mcr-3*, respectively.

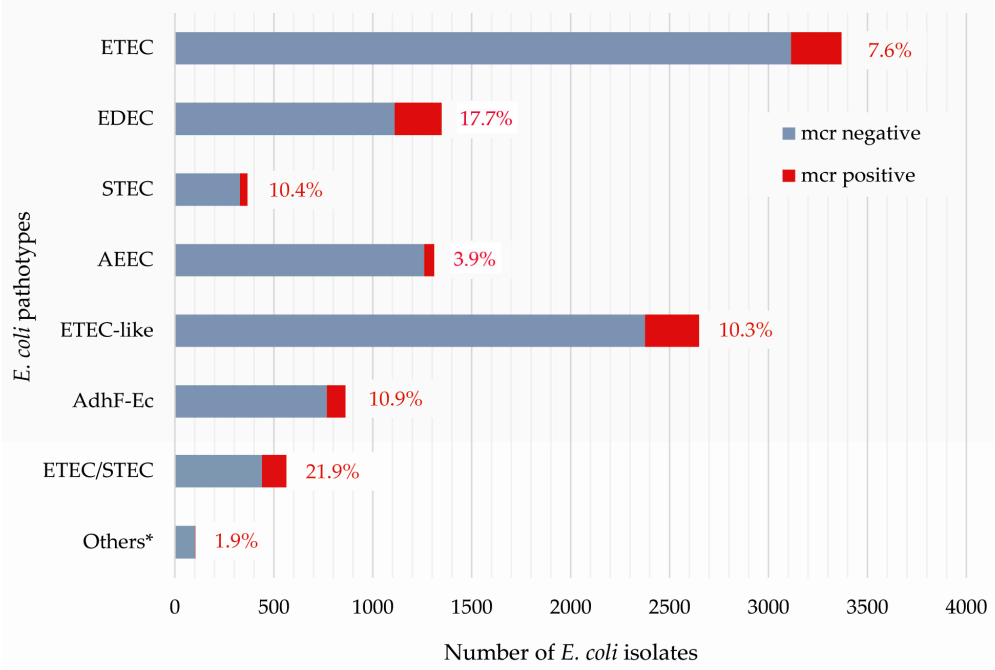

Figure 1. Presence of *mcr* genes among 10,573 porcine *E. coli* isolates based on their association to a distinct pathotype. * Others include 104 *E. coli* isolates defined as ETEC-like/STEC (n = 75), AEEC/STEC (n = 23), ETEC-like/AEEC (n = 3), AdhF-*Ec*/EDEC (n = 2), and AdhF-*Ec*/AEEC (n = 1).

The overall frequency of *mcr-1*, either as a single gene or in combination with other *mcr* genes, was highest (8.4%), followed by *mcr-4* (1.6%) and *mcr-5* (0.3%) (both either as a single gene or in combination), *mcr-3* (0.03%), and *mcr-2* (0.02%). None of the isolates carried *mcr-6*, *mcr-7*, *mcr-8*, *mcr-9*, or *mcr-10*.

In terms of different time periods of sample collection and *E. coli* isolation, the prevalence of *mcr*-positive isolates differed as follows: 2000 to 2005 (0.8% among 1022 isolates obtained from this time period), 2006 to 2010 (5.9%/1266), 2011 to 2015 (15.8%/3270), and 2016 to 2021 (9.4%/5015). The earliest time points of *mcr* gene detection were 2001 (*mcr-5*, ETEC/STEC), 2005 (*mcr-4*, ETEC-like), 2006 (*mcr-1*, ETEC), 2014 (*mcr-2*, ETEC-like), and 2014 (*mcr-3*, EDEC).

Among 1075 *mcr*-positive porcine *E. coli* isolates, 220 isolates, representing different pathotypes, isolation dates, and *mcr* genes, were selected for whole genome sequencing. In detail, we chose (i) pathotypes (ETEC (n = 56), EDEC (n = 48), AEEC (n = 41), ETEC-like (n = 30), ETEC/STEC (n = 23), STEC (n = 14), and AdhF-*Ec* (n = 8)); (ii) isolation dates (2001–2005 (n = 5), 2006–2010 (n = 34), 2011–2015 (n = 113), and 2016–2021 (n = 68); and (iii) *mcr* genes (*mcr-1* (n = 125), *mcr-3* (n = 1), *mcr-4* (n = 67), *mcr-5* (n = 17), *mcr-1* and *mcr-4* (n = 6), *mcr-1* and *mcr-5* (n = 1), and *mcr-4* and *mcr-5* (n = 3) for whole genome sequencing.

Of 132 *mcr-1*-positive isolates, the majority (96.2%) carried the *mcr-1.1* gene variant. One ST29-AEEC isolate obtained from a seven-week-old pig suffering from diarrhea in 2018 carried an *mcr-1.26* allele. A novel *mcr-1* variant, termed *mcr-1.36* (NCBI Reference Sequence: NG_231577.1), was identified in an ST48-AEEC isolate which was provided as *E. coli* isolate from another laboratory in 2016. The *mcr-1.36* gene variant differed from *mcr-1.1* by a nucleotide substitution at position 1588 (G → T), resulting in an amino acid change at position 530 (alanine → serine) of MCR-1.36 compared to MCR-1. Another three isolates revealed *mcr-1.1*-like genes that were either disrupted by an *IS*26 element (ST29-AEEC obtained from a pig with watery diarrhea in 2015) or showed alternative start codons (ST1-EDEC, 2008; ST29-AAEC, 2016, both obtained from pigs with clinical signs of edema disease). In 53 isolates, the genomic contig carried both a plasmid replicon gene and an *mcr* gene, which allowed us to determine the location of *mcr-1* genes on plasmids of incompatibility groups IncX4 (n = 44), IncHI2 (n = 7), and IncI2 (n = 2).

The *mcr-3* gene variant shared 99.8% nucleotide sequence similarity and 100% deduced amino acid sequence identity to *mcr-3.12* and MCR-3.12, respectively. The *mcr-3*-containing contig was 18,883 bp in length and was predicted as a plasmidial sequence (95.5%) using mlplasmids v2.1.0 (https://sarredondo.shinyapps.io/mlplasmids/, accessed on 28 November 2023). We observed co-localization of *mcr-3.12* with antimicrobial resistance genes *aadA5*, *dfrA1*, *sul1*, *tet(A)*, *blaOXA-1*, and *catB3* on the same contig.

The majority of 70 sequenced *mcr-4*-positive isolates carried *mcr-4.6* (*n* = 43; 61.4%) and *mcr-4.2* (*n* = 21; 30.0%), followed by *mcr-4.1* and *mcr-4.3* (*n* = 1 each). In addition, a novel *mcr-4* gene variant, termed *mcr-4.8* (NCBI Reference Sequence: NG_231578.1), was identified in three ETEC and one ETEC-like isolate collected in the years 2009, 2015, 2017, and 2019. The *mcr-4.8* gene differs from *mcr-4.1* by a nucleotide substitution at position 706 (G → T), resulting in an amino acid change at position 331 (glutamine → arginine) (Table 2) of the gene product. In nearly all cases (97.1%), *mcr-4* genes were located on ColE10 plasmids.

Table 2. Overview of *mcr-4*/MCR-4 alleles and depiction of nucleotide/amino acid sequence changes compared to *mcr-4.1*.

Year	Species	Source	Country	Allele	Nucleotides at Signature Positions *						AA Sequence Change and Position **	NCBI Reference Sequence
					329	536	613	706	992	1453		
2011	*E. coli*	pig	DE	*mcr-4.1*	C	T	C	G	A	G	-	NG_057470.1
2011	*E. coli*	pig	DE	*mcr-4.2*	C	T	C	G	G	G	Q331R	NG_057471.1
2014	*Ent. cloacae*	human	n.p.	*mcr-4.3*	C	G	C	T	A	G	V179G, V236F	NG_057461.1
n.p.	*E. coli*	pig	ES	*mcr-4.4*	C	T	A	G	G	G	H205N, Q331R	NG_057465.1
n.p.	*E. coli*	pig	ES	*mcr-4.5*	T	T	C	G	G	G	P110L, Q331R	NG_057464.1
2016	*S. enterica*	pig	ES	*mcr-4.6*	C	T	C	T	A	G	V236F	NG_061608.1
2009	*A. baumannii*	pulp	FI	*mcr-4.7*	C	G	C	T	G	A	V179G, V236F, Q331R, V485I	NG_088453.1
2017	*E. coli*	pig	DE	*mcr-4.8*	C	T	C	T	G	G	Q331R	NG_231578.1

A. = *Acinetobacter*; *E.* = *Escherichia*; *Ent.* = *Enterobacter*; *S.* = *Salmonella*; DE = Germany; ES = Spain; FI = Finland; n.p. = not provided; * 1-letter code nucleotides: A = adenine; C = cytosine; G = guanine; T= thymine. Nucleotide changes in comparison to *mcr-4.1* are highlighted in gray. ** 1-letter code amino acids: F = phenylalanine; G = glycine; H = histidine; I = isoleucine; L = leucine; N = asparagine; P = proline; Q = glutamine; R = arginine; V = valine; AA = amino acid.

Of 17 sequenced *mcr-5*-positive isolates, the majority revealed *mcr-5.1* (94.1%). One ST29-AEEC isolate, which was obtained from an eight-week-old pig with diarrhea, carried a novel *mcr-5* variant termed *mcr-5.5* (NG_231579.1). As illustrated in Table 3, the *mcr-5.5* gene carried one missense mutation at position 522 (T → G), in comparison to *mcr-5.1*, resulting in a codon change at position 498 (aspartic acid → asparagine). No plasmid-related genes were identified in the approximately 7.3 to 10.9 kb contigs containing the *mcr-5* genes.

Table 3. Overview of *mcr-5*/MCR-5 alleles and depiction of nucleotide/amino acid sequence changes compared with *mcr-5.1*.

Year	Species	Source	Country	Allele	Nucleotides at Signature Positions *				AA Sequence Change and Position **	NCBI Reference Sequence or GenBank No.
					313	522	698–700	1240		
2012	*S. enterica*	chicken meat	DE	*mcr-5.1*	C	T	AAG	G	-	NG_055658.1
2011	*E. coli*	pig	DE	*mcr-5.2*	C	T	del	G	E234del	MG384740.1
2012	*E. coli*	horse	BR	*mcr-5.3*	C	T	AAG	T	A414S	MH062179.1
2017	*E. coli*	hosp. tap water	NL	*mcr-5.4*	T	T	AAG	G	L105F	NG_065945.1
2006	*E. coli*	pig	DE	*mcr-5.5*	C	G	AAG	G	D498N	NG_231579.1

E. = *Escherichia*; *S.* = *Salmonella*; BR = Brazil; DE = Germany; NL = Netherlands; * 1-letter code nucleotides: A = adenine; C = cytosine; G = guanine; T= thymine. Nucleotide changes in comparison to *mcr-5.1* are highlighted in gray. ** 1-letter code amino acids: A = alanine; D = aspartic acid; E = glutamic acid; F = phenylalanine; L = leucine; N = asparagine; S = serine; AA = amino acid; del = deletion; hosp. = hospital.

3.3. Presence of ESBL, AmpC, Carbapenemase, and Other Antimicrobial Resistance Genes and Chromosomal Mutations among Whole Genome Sequenced mcr-Positive E. coli Isolates

Only a few (3.2%) of the 220 sequenced *mcr*-positive isolates co-harbored extended spectrum β-lactamase genes. ESBL gene $bla_{\text{CTX-M-1}}$ was determined in AEEC (n = 2; both ST29), EDEC (n = 1; ST744), ETEC (n = 1; ST772), and ETEC/STEC (n = 1; ST10); $bla_{\text{CTX-M-14}}$ in an ST29-AEEC isolate; and $bla_{\text{TEM-52}}$ in an ST10-ETEC isolate. Acquired AmpC β-lactamase genes and carbapenemase genes were not identified. Two ST86-ETEC/STEC isolates revealed *ampC* promoter mutations (42C → T), which are known to increase *ampC* transcription rates and play an important role in *E. coli* resistance to β-lactams [24]. Broad-spectrum beta lactamase genes detected were $bla_{\text{TEM-1}}$: (87.7%), $bla_{\text{OXA-1}}$ (0.5%), and $bla_{\text{CARB-16}}$ (1.4%).

The 220 isolates harbored several aminoglycoside resistance genes in different frequencies, such as *aadA1* (63.6%), *aadA2* (25.9%), *aadA5* (3.6%), *aadA12* (0.5%), *aadA13* (2.7%), *aadA24* (0.9%), *aph(3′)-Ia* (30.0%), *aph(6)-Id* (70.7%), *aph(3′)-IIa* (1.4%), *aac(3)-IIa* (3.6%), *aac(3)-IV* (10.9%), *aph(4)-Ia* (10.9%), *ant(3″)-Ia* (2.3%), and *ant(2″)-Ia* (0.5%). Moreover, the isolates carried tetracycline resistance genes *tet*(*A*) (76.8%), *tet*(*B*) (24.5%), *tet*(*C*) (4.1%), and *tet*(*M*) (3.6%), folate pathway antagonist genes *sul1* (40.5%), *sul2* (69.5%), *sul3* (38.2%), *dfrA1* (47.3%), *dfrA5* (1.4%), *dfrA8* (3.2%), *dfrA12* (5.5%), *dfrA14* (9,5%), *dfrA16* (0.5%), and *dfrA36* (0.5%), chloramphenicol resistance genes *catA1* (19.1%), *catB2* (0.9%), *catB3* (3.2%), and *floR* (6.8%), macrolide resistance genes *mph(A)* (4.1%), *mph(B)* (7.7%), *mph(E)* (0.9%), *mph(G)* 1.4%), *msr(E)* (1.4%), *erm(B)* (1.4%), *mef(B)* (1.4%), and *mef(C)* (1.4%), as well as lincomycin resistance gene *Inu(F)* (2.7%). Genetic determinants associated with quinolone resistance included genes *qnrB19* (0.5%) and *qnrS1* (2.3%), as well as chromosomal mutations in *gyrA*, *parC,* and *parE* genes, which were identified in 19.1% of the isolates (*gyrA* S83L, 10.0%; *gyrA* D87N, 0.5%; *gyrA* D87G, 0.5%; *gyrA* D87Y, 1.8%; *gyrA* S83L and *parE* I355T, 2.5%; *gyrA* S83L and *gyrA* D87N and *parC* A56T and *parC* S80I, 0.5%; *gyrA* S83L and *gyrA* D87G and *parC* S80R, 1.4%; *gyrA* S83L and *parE* I529L, 0.5%; *parE* I529L, 1.4%). Eleven isolates, thereof five *mcr-1.1* (ETEC and ETEC-like), four *mcr-4.2* (ETEC and ETEC-like), one *mcr-4.6* (ETEC), and one *mcr-5.1* (ETEC/STEC) isolate, additionally revealed a mutation in the *pmrB* gene (V161G), which is known to confer resistance to colistin.

The majority of the isolates (55.0%) harbored between six and ten AMR genes, while almost one-third (30.0%) possessed 11–15, and 12.7% carried 1–5 AMR genes. The highest number of AMR genes, namely 16 to 19, was observed in 2.3% of the isolates, represented by *mcr-1.1* positive ETEC/STEC (n = 2), EDEC (n = 1), ETEC (n = 1), and AEEC (n = 1). The number of antimicrobial resistance genes and the pathotype were not correlated.

3.4. Multi Locus Sequence Types, Phylogenetic Groups, and Clonotypes

Overall, 30 known and seven novel STs (ST15336–ST15342) were identified among the 220 whole genome sequenced *E. coli* isolates. Predominant STs were ST10 (n = 56), ST1 (n = 48), ST29 (n = 25), ST100 (n = 21), ST42 (n = 7), and ST86 (n = 6), as well as ST131, ST641, and ST763 (n = 4 isolates each). ETEC isolates were mainly assigned to ST100 (37.5%), ST10 (21.4%), ST42 (10.7%), and ST131 (7.1%), while EDEC isolates mostly belonged to ST1 (79.2%) and ST10 (12.5%). Predominant STs of AEEC isolates were ST29 (61.0%) and ST20 (7.3%), while most of the sequenced ETEC/STEC, STEC, and ETEC-like isolates belonged to ST10 (69.6%, 64.3%, and 33.3%, respectively).

The most frequent phylogroup was group A (n = 92), predominantly associated with ST10 (n = 56) and ST100 (n = 21), followed by D (n = 60), among others associated with ST1 (n = 48) and ST42 (n = 7), B1 (n = 51; mostly ST29 (n = 25), ST86 (n = 6), ST641 (n = 4), and ST763 (n = 4)), E (n = 7; mostly ST118 (n = 3)and ST5759 (n = 3)), B2 (n = 4; all ST131), C (n = 3; all ST23), and clade I (n = 3) (Figure 2).

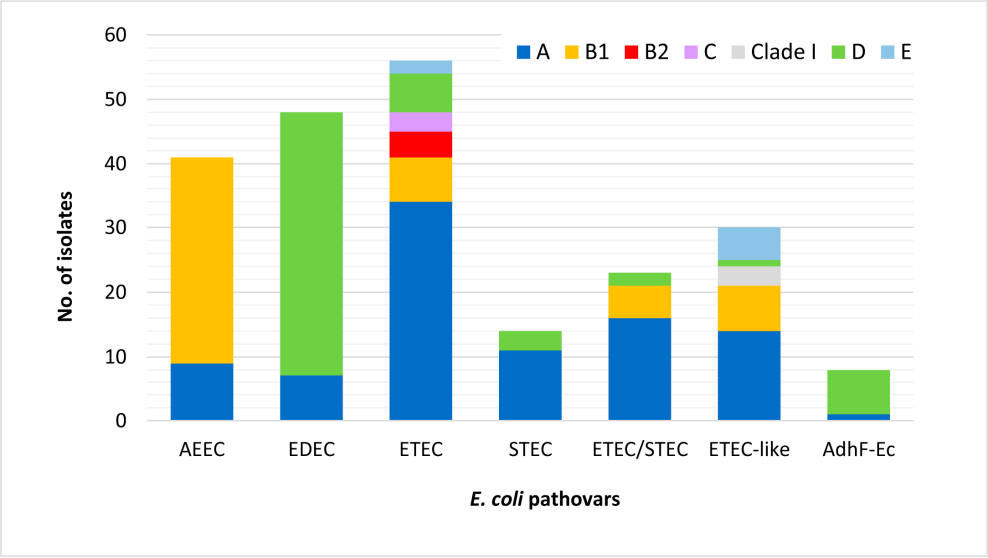

Figure 2. Distribution of phylogenetic groups among 220 *mcr*-positive porcine *E. coli* isolates according to pathotypes.

Thirty-six different clonotypes, i.e., combinations of fumC and fimH alleles, were determined. The most frequent CH types were 2–54 (51; (ST1-ETEC), 11–24 and 11–23 (29/17) (ST10), 4–24 (23) ST29, and 27–0 (20, all ST100). ST131 ETEC isolates carrying the mcr-1.1 gene revealed CH types 40–22 (n = 1) and 40–683 (n = 3).

3.5. Sero(geno)types

Overall, 24 distinct sero(geno)types could be detected among 220 whole genome sequenced *mcr*-positive *E. coli* isolates. About half of the isolates (47.3%), almost equally distributed among the different pathotypes, were not typable (Ont). The most frequent serotype observed was O139 (16.8%; in 86.5% of the isolates associated with H-antigen H4), followed by O123:H11 (5.5%), O141:H4 (5.0%), and O149 (4.5%; 80.0% associated with H10). Genes encoding O types O8, O26, O35, O45, O50, O103, and O182 were each present in three to six of the isolates. O139 was associated with the EDEC pathotype, and O123:H11 and O149 were exclusively detected in AEEC and ETEC isolates, respectively. O141 occurred in four different pathotypes (ETEC, EDEC, ETEC/STEC, and AdhF-*Ec*). The four ST131 isolates belonged to the O25b:H4 serogroup.

3.6. Intimin Subtypes

The complete *eae* sequences were obtained from the genomes of 41 sequenced AEEC genomes; two strains which failed to yield the *eae* sequence were excluded from subtyping analysis. Four *eae* subtypes, namely β1 (n = 28), ε1 (n = 8), θ2 (n = 2), and ξ, (n = 1), were assigned. Sequence polymorphisms in the *eae* gene, also known as genotypes (GTs), were examined to determine the diversity within each *eae* subtype. Subtypes that were represented by at least two isolates were explored. While ε1 and θ2 subtypes consisted only of one genotype each, the β1 subtype contained five genotypes, namely GT1 (n = 20; all ST29), GT2 (n = 4; 2 × ST29, 2 × ST20), and GT3 to GT6 (n = 1 each; 3 × ST29, 1 × ST20).

4. Discussion

E. coli neonatal and post-weaning diarrhea affecting pigs during the first weeks after birth and edema disease, an acute, often fatal enterotoxemia that affects primarily healthy, rapidly growing nursery pigs, are economically important diseases for the swine industry worldwide [25]. In the present study, we performed a comprehensive study on a collection of 10,573 fecal or intestinal *E. coli* isolates recovered from pigs with diarrheal disease or edema disease as well as from healthy swine sent to our laboratory to determine the

presence of potentially pathogenic *E. coli* strains. The distribution of *mcr* genes among *E. coli* isolates linked with diarrhea or edema diseases in pigs and/or subtyped at the pathotype level has rarely been studied so far.

An epidemiological study from Spain analyzed 481 *E. coli* isolates obtained from 179 diarrheagenic outbreaks in pigs [26]. The most prevalent pathotypes found were ETEC (57.6%), aEPEC (32.4%, in this study referred to as AEEC), hybrid ETEC/STEC (6.9%), and STEC (3.1%). While we report similar prevalences for hybrid ETEC/STEC and STEC pathotypes in our study, the detected occurrence of ETEC and aEPEC was lower. Instead, we report the detection of additional pathotypes including EDEC, ETEC-like, and AdhF-*Ec*.

High prevalences of *mcr*-positive non-pathogenic *E. coli* were previously reported in the surroundings of fattening pig farms and pig slaughterhouses in Germany [27,28]. Our study is based exclusively on the investigation of *mcr* prevalences in pathogenic *E. coli*. The frequency of *mcr*-positive *E. coli* strains was 10.2%, which is lower than previous reports from other countries suggest [26,29]. Fukuda et al. (2018, 2022) found that *mcr-1*, *mcr-3*, and *mcr-5* were prevalent in *E. coli* derived from diseased pigs in Japan [30,31]. Among 120 strains isolated from pigs with PWD on 40 farms in 2012, the *mcr-1* (30.0%), *mcr-3* (8.3%), *mcr-5* (28.3%), and *mcr-9* (0.8%) genes were detected, while *mcr-2*, *mcr-4*, *mcr-6* to *mcr-8* and *mcr-10* were not reported. Coexistence of *mcr-1* and *mcr-5* (4.2%; 5/120) in the same strain was observed, but other combinations were not. Another study investigated 200 pathogenic *E. coli* isolated from swine enteric clinical cases between 1999 and 2018 in Spain [32]. The *mcr-4* gene was the most frequently detected mobile colistin resistance gene (13%), followed by *mcr-1* (7%) and *mcr-5* (3%). These reports are in line with our prevalence data.

In our study, the proportion of *mcr*-positive *E. coli* was highest in ETEC/STEC hybrid isolates and in EDEC isolates. The significantly higher prevalence of *mcr*-positive ETEC/STEC isolates could indicate a more frequent use of colistin to treat diarrheal diseases caused by ETEC/STEC in Germany [33].

The majority of the 132 representative *mcr-1*-positive isolates carried the *mcr-1.1* variant, which has been commonly reported worldwide. One isolated ST29-AEEC from 2018 carried the *mcr-1.26* variant, which was first detected in an *E. coli* strain isolated from the blood culture of a 79-year-old patient with fever in Germany [34]. At present, mobile colistin resistance genes 3 (*mcr-3.1*–*mcr-3.42*) and 1 (*mcr-1.1*–*mcr-1.36*) exhibit the highest numbers of reported variants (https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/, accessed 3 December 2023). We here report the newest *mcr-1* variant (termed *mcr-1.36*).

To the best of our knowledge, we report the first detection of *mcr-3* in EDEC isolates which were obtained from three pigs on one farm in Germany in 2014. While the occurrence of *mcr-3* has not yet been reported in pathogenic *E. coli* isolates from pigs in Europe, studies from Japan, Korea, and Thailand described high prevalences (Table S3) [35–39].

Soon after detecting and identifying the *mcr-1*–*mcr-3* genes, the discovery of another *mcr* gene (*mcr-4*) was reported. Carattoli et al. (2017) published the finding of a novel *mcr-4* gene harbored by a *Salmonella enterica* strain, which originated from the caecal content of a healthy pig at slaughter in Italy in 2013 [12]. This new gene was located on an 8749 bp ColE10 plasmid. Until now, seven *mcr-4* variants (*mcr-4.1*–*mcr-4.7*) have been reported in several countries, including Germany, Italy, Singapore, South Korea, and Australia. While *mcr-4* has been associated mainly with ColE10 or ColE10-like type plasmids, several novel or non-typeable *mcr-4*-harboring plasmids have been identified, e.g., in one *Acinetobacter baumannii* isolate obtained from frog legs in Vietnam and in a *Shewanella baltica* strain isolated from the gut contents of a wild Atlantic mackerel [10,40–42]. In this study, we report a novel *mcr-4* variant (termed *mcr-4.8*) on a ColE10 plasmid in an ETEC-like *E. coli* strain isolated from a pig in April 2019. The most predominant *mcr-4.6* variant found in our study was first detected in a *Salmonella enterica* strain originally found in a pig carcass in Spain [43]. Our study details the identification of the novel *mcr-5.5* variant, which was detected in an isolate from a pig in Germany in 2006 and represents the third *mcr-5* variant discovered in Germany. Our investigation revealed that an *mcr-5.1*-positive ETEC (ST5759)

was isolated from a pig with diarrhea in 2004, making it the earliest detection of *mcr* genes within this study. Nevertheless, earlier observations of the *mcr-5* gene were reported in diseased swine in Spain in 2000 [32].

None of the isolates tested in our study carried *mcr-6* to *mcr-10*, which is in accordance with previous studies in commensal isolates [7]. The majority of prior studies investigating the presence of *mcr* genes in *E. coli* isolates from diseased or healthy pigs with defined *E. coli* pathotypes/VAG-typed *E. coli* did not test for *mcr-6* to *mcr-10* (Table S3) [44–48]. Only two out of seven studies investigating for *mcr-6* to *mcr-10* reported *mcr-9*-positive *E. coli* from pigs with diarrhea (Table S3) [49–52]. The first occurrence and distribution of *mcr-1–mcr-10* were reviewed recently [6]. No specific differences of *mcr-1* to *mcr-5* versus *mcr-6* to *mcr-10* were described with regard to source or bacterial species.

The location of specific *mcr* genes on different plasmids was studied by García-Meniño et al. (2019). They investigated 35 *E. coli* isolates obtained from pigs with diarrhea from 2006 to 2016 in Spain for different *mcr* variants [10]. Among 18 *mcr-1.1* variants located mainly on IncHI2 and IncX4-type plasmids, *mcr-4.2* (n = 13) and *mcr-4.5* (n = 2) were located on ColE10-like plasmids, while one *mcr-4.1* variant was found on a Col8282-like plasmid. A more recent study associated plasmid ColE10 with *mcr-4.6* genes isolated from eight colistin-resistant *E. coli* obtained from veal calves in Belgium from 2012 to 2015 [40]. These reports support our findings on the location of *mcr* genes in pathogenic *E. coli* isolates from pigs.

We observed high diversity in the phylogeny of the 220 *mcr*-positive isolates; however, some phylogroups and STs, including A-ST10 (25.5%), A-ST100 (9.5%), D-ST1 (21.8%), and B1-ST29 (11.4%), were predominant. Other STs detected in the present study such as ST42, ST118, and ST131 have been previously associated with intestinal pathogenic *E. coli* in pigs [26,53].

Four *mcr-1.1*-positive ST131 ETEC were isolated from three farms at four different time points between 2009 and 2014, all from pigs with post-weaning diarrhea. In addition to ETEC-typical genes *fedAac*, *estap*, and *estb*, they harbored genes typical of extraintestinal pathogenic *E. coli* (ExPEC), including *chuA*, *fyuA*, *ibeA*, *iroN*, *iucD*, *iutA*, *kpsMTII*, *papA*, *papC*, *sitA*, and *yfcV* (fimbrial-like gene; used by Spurbeck as UPEC-predictor, together with *chuA*, *fyuA*, *vat*) and belonged to clonal types (*fumC-fimH* allele) 40–22 (n = 1) (previously detected in *mcr-1*-positive ST131 from Spain, Garcia-Menino, ST131) and 40–683 (n = 3). All belonged to serogroup O25b:H4. One strain was identified as being part of subclone *H*22 (clade B), which is known as the predecessor to subclone *H*30 that is commonly linked to fluoroquinolone resistance [54,55]. This clonal type (40–22) has been previously detected in *mcr-1*-positive ST131 strains isolated from pigs with diarrhea in Spain [26].

Based on amino acid sequence differences at the C-terminus, at least 30 intimin subtypes have been defined, namely, α1, α2, α8, β1, β2, β3, γ1, γ2, ε1, ε2, ε3, ε4, ξ, z, z3, η, η2, θ, τ, ι1, ι2, κ, λ, μ, ν, υ, ο, π, ρ, and σ [56]. Intimin subtypes are correlated with host specificity and tissue tropism [57]. In the 39 AEEC isolates analyzed in our study, only *eae* genes encoding for subtypes β1, ε1, θ2, and ξ were detected. The β1 subtype was the most prevalent, consistent with previous findings of intimin subtypes in pigs, cattle, birds, and diarrheal patients [58–60]. The β1_GT1 genotype, which predominated in our study, was first detected in an *E. coli* isolate from yak feces in Qinghai, China in 2012 [60].

Our study has several limitations. Our analysis is based exclusively on the investigation of *mcr* prevalences in pathogenic *E. coli*, and therefore no statement can be made about the occurrence of colistin resistance genes in non-pathogenic isolates. We also lack information regarding pig housing conditions, hygiene standards, previous medical treatment, and animal trafficking. On the other hand, our study has several strengths. A total of 10,573 putative pathogenic porcine *E. coli* strains were screened for VAGs and plasmid-mediated colistin resistance genes (*mcr-1* to *mcr-10*). Moreover, 220 representative *mcr*-positive isolates underwent whole genome sequencing and were examined for various aspects, including *mcr*-carrying plasmids, genes associated with antimicrobial resistance, and STs, which offers deep insight into the molecular characteristics of *mcr*-positive pig-associated *E. coli* pathotypes.

5. Conclusions

To the best of our knowledge, we here present for the first time prevalence data of all ten currently known *mcr* genes in a large set of pathogenic porcine *E. coli* isolates collected over a period of 20 years in Germany. Regarding the distribution of *mcr* genes in pig-associated pathotypes in Germany, ETEC/STEC hybrid strains revealed the highest prevalence of *mcr*, in particular *mcr-1*, *-4,* and *-5*. If this might be associated with the antimicrobial treatment strategy of diarrheal diseases in pigs or with the potential ability of these hybrid strains to acquire mobile AMR genes and/or plasmids more easily than other pathotypes remains an interesting point for future research.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/applmicrobiol4010005/s1, Table S1: primers and positive control strains or DNA used for multiplex PCRs to detect virulence-associated genes and *mcr-1* to *mcr-10* genes [61]; Table S2: overview of all porcine *E. coli* isolates included in this study and their classification into pathotypes according to the VAG pattern; Table S3: occurrence of *mcr* genes in *E. coli* isolates from samples obtained from (i) diseased pigs or (ii) healthy pigs with defined *E. coli* pathotypes/VAG-typed *E. coli*. Studies involving screening of other animals/humans were included only for data as previously defined.

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Data Availability Statement: Data are contained within the article and supplementary materials. NCBI reference sequence numbers for novel *mcr* variants are NG_231577.1 (*mcr-1.36*), NG_231578.1 (*mcr-4.8*), and NG_231579.1 (*mcr-5.5*). The sequences are available in the NCBI Reference Sequence Database (https://www.ncbi.nlm.nih.gov/refseq/, accessed on 3 December 2023). Further raw data can be made available on reasonable request.

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