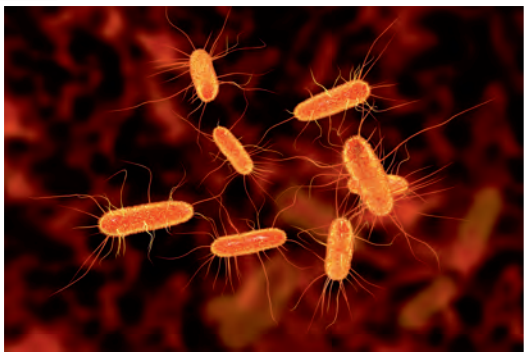
LISA GÖPEL

Untersuchungen zum Vorkommen und zur Stabilität von Colistin-Resistenzdeterminanten in pathogenen *Escherichia coli*-Isolaten aus klinischem Untersuchungsgut von Schweinen



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der Justus-Liebig-Universität Gießen

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LISA GÖPEL

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II Abkürzungsverzeichnis

Abkürzung	Bedeutung
AEEC	Attaching and effacing *E. coli*
aEPEC	atypische enteropathogene *E. coli*
APEC	Aviäre pathogene *E. coli*
BMD	Broth microdilution
Ca^{++}	Calcium-Ion
CFTR	cystic fibrosis transmembrane regulator
cGMP	cyclisches Guanosinmonophosphat
Cl^-	Chlorid-Ion
CLSI	Clinical & Laboratory Standards Institute
E.	*Escherichia*
EDEC	Edema disease *E. coli*/Ödemkrankheit auslösende *E. coli*
EMA	European Medicines Agency
EPEC	Enteropathogene *E. coli*
ETEC	Enterotoxische *E. coli*
EUCAST	European Committee on Antimicrobial Susceptibility Testing
ExPEC	Extraintestinal pathogene *E. coli*
HCO_3^-	Hydrogencarbonat-Ion
InPEC	Intestinal pathogene *E. coli*
L-Ara4N	L-4-Amino-4-desoxyarabinose
LPS	Lipopolysaccharide
mcr	mobile colistin resistance
mg/PCU	Milligramm Wirkstoff je Population Correction Unit
MHK	Minimale Hemmstoffkonzentration
PCR	Polymerase Chain Reaction
pEtN	Phosphoethanolamin
PWD	Post weaning disease
STEC	Shigatoxin-bildende *E. coli*
Stx2e	Shigatoxin 2e
VAG	Virulenz-assoziierte Gene
WHO	World Health Organization

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- **Ewers, C., Göpel, L., Prenger-Berninghoff, E., Semmler, T., Kerner, K., Bauerfeind, R. (2022)**
  Occurrence of *mcr-1* and *mcr-2* colistin resistance genes in porcine *Escherichia coli* isolates (2010-2020) and genomic characterization of *mcr-2*-positive *E. coli*; Frontiers in Microbiology 13, Article 1076315; doi: 10.3389/fmicb.2022.1076315
- **Göpel, L., Prenger-Berninghoff, E., Wolf, S. A., Semmler, T., Bauerfeind, R., Ewers, C. (2024)**
  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; Applied Microbiology 4 (1), 70-84; doi: 10.3390/applmicrobiol 4010005
- **Göpel, L., Prenger-Berninghoff, E., Wolf, S. A., Semmler, T., Bauerfeind, R., Ewers, C. (2024)**
  Repeated occurrence of mobile colistin resistance gene - carrying plasmids in pathogenic *Escherichia coli* from German pig farms; Microorganisms 12 (4), 729; doi: 10.3390/microorganisms12040729

VORTRÄGE

- **Göpel, L., Prenger-Berninghoff, E., Semmler, T., Bauerfeind, R., Ewers, C. (2023)**
  Retrospective study of three pig farms in Germany with recurring colistin resistance gene *mcr-1* in porcine pathogenic *Escherichia coli*; Tagung der DVG (Deutsche Veterinärmedizinische Gesellschaft), Fachgruppe Bakteriologie und Mykologie, Berlin, 22.05. – 24.05.2023

1 Einleitung

Escherichia (*E.*) *coli* ist ein Gram-negatives, fakultativ anaerobes Bakterium, das weltweit im Darm von Säugetieren vorkommt. Es wurde ebenfalls im Darm von Vögeln, Reptilien und Fischen, im Boden, Wasser und in Pflanzen sowie Lebensmitteln nachgewiesen. Durch die intestinale Ausscheidung gelangen *E. coli*-Bakterien in die Umwelt, wo sie über einen langen Zeitraum lebensfähig bleiben können (Blount 2015). Dabei wird in kommensale *E. coli* und intestinal (InPEC) wie auch extraintestinal (ExPEC) pathogene *E. coli* unterschieden. InPEC und ExPEC sind sowohl bei Menschen als auch bei Tieren wichtige Infektionserreger (Kaper et al. 2004). Auf der Basis ihrer unterschiedlichen Virulenzgenprofile werden diese in verschiedene Pathovare eingeteilt (Bok et al. 2020).

ExPEC gelangen durch Translokation aus dem Darm in andere Körperregionen und lösen dort Infektionen aus. Beim Menschen handelt es sich vor allem um Harnwegsinfektionen (uropathogene *E. coli*, UPEC) (Kot 2019), sowie Pneumonien, Blutstrom- und Wundinfektionen (Riley 2020). In der Veterinärmedizin spielen ExPEC in erster Linie eine Rolle als Auslöser von Infektionen bei Kleintieren, darunter Harnwegsinfektionen, Pneumonien und Wundinfektionen (Soonthornsit et al. 2022; Yun et al. 2023). In der Nutztierhaltung sind durch ExPEC ausgelöste Infektionen weniger häufig und können bei Schweinen zu einer Erkrankung des Urogenitaltrakts, Mastitis und Sepsis führen (Tan et al. 2012).

Intestinal pathogene *E. coli* lösen im Gegensatz dazu vor allem Durchfallerkrankungen bei Menschen (z. B. enteropathogene *E. coli* als Erreger der Reisediarrhö) und Tieren aus (Kaper et al. 2004; Mueller und Tainter 2024). Die Folgen dieser Infektionserkrankungen führen insbesondere in der Schweinemast jährlich weltweit zu wirtschaftlichen Verlusten durch erhöhte Morbiditäts- und Mortalitätsraten, geringere Gewichtszunahmen und steigende Kosten für die Behandlung von erkrankten Tieren (Luppi 2017). Die zwei häufigsten Pathovare sind dabei enterotoxische *E. coli* (ETEC) und Ödemkrankheit auslösende *E. coli* (EDEC) (Barros et al. 2023). Charakteristische Symptome einer ETEC-Infektion bei Saug- und Absetzferkeln sind wässrig-breiiger Durchfall sowie Abmagerung und Dehydratation. EDEC-Infektionen können sowohl Absetzferkel als auch wie zunehmend berichtet ältere Läufer betreffen und äußern sich durch Ödembildung an verschiedenen Organen und gegebenenfalls durch ZNS-Symptome, wie Ataxie und Paresen (Selbitz et al. 2023).

Zur Behandlung dieser durch EDEC oder ETEC verursachten Erkrankungen bei Schweinen wird häufig Colistin (Polymyxin E) eingesetzt, ein bakterizid wirkendes Antibiotikum aus der Gruppe der Polypeptidantibiotika, das zur Gruppe der Polymyxine gehört. Aufgrund seiner hohen Wirksamkeit gegen multiresistente Gram-negative Bakterien hat das in der Humanmedizin eingesetzte Colistin als Reserveantibiotikum in den letzten Jahren immer mehr an Bedeutung gewonnen (WHO 2017; Sahoo et al. 2023). Im November 2015 wurde erstmals das horizontal übertragbare, Plasmid-kodierte Resistenzgen *mcr-1* (mobile colistin resistance gene 1) in *E. coli*-Stämmen von Schweinen nachgewiesen (Liu et al. 2016). Bis 2021 wurden neun weitere *mcr*-Gene (*mcr-2* bis *mcr-10*) in verschiedenen Bakterienisolaten von Tieren, Lebensmitteln, Menschen und der Umwelt festgestellt (Hussein et al. 2021). Die Nutztierhaltung stellt nach derzeitigem Kenntnisstand das Hauptreservoir für *mcr* dar, was im Wesentlichen auf die Anwendung von Colistin zur Therapie insbesondere bakteriell bedingter Darmerkrankungen, aber auch anderer Infektionen zurückzuführen ist (Kumar et al. 2020; Wang et al. 2020b). Darüber hinaus wird Colistin in einigen Ländern wie Pakistan und Vietnam auch heute noch als Leistungsförderer bei Nutztieren eingesetzt (Umair et al. 2023), nationale Verbote für die Verwendung von Colistin als Futtermittelzusatzstoff bestehen nur in wenigen Ländern, wie z. B. seit 2016 in China (Walsh und Wu 2016).

Ziel der Arbeit war es, einen Einblick in die deutschlandweite Verbreitung der Colistin-Resistenzgene *mcr-1* bis *mcr-10* bei pathogenen porcinen *E. coli*-Isolaten zu gewinnen, sowie die Lokalisierung der *mcr*-Gene in diesen Bakterien zu untersuchen. Das Wissen zum Vorkommen von übertragbaren Antibiotikaresistenzen in Schweinebetrieben ist ein wichtiger Baustein bei der Verfolgung des One-Health-Ansatzes. Das Bundesministerium für wirtschaftliche Zusammenarbeit und Entwicklung definiert dieses Konzept wie folgt: „Der One-Health-Ansatz basiert auf dem Verständnis, dass die Gesundheit von Mensch, Tier und Umwelt eng miteinander zusammenhängt. Der One-Health-Ansatz dient der Vorbeugung und fördert die interdisziplinäre Zusammenarbeit, insbesondere zwischen Humanmedizin, Veterinärmedizin und Umweltwissenschaften“ (BMZ 2022). Dies gilt insbesondere für den Bereich der antimikrobiellen Resistenzen und der Entstehung und Ausbreitung von antibiotikaresistenten Bakterien (Aslam et al. 2021). Schweine, die als landwirtschaftliche Nutztiere weltweit gehalten werden (Kim et al. 2023), wurden in den letzten Jahren bereits global als Träger von *mcr*-positiven *E. coli*-Isolaten identifiziert (Valiakos und Kapna 2021). Sowohl bei gesunden als auch klinisch erkrankten

Schweinen wurden dabei vorwiegend *mcr-1*-positive *E. coli* aus Kotproben isoliert. Dabei bezogen sich wenige Studien auf das Vorkommen von *mcr*-Genen bei weltweit vorkommenden InPEC-Pathovaren, wie ETEC oder EDEC. In der vorliegenden Arbeit wurde das Vorkommen von *mcr-1*- bis *mcr-10*-Genen in porcinen *E. coli*-Pathovaren untersucht, die über einen Zeitraum von 20 Jahren von Schweinen in Deutschland im Rahmen von mikrobiologischen diagnostischen Untersuchungen gewonnen werden konnten. Für ausgewählte Isolate wurde die Lokalisierung der nachgewiesenen *mcr*-Gene auf Plasmiden oder dem Chromosom bestimmt. Basierend auf Ganzgenomsequenzen wurden zudem phylogenetische Zusammenhänge der gewonnenen Isolate, Virulenz-assoziierte Gene (VAGs) und Antibiotikaresistenzgene mittels bioinformatischer Analysen bestimmt. Des Weiteren wurden *E. coli*-Isolate aus drei ausgewählten Schweinebetrieben in Deutschland hinsichtlich des Vorkommens von *mcr*-Genen über mehrere aufeinanderfolgende Jahre untersucht. Die Lokalisation der Resistenzgene auf Plasmiden wurde über die Jahre hinweg verglichen, um die Kontinuität des Auftretens von Plasmid-vermittelter Colistin-Resistenz in diesen Betrieben und den potentiellen Eintrag von neuen Colistin-resistenten *E. coli*-Isolaten bzw. *mcr*-tragenden Plasmiden in die Betriebe beurteilen zu können. Die Daten sollen dazu dienen, das Potential der Verbreitung und eines möglicherweise längerfristigen Auftretens der Plasmid-vermittelten Colistin-Resistenz in pathogenen *E. coli*-Isolaten aus Schweinebeständen in Deutschland einschätzen zu können.

2 Literaturübersicht

2.1 Taxonomie

Die Gattung *Escherichia* (*E.*) gehört zur Familie der *Enterobacteriaceae* innerhalb der Ordnung der Enterobacterales. Im Jahr 1885 beschrieb der bayerische Kinderarzt Theodor Escherich erstmals das später nach ihm benannte Bakterium *Escherichia coli* (damals *Bacterium coli commune*), welches er aus dem Stuhl von Säuglingen isolierte (Escherich 1885).

Derzeit umfasst die Gattung *Escherichia* (Stand August 2024) sieben gültig benannte Arten (https://lpsn.dsmz.de/genus/escherichia). *E. albertii* und *E. fergusonii* wurden sporadisch als Auslöser von Durchfallerkrankungen bei Menschen und verschiedenen Tierarten beschrieben (Huys et al. 2003; Hariharan et al. 2007; Okuno et al. 2023), während *E. hermannii* als Infektionserreger bei Patienten mit Sepsis identifiziert werden konnte (Lu et al. 2023; Rank et

al. 2016). Von größter infektionsmedizinischer Bedeutung ist *E. coli*, das als Erreger sowohl intestinale als auch extraintestinale Erkrankungen bei Menschen und Tieren auslösen kann (Riley 2020).

2.2 Virulenzfaktoren und intestinal pathogene *E. coli*-Pathovare beim Schwein

Intestinal pathogene *E. coli* lösen beim Menschen je nach Pathovar eine Vielzahl an unterschiedlichen Durchfallerkrankungen aus. So sind beispielsweise ETEC (enterotoxische *E. coli*) Auslöser der Reisediarrhö, EPEC (enteropathogene *E. coli*) verursachen eine Diarrhö bei Säuglingen und Kleinkindern und EHEC/STEC (enterohämorrhagische *E. coli*/Shigatoxin-bildende *E. coli*) verursachen blutige Durchfälle sowie das lebensbedrohliche hämolytisch-urämische Syndrom (Mueller und Tainter 2024). Bei Tieren können Infektionen mit *E. coli*-Pathovaren sowohl bei Nutz- als auch Kleintieren Durchfallerkrankungen hervorrufen, wobei insbesondere Jungtiere betroffen sind (Selbitz et al. 2023). Bei Kälbern konnten ETEC (positiv für F5-, F17- und F41-Fimbrien sowie hitzestabile Enterotoxine) als Auslöser von *E. coli*-Diarrhö identifiziert werden (Kolenda et al. 2015). Da in dieser Arbeit nur porcine InPEC untersucht wurden, werden im Folgenden *E. coli*-Pathovare detailliert beschrieben, die im Zusammenhang mit Krankheitsgeschehen bei Schweinen stehen.

2.2.1 Enterotoxische *E. coli* (ETEC)

Das Pathovar ETEC ist das bedeutendste Pathovar bei Nutztieren außer dem Geflügel (Nagy und Fekete 2005). Bei einer Infektion von Schweinen kann es zu sekretorischem Durchfall führen (Fairbrother und Nadeau 2019). ETEC zeichnen sich durch das Vorhandensein von zwei unterschiedlichen Typen von Virulenzfaktoren aus: Adhäsine, die die Bindung und Besiedlung des Darmepithels fördern, sowie Enterotoxine, die für die gesteigerte Sekretion von Flüssigkeit in das Darmlumen verantwortlich sind (Kopic und Geibel 2010). Typische ETEC-Adhäsine, die bei klinischen Isolaten von Tieren mit Durchfallerkrankung nachgewiesen werden konnten, sind dabei die *E. coli*-Fimbrien F4, F5, F6, F17ab, F18ac und F41 (Dubreuil et al. 2016; Selbitz et al. 2023). Diese Fimbrien bestehen aus Proteinstrukturen, die auf der Bakterienzelloberfläche lokalisiert sind und die wirts- und rezeptorspezifische Anheftung an Dünndarmepithelzellen vermitteln (Fairbrother und Nadeau 2019). Im Dünndarm von Tieren produzieren ETEC Enterotoxine, die zu Durchfall führen. Die zwei Hauptklassen, die von ETEC

produziert werden, sind hitzelabile (LT) und hitzestabile Enterotoxine (ST). Über eine Bindung der LT-B-Untereinheit mit dem zellulären Gangliosid GM1 erfolgt die Aufnahme des hitzelabilen Enterotoxins in die Darmepithelzelle (Lencer et al. 1999). Hier führt das Toxin über die Phosphorylierung des „cystic fibrosis transmembrane regulators" (CFTR) und eine daraus resultierende Aktivierung dieses Regulators zur Sekretion von Chlorid-Ionen (Cl^-) und damit zu sekretorischem Durchfall (Vaandrager et al. 1997). Die hitzestabilen Enterotoxine werden unterschieden in das Peptid STa und das Protein STb. STa bindet an die extrazellulär lokalisierte Guanylatzyklase C und steigert damit die Konzentration von cyclischem Guanosinmonophosphat (cGMP) in der Zelle. Über die erhöhte cGMP-Konzentration werden Kinasen aktiviert, die zur Sekretion von Chlorid-Ionen und Hydrogencarbonat-Ionen (HCO_3^-) führen (Dubreuil 2012). Dagegen bindet das STb-Toxin innerhalb der Zelle an ein G-Protein, was zu einem Anstieg von Calcium-Ionen (Ca^{++}) führt, welcher die Aktivierung einer Calmodulin-abhängigen Proteinkinase II (CAMKII) nach sich zieht. Diese phosphoryliert CFTR, woraufhin Cl^- und HCO_3^- sezerniert werden. Über zusätzliche Aktivierungen von Kinasen sowie die Freisetzung von Serotonin und Prostaglandin E_2 durch den erhöhten Ca^{++}-Spiegel wird der Verlust von Elektrolyten und Wasser aus den Darmzellen ergänzend gefördert (Peterson und Whipp 1995; Dubreuil 2012).

Bei Schweinen lösen Infektionen mit ETEC vor allem zwei bekannte Krankheitsbilder aus, deren Verlauf sich je nach Alter der Ferkel, altersabhängig unterschiedlicher Ausbildung der Fimbrien-Rezeptoren, sowie immunologischem Status der Tiere unterscheiden. Die neonatale *E. coli*-Diarrhö betrifft Ferkel von der Geburt bis zum vierten Lebenstag und wird durch ETEC-Isolate ausgelöst, die die Ferkel oft noch vor dem ersten Säugen über die kontaminierte Umgebung aufnehmen (Fairbrother und Nadeau 2019). Diese ETEC besiedeln meist das vordere Jejunum und Ileum und können innerhalb der ersten Stunden nach der Geburt bereits zu klinischen Symptomen wie wässrig-breiigen Durchfall führen. Bei einem milden Krankheitsverlauf können die Tiere Abmagerung und ein struppiges Borstenkleid aufweisen. Schwerere Verläufe sind von Dehydrierung, Apathie und in manchen Fällen sogar vom Tod noch vor der Entwicklung klinischer Symptome gekennzeichnet. Es können sowohl einzelne Ferkel als auch ganze Würfe betroffen sein. Die Morbidität und Mortalität schwankt abhängig von der Schwere der Erkrankung und den getroffenen Hygienemaßnahmen stark (Selbitz et al. 2023).

Die ETEC-bedingten Durchfallerkrankungen bei älteren Ferkeln treten häufig kurz nach dem Absetzen auf (daher im angloamerikanischen Raum: Post weaning diarrhea; PWD). Da die lokale Darmimmunität der Ferkel zum Zeitpunkt des frühen Absetzens noch nicht ausreichend ausgebildet ist und die Versorgung mit maternalen Antikörpern wegfällt, erhöht sich die Anfälligkeit von Schweinen an PWD zu erkranken. Die Krankheitssymptome ähneln denen der neonatalen Diarrhö, sind jedoch weniger schwerwiegend. Aufgrund der oft tagelang anhaltenden Durchfallsymptomatik kommt es zu Dehydrierung und Auszehrung der Absetzferkel, die Mortalität kann bis zu 25 % betragen (Fairbrother und Nadeau 2019). Durch geeignete Hygienemaßnahmen und Fütterungsprogramme können präventiv Maßnahmen ergriffen werden, um das Risiko eines Krankheitsgeschehens in Schweinebeständen zu verringern (Goodman et al. 2023).

ETEC, die eine neonatale Diarrhö verursachen, sind überwiegend positiv für F4- oder F6-Fimbrien. Dabei gehören die F4-ETEC meist zu den Serovaren O6, O8, O101 und O149 (Do et al. 2006; García et al. 2020) während die F6-ETEC mit den Serovaren O9 und O64 assoziiert sind (Vu Khac et al. 2006).

ETEC, die Krankheitsausbrüche bei älteren Ferkeln verursachen, produzieren typischerweise F4- und F18-Fimbrien (Zhang et al. 2007; Luppi et al. 2016), wobei die prävalenten Subgruppen F4ac und F18ac im Zusammenhang mit ETEC-bedingten Durchfallerkrankungen bei Absetzferkeln festgestellt werden konnten (Dean-Nystrom et al. 1997; Luppi 2017). F4-ETEC waren hauptsächlich den Serovaren O149 und O6 sowie den Multilokus-Sequenztypen ST100 und ST48 zugeordnet, während F18-ETEC hauptsächlich den Serovaren O8 und O147 und den Sequenztypen ST10, ST23 und ST42 angehörten (García et al. 2020). Die Multilokus-Sequenztypisierung ermöglicht dabei die Einordnung von *E. coli*-Isolaten in bestimmte Sequenztypen und damit die Identifizierung klonaler Linien im Rahmen epidemiologischer Analysen von Krankheitsausbrüchen. Neben den bereits beschriebenen typischen ETEC-VAGs besitzen einige ETEC-Stämme zusätzlich das Shigatoxin 2e (Stx2e) (Baldo et al. 2020). Dieses Toxin ist für die Pathogenese der Ödemkrankheit (siehe Kapitel 2.2.2) verantwortlich, die häufig auch bei Absetzferkeln beobachtet wird (Fairbrother und Nadeau 2019).

2.2.2 Ödemkrankheit auslösende *E. coli* (EDEC)

Die Ödemkrankheit (edema disease, abgekürzt ED) wird durch EDEC ausgelöst, die durch das Vorkommen der Virulenzfaktoren F18 und Stx2e charakterisiert sind. Im Gegensatz zu ETEC ist der Fimbrien-Subtyp F18ab bei EDEC-Isolaten vorherrschend (Moxley 2000; Barth et al. 2011; Göpel et al. 2024b). Durch die F18-vermittelte Kolonisierung von EDEC im Dünndarm gelangen große Mengen des exprimierten Toxins Stx2e durch einen nicht geklärten Mechanismus in den Blutkreislauf. Dort verursacht es Schäden an den Endothelzellen, was typischerweise Ödeme an den Augenlidern, am Nasenrücken oder am Unterbauch zur Folge hat. Wenn Stx2e die Blut-Hirn-Schranke passiert, kann es zu Hirnödemen kommen, die zu ZNS-Symptomen wie Ataxie, Paresen oder ungewöhnlichen Lautäußerungen führen können. Subklinische Verläufe der Ödemkrankheit sind durch eine Unterentwicklung der Ferkel gekennzeichnet. Bei akuten Krankheitsausbrüchen kann die Mortalität bis zu 90 % betragen, ohne dass Fieber auftritt. Um einen Krankheitsausbruch zu vermeiden, sollte in den ersten Wochen nach dem Absetzen der Ferkel die intestinale Mikrobiota möglichst stabil erhalten werden. Dies kann z. B. durch ein restriktives Fütterungsmanagement erreicht werden, um die übermäßige Vermehrung von *E. coli* im Darm zu verhindern (Fairbrother und Nadeau 2019; Selbitz et al. 2023). Durch die parenterale Verabreichung von rekombinanten Stx2e-Antigenen konnte die Mortalität bei erkrankten Ferkeln signifikant reduziert werden (Fricke et al. 2015). Zudem besteht die Möglichkeit einer parenteralen Muttertierimpfung (Enterocolix®). Der Impfstoff enthält unter anderem die *E. coli*-Fimbrienantigene F4ac, F5, F6, F18ab, F18ac und F41 und kann somit zur passiven Immunisierung der Ferkel gegen die *E. coli*-Diarrhö und die Ödemkrankheit beitragen (StIKo Vet 2023). Häufig festgestellte Serovare bei EDEC sind O138, O139 und O141, während der vorherrschende Sequenztyp in EDEC ST1 ist (Kusumoto et al. 2016a; Selbitz et al. 2023; Göpel et al. 2024b).

2.2.3 Enteropathogene *E. coli* (EPEC)

Infektionen mit enteropathogenen *E. coli* können bei Schweinen Diarrhö verursachen und sind unter anderem charakterisiert durch das Vorhandensein des Virulenzfaktors Intimin, während klassische ETEC- und EDEC-VAGs nicht zwingend vorhanden sind (Zhu et al. 1994). Dabei können EPEC in typische und atypische EPEC unterschieden werden. Bei atypischen EPEC (aEPEC) erfolgt die initiale Adhäsion durch das von *E. coli*-sezernierte Protein A (EsPA). Anschließend

bindet das bakterielle äußere Membranprotein Intimin an den bakteriell sezernierten Rezeptor Tir (translocated intimin receptor), welcher zuvor über den Typ III-Sekretionsmechanismus in die Wirtszelle injiziert wurde. Dadurch entstehen sogenannte Attaching-and-effacing-Läsionen (AE-Läsionen), die sich durch das Verstreichen der Mikrovilli im Bereich der Bakterienanheftung darstellen. Typische EPEC exprimieren zusätzlich das Plasmid-kodierte Protein „bundle-forming pilus“, welches die Anheftung zwischen Bakterien ermöglicht und eine entscheidende Rolle bei der initialen Adhäsion an Darmepithelzellen spielt (Cleary et al. 2004).
EPEC, die AE-Läsionen induzieren, werden auch als Attaching and effacing *E. coli* (AEEC) bezeichnet. Durch die Sekretion von Proteinen in die Wirtszelle kommt es bei infizierten Schweinen zu Durchfall, der genaue Wirkungsmechanismus ist noch nicht geklärt (Fairbrother und Nadeau 2019; Selbitz et al. 2023). In einer epidemiologischen Studie aus Spanien wurden 481 *E. coli*-Isolate analysiert, die aus dem Kot von klinisch erkrankten Schweinen mit Durchfall gewonnen wurden. In 60,3 % der erkrankten Saugferkel und bei 21,7 % der erkrankten Absetzferkel konnte das Vorhandensein von EPEC nachgewiesen werden (García-Meniño et al. 2018). Dabei wurden die Serovare O26, O49, O80 und O111 bei den untersuchten EPEC-Isolaten vermehrt festgestellt (García-Meniño et al. 2018).

2.3 Resistenz von *E. coli* gegen Colistin

2.3.1 Colistin als antimikrobieller Wirkstoff

Die Wirkstoffgruppe der Polymyxine, eine Gruppe der Polypeptid-Antibiotika, welche aus fünf antimikrobiellen Substanzen (Polymyxin A - E) besteht, wurde im Jahr 1947 entdeckt (Stansly et al. 1947). Colistin wurde erstmals als Sekundärmetabolit des grampositiven Bodenbakteriums *Paenibacillus polymyxa* subsp. *colistinus* in Japan beschrieben (Koyama et al. 1950). Später konnte festgestellt werden, dass Colistin in seiner chemischen Struktur mit Polymyxin E identisch ist (Suzuki et al. 1965). Aufgrund von geringeren nephrotoxischen Nebenwirkungen, aber ähnlicher antibakterieller Aktivität von Polymyxin B und Colistin gegenüber den anderen Vertretern ihrer Wirkstoffklasse, wurden nur Polymyxin B und Colistin für den medizinisch therapeutischen Einsatz weiterentwickelt (Brownlee et al. 1952). Seit 1959 ist Colistin erhältlich zur Therapie von Infektionskrankheiten ausgelöst durch Gram-negative Bakterien (El-Sayed Ahmed et al. 2020). Als jedoch erste klinische Berichte auf eine hohe Toxizität von Colistin hinwiesen (Ryan et al. 1969; Koch-Weser et al. 1970), wurde die

Anwendung in der Humanmedizin eingeschränkt und der Einsatz von besser verträglichen Antibiotika, wie z. B. Aminoglykosiden, bevorzugt. Die Zunahme von multiresistenten Gram-negativen Bakterien, wie z. B. *Pseudomonas aeruginosa* als Verursacher von nosokomialen Infektionen, Sepsis und Pneumonien sowie von Carbapenem-resistenten *E. coli* im Zusammenhang mit Urogenitalinfektionen und Sepsis in den letzten Jahren, reduzierte die Auswahl therapeutisch wirksamer Antibiotika drastisch (van Duin und Paterson 2020). Erst die Wirksamkeit von Colistin gegen *Pseudomonas aeruginosa*-Besiedelung der Lunge bei Mukoviszidose-Patienten (Beringer 2001) und weitere Berichte über die alternative Behandlungsmöglichkeit mit Colistin bei multiresistenten bakteriellen Infektionen (Garnacho-Montero et al. 2003; Markou et al. 2003) führten zu einem verstärkten Einsatz von Colistin in der Humanmedizin. Dabei kommen zwei Formen von Colistin zum Einsatz: Colistinsulfat wird für die orale und topische Anwendung genutzt, während die Prodrug Colistin-Methansulfonat zur parenteralen und inhalativen Therapie verwendet wird, da es weniger toxisch als Colistinsulfat wirkt (El-Sayed Ahmed et al. 2020).

Colistin besteht aus einem zyklischen Heptapeptidring mit einem kovalent verbundenen Tripeptidschwanz (Lewis und Lewis 2004; Li et al. 2005). Entscheidend für die bakterizide Wirkung von Colistin ist dabei der Aufbau der Zellwand von Gram-negativen Bakterien (El-Sayed Ahmed et al. 2020). Die äußere Membran Gram-negativer Bakterien stellt durch das Vorhandensein von negativ geladenen Lipopolysacchariden (LPS) an der Zelloberfläche eine Permeabilitätsbarriere dar, die das Eindringen von schädlichen Wirkstoffen, darunter auch antimikrobielle Substanzen, verhindern kann (Velkov et al. 2013). Die Struktur des LPS besteht aus drei Domänen: einer O-Antigenkette, einem Kernpolysaccharid und einem konservierten Lipid A, das als hydrophober Anker in der äußeren Membran fungiert (Velkov et al. 2013). Der kationische Peptidring von Colistin bindet an die negativ geladene Lipid A-Einheit von LPS in der äußeren Membran. Dadurch wird die Membran destabilisiert und es kommt zu einer Interaktion des Tripeptidschwanzes von Colistin mit den Fettsäureschwänzen von Lipid A. Dies führt zur weiteren Destabilisierung des LPS und einer Schädigung der äußeren Membran (Velkov et al. 2010). Durch diesen Mechanismus fördert Colistin seine eigene Aufnahme in den periplasmatischen Raum und kann in der Folge die Integrität der inneren Membran zerstören, was schlussendlich zum Zelltod führt (Newton 1956; Velkov et al. 2010).

In der Veterinärmedizin wird Colistin seit Jahrzenten weltweit vor allem bei Nutztieren, wie Schweinen, Geflügel, Rindern, Schafen, Ziegen und Kaninchen verwendet (Catry et al. 2015). Hauptindikation für den Einsatz von Colistin ist die Prävention und Behandlung von Infektionen durch *Enterobacteriaceae* (vor allem *E. coli* und *Salmonella* spp.). Darüber hinaus wird bzw. wurde Colistin in einigen Ländern, darunter China, Indien und Vietnam, in großem Umfang als Wachstumsförderer eingesetzt, um die Futterverwertung und Gewichtszunahme bei Nutztieren zu verbessern (Kempf et al. 2016). Der Großteil der Verabreichung von Colistin in der Veterinärmedizin erfolgt oral, häufig dargereicht mit dem Trinkwasser oder Futter, teilweise wird es auch injiziert oder, und dies eher in der Kleintiermedizin, topisch (als Augen- oder Ohrentropfen) appliziert. Nach der oralen Verabreichung werden nur sehr geringe Mengen an Colistin resorbiert, die Elimination erfolgt renal (Abraham 2016). Aufgrund der schlechten Absorption von Colistin im Gastrointestinaltrakt wird ein Großteil des von den Tieren mit dem Trinkwasser oder Futter aufgenommenen Colistins über den Urin und Kot in die Umgebung ausgeschieden (Peng et al. 2021).

In veterinärmedizinischen Verkaufsdaten aus 25 Europäischen Union-/Europäischen Wirtschaftsraum-Staaten (inklusive Deutschland) für lebensmittelliefernde Tiere aus dem Jahr 2011 (European Medicines Agency 2014), wurde die Wirkstoffklasse der Polymyxine als fünftmeist verkaufte Gruppe antimikrobieller Mittel (7 %), nach Tetracyclinen (37 %), Penicillinen (23 %), Sulfonamiden (11 %) und Makroliden (8 %), beschrieben. Nach der Entdeckung des horizontal übertragbaren Resistenzgens *mcr* (mobile colistin resistance) bei Bakterienisolaten von Schweinen und Menschen (Liu et al. 2016) und ersten Hinweisen auf die Übertragung von *mcr*-Genen vom Tier auf den Menschen (Liu et al. 2016; Webb et al. 2016) reagierte die Europäische Arzneimittelagentur mit der Empfehlung, den Einsatz und Verkauf von Colistin in den EU-Ländern in den kommenden Jahren zu minimieren (European Medicines Agency 2016). Aufgrund der wachsenden Bedeutung von Colistin als Reserveantibiotikum in der Humanmedizin wurde es zudem im Jahr 2017 von der Weltgesundheitsorganisation (WHO) in die Gruppe der besonders wichtigen Antibiotika mit höchster Priorität („highest priority critically important antimicrobials“) eingestuft. Gemäß den Empfehlungen der WHO sollten Antibiotika dieser Kategorie bei lebensmittelliefernden Tieren nicht zur Wachstumsförderung oder zur Vorbeugung von Infektionskrankheiten eingesetzt werden. Die Behandlung diagnostizierter Infektionskrankheiten mit Antibiotika dieser Kategorie bei lebensmittelliefernden Tieren

sollte nur in begrenztem Umfang erfolgen, beispielsweise wenn keine alternativen Arzneimittel zur Behandlung verfügbar sind (WHO 2017).

Aus dem jüngsten Bericht der Europäischen Arzneimittelagentur (EMA) geht hervor, dass der Gesamtumsatz aller Antibiotika in Europa im veterinärmedizinischen Bereich von 2011 bis 2022 um 52 % und der Umsatz von Polymyxinen um 81 % zurückgegangen ist (European Medicines Agency 2022). Damit bildet die Wirkstoffklasse der Polymyxine mit 2,8 % zusammen mit Fluorquinolonen nur noch die achtmeist verkaufte Antibiotikaklasse in 31 Europäischen Ländern. Die EMA erfasst die abgegebenen Antibiotikamengen als sogenannte PCU (Population correction unit). Diese technische Einheit beschreibt die Antibiotikamenge in Milligramm pro Kilogramm Körpergewicht der wichtigsten Nutztierarten pro Land und Jahr. Während in Dänemark, Finnland, Island, Irland, Norwegen und dem Vereinigten Königreich keine Polymyxine verkauft wurden, waren die höchsten Abgabemengen in Polen mit 10,2 mg/PCU sowie in Deutschland, Litauen und Ungarn mit jeweils 5,8 mg/PCU zu verzeichnen. Insgesamt wurden zu 90,6 % orale Polymyxinlösungen abgegeben, gefolgt von oralen Pulvern (4,7 %), Vormischungen (4,1 %) und Injektionslösungen sowie sonstigen Darreichungsformen (u. a. Boli, orale Pasten; 0,6 %). Informationen über die Verwendung der verkauften Polymyxine, z. B. behandelte Tierart oder Indikation, liegen nicht vor. Weitere Zahlen zu Polymyxinen, wie z. B. in außereuropäischen Ländern vermarktete Mengen, behandelte Tierarten oder Indikationen, sind nicht Gegenstand umfassender Erhebungen. Aktuelle Verkaufsdaten weisen jedoch darauf hin, dass Colistin als Tierarzneimittel und Wachstumsförderer in Ländern mit niedrigem und mittlerem Einkommen, wie Pakistan und Vietnam, trotz aktueller Empfehlungen unvermindert eingesetzt wird. Die Autoren der Studie fordern eine strenge nationale und internationale Gesetzgebung, um den Handel und den Einsatz von Colistin in der Veterinärmedizin und Tierzucht zu regulieren (Umair et al. 2023). Bisher gibt es nur vereinzelte nationale Verbote oder Vereinbarungen über ein Verbot der Verwendung von Colistin als Futtermittelzusatzstoff zur Wachstumsförderung in Argentinien, Brasilien, China, Indien, Japan, Malaysia und Thailand (Olaitan et al. 2021).

Die Hauptindikation für den Einsatz von Colistin bei Schweinen ist die Behandlung von Infektionen des Verdauungstrakts ausgelöst durch *Enterobacteriaceae*, insbesondere durch *E. coli* (Rhouma et al. 2016; Kempf et al. 2016; Rhouma et al. 2017). Colistin findet in erster Linie Anwendung bei der durch ETEC-verursachten Diarrhö der Absetzferkel (Kempf et al. 2013).

2.3.2 Mechanismen der Colistin-Resistenz

Der am häufigsten beschriebene Mechanismus der Colistin-Resistenz bei Gram-negativen Bakterien ist eine Veränderung der Lipid-A-Struktur. Durch eine Reduzierung der negativen Ladung von LPS durch verschiedene Mechanismen wird die Affinität von Colistin zu Lipid A verringert und die Insertion von Colistin in die äußere Membran der Bakterienzelle gehemmt. Einige Gram-negative Bakterien, wie z. B. *Proteus mirabilis*, *Neisseria meningitidis* oder *Burkholderia*-Spezies, können durch die Expression von bestimmten Genen, wie *arnBCADTEF* und *eptB*, eine Anlagerung von kationischen Phosphoethanolamin- (pEtN) oder L-4-Amino-4-desoxyarabinose- (L-Ara4N) Gruppen an das LPS bewirken. Dadurch sind diese Bakterien intrinsisch resistent gegen Colistin (Gogry et al. 2021).

2.3.2.1 Chromosomal vermittelte Resistenz gegenüber Colistin in *E. coli*

Die chromosomal vermittelte Resistenz bei Gram-negativen Bakterien ähnelt in ihrem Mechanismus dem bei intrinsisch resistenten Bakterien. Dabei sind zahlreiche Gene und Operons beteiligt, deren Produkte entweder für die Synthese oder auch Anlagerung kationischer Gruppen an das LPS verantwortlich sind (Poirel et al. 2017). In *E. coli* sind insbesondere chromosomale Mutationen in den Zweikomponentensystemen PmrAB und PhoPQ für die Colistin-Resistenz verantwortlich (Quesada et al. 2015; Olaitan et al. 2015).

Das Zweikomponentensystem PmrAB besteht aus einer Histidin-Kinase (kodiert durch das *pmrB*-Gen) und einem Regulator (kodiert durch das *pmrA*-Gen). Der Regulator PmrA aktiviert nach Phosphorylierung durch PmrB die Operone *pmrHFIJKLM* und *pmrCAB*, sowie das *pmrE*-Gen. Während PmrCAB die Modifizierung von LPS mit pEtN bewirkt (Lee et al. 2004), induzieren PmrHFIJKLM und PmrE die Synthese von L-Ara4N und die Bindung an die Lipid-A-Einheit des LPS (Yan et al. 2007). Es konnte gezeigt werden, dass spezifische Mutationen im *pmrA*- oder *pmrB*-Gen (*pmrA* R81S und *pmrB* V161G) das PmrAB-Zweikomponentensystem dauerhaft aktivieren und somit die Colistin-Resistenz in *E. coli* verursachen können (Quesada et al. 2015; Wang et al. 2024).

Zahlreiche Mutationen im Zweikomponentensystem PhoPQ sind verantwortlich für die Resistenz gegen Colistin bei Isolaten der Gram-negativen Bakterienspezies *Klebsiella pneumoniae*. Die Mutations-bedingte Aktivierung dieses Systems führt ebenfalls zur Aktivierung des bereits beschriebenen *pmrHFIJKLM*-Operons sowie des *pmrA*-Gens und der LPS-Modifizierung durch

L-Ara4N und pEtN. Obwohl eine Mutation im PhoPQ-Zweikomponentensystem beschrieben wurde, die möglicherweise für die Colistin-Resistenz in *E. coli* verantwortlich ist (Olaitan et al. 2015), deutet eine aktuelle Studie daraufhin, dass genetische Veränderungen im PhoPQ-Zweikomponentensystem keinen Einfluss auf die Empfindlichkeit von *E. coli* gegenüber Colistin haben (Wang et al. 2024).

2.3.2.2 Plasmid-vermittelte Resistenz gegenüber Colistin in *E. coli*

Im November 2015 wurde in China erstmals das horizontal übertragbare, Plasmid-kodierte Resistenzgen *mcr-1* in *E. coli*-Isolaten von Schweinen nachgewiesen (Liu et al. 2016). Das kodierte MCR-1-Protein besteht aus 541 Aminosäuren und bewirkt die Anlagerung von pEtN an die Lipid-A-Einheit der äußeren Bakterienmembran. Damit ist der Wirkmechanismus dem der chromosomalen Mutationen sehr ähnlich. Die Produktion des MCR-1-Proteins führt zu einer vier- bis achtfachen Erhöhung der minimalen Hemmstoffkonzentration (MHK) von Colistin in *E. coli* und kann somit zu einer Resistenz gegen diesen Wirkstoff führen (Poirel et al. 2017). Nach der Entdeckung von *mcr-1* konnten in den letzten Jahren eine Vielzahl von weiteren *mcr*-Genen in unterschiedlichen Bakterienspezies identifiziert werden, die sowohl Plasmid-kodiert als auch chromosomal lokalisiert waren (siehe **Tabelle 1**).

Tabelle 1: **Übersicht der Erstbeschreibung von bekannten *mcr*-Genen und Anzahl der bekannten *mcr*-Varianten**

Gen	Lokalisation*	Art	Wirt	Land	Referenz	Varianten**
mcr-1	P (IncI2)	*Ec*	Schwein	CN	(Liu et al. 2016)	*mcr-1.1 – mcr-1.37*
mcr-2	P (IncX4)	*Ec*	Schwein	BE	(Xavier et al. 2016)	*mcr-2.1 – mcr-2.8*
mcr-3	P (IncHI2)	*Ec*	Schwein	CN	(Yin et al. 2017)	*mcr-3.1 – mcr-3.42*
mcr-4	P (ColE10)	*Ec*	Schwein	BE, ES, IT	(Carattoli et al. 2017)	*mcr-4.1 – mcr-4.9*
mcr-5	C, P (ColE)	*Se*	Geflügel	DE	(Borowiak et al. 2017)	*mcr-5.1 – mcr-5.5*
mcr-6	C	*Mp*	Schwein	GB	(AbuOun et al. 2018)	*mcr-6.1*
mcr-7	P (IncI2)	*Kp*	Huhn	CN	(Yang et al. 2018)	*mcr-7.1*
mcr-8	P (IncFII)	*Kp*	Schwein	CN	(Wang et al. 2018b)	*mcr-8.1 – mcr-8.4*
mcr-9	P (IncHI2/IncHI2A)	*Se*	Mensch	US	(Carroll et al. 2019)	*mcr-9.1 – mcr-9.3*
mcr-10	P (IncFIA)	*Er*	Mensch	CN	(Wang et al. 2020a)	*mcr-10.1 – mcr-10.5*

C = Chromosom, **P** = Plasmid; ***Ec*** = *Escherichia coli*, ***Er*** = *Enterobacter roggenkampii*, ***Kp*** = *Klebsiella pneumoniae*, ***Mp*** = *Moraxella pluranimalium*, ***Se*** = *Salmonella enterica*; **BE** = Belgien, **CN** = China, **DE** = Deutschland, **ES** = Spanien, **GB** = Großbritannien, **IT** = Italien, **US** = USA.
*Identifizierte Plasmid Inc-Typen der jeweiligen Erstnachweise der *mcr*-Gene sind in Klammern angegeben.
**Stand August 2024; Recherchequelle: https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/.

Die Lokalisation der *mcr*-Gene auf Plasmiden ist von entscheidender Bedeutung für die Verbreitung der antimikrobiellen Resistenz gegen Colistin durch den konjugativen Transfer der Plasmide zwischen Bakterien (Mmatli et al. 2022). Das am weitesten verbreitete *mcr*-Gen, *mcr-1*, konnte bereits auf einer Vielzahl von unterschiedlichen Plasmiden nachgewiesen werden, darunter solche der Inkompatibilitätsklassen (Inc) IncF, IncHI1, IncHI2, IncI2, IncN, IncP, IncX4 und IncN1-IncHI2 (Lima et al. 2022; McGann et al. 2016; Mei et al. 2022; Göpel et al. 2024a). Die Resistenzgene *mcr-2*, *mcr-3*, *mcr-6*, *mcr-7*, *mcr-8*, *mcr-9* und *mcr-10* konnten jeweils häufig auf den Plasmidtypen IncX4, IncHI2, IncHI2, IncI2, IncF-Typ, IncHI2/HI2A und IncF nachgewiesen werden (Mmatli et al. 2022). Weitere Details zur Epidemiologie von *mcr*-tragenden Plasmiden finden sich in Kapitel 2.4.

2.3.3 Testung der antimikrobiellen Resistenz gegen Colistin

Die Untersuchung der Resistenz von Gram-negativen Bakterien gegen Colistin kann mittels einer Vielzahl an Methoden erfolgen, die sich jedoch hinsichtlich der Vergleichbarkeit der Ergebnisse teilweise stark unterscheiden. Im Folgenden werden die wichtigsten und am häufigsten eingesetzten Methoden beschrieben.

2.3.3.1 Mikrodilution

Der Goldstandard für die Bestimmung der MHK von Colistin bei Bakterien ist die Mikrodilution unter Verwendung von kationenangepasster Müller-Hinton-Bouillon (auch broth microdilution = BMD) (CLSI 2022; EUCAST 2024). Dabei wird eine Verdünnungsreihe mit Colistinsulfat hergestellt und ein Bakterieninokulum zugegeben, wobei die Endkonzentration der Bakterien etwa 5 x 10^5 Kolonie bildende Einheiten pro Milliliter (KBE/mL) betragen sollte. Die Inkubation erfolgt über 18 Stunden bei 37 °C. Die MHK wird als die niedrigste Konzentration abgelesen, bei der kein Bakterienwachstum beobachtet wird. Nach Empfehlung des European Committee on Antimicrobial Susceptibility Testing (EUCAST) gelten Enterobacterales mit einer MHK von ≤ 2 mg/L als Colistin-empfindlich, während sie bei einer MHK von > 2 mg/L als resistent einzustufen sind (EUCAST 2024). Das Clinical and Laboratory Standards Institute (CLSI) unterscheidet in seiner Richtlinie hingegen die Kategorien intermediär (≤ 2 mg/L) oder resistent (> 2 mg/L) (CLSI 2022).
Da die empfohlene Resistenzbestimmung mittels BMD zeitaufwendig ist, eignet sie sich wenig für klinisch-mikrobiologische Labore z. B. in öffentlichen Krankenhäusern. Für die antimikrobielle Empfindlichkeitsprüfung stehen Mikrotitrationsplatten mit Colistin in dehydratisierter Form von unterschiedlichen Herstellern zur Verfügung. Das Colistin wird durch die Zugabe der hergestellten Bakteriensuspension gelöst und nach der angegebenen Inkubationszeit bei 37 °C entweder photometrisch oder optisch ausgewertet. Im Rahmen einer im Jahr 2018 durchgeführten Studie wurde die MHK von insgesamt 75 Gram-negativen Bakterien (22 *Acinetobacter* spp., 21 *Pseudomonas aeruginosa*, 18 *Klebsiella pneumoniae*, 14 *E. coli*) mithilfe von fünf kommerziell erhältlichen BMD-Produkten von vier unterschiedlichen Herstellern vergleichend bestimmt (Matuschek et al. 2018). Die Untersuchungen ergaben, dass bei drei von

fünf Produkten mindestens 96 % der erhaltenen MHKs gleich oder innerhalb einer Verdünnungsstufe im Vergleich zur Referenzmethode lagen. Damit stellen ausgewählte kommerziell erhältliche Mikrodilutionsplatten eine gute Alternative zur klassischen BMD dar.

2.3.3.2 Epsilometertest

Der Epsilometertest (E-Test) ist eine Variante des Agardiffusionstests, der die Ermittlung einer MHK zulässt. Dabei wird eine Bakteriensuspension von 0,5 McFarland hergestellt und diese mit einem Wattetupfer auf einer Müller-Hinton E-Agarplatte verteilt. Anschließend wird ein Kunststoffstreifen, der mit einem definierten Antibiotikumgradienten beschichtet ist, auf den bestrichenen Nährboden gelegt und für 24 Stunden bei 37 °C inkubiert. Durch die Diffusion des Antibiotikums vom getränkten Papierstreifen in den Agar wird das Bakterienwachstum auf der Agarplatte je nach Empfindlichkeit des getesteten Keimes um den Kunststoffstreifen gehemmt. Anhand des skalierten Kunststoffstreifens wird die MHK an der Stelle abgelesen, an der der Hemmhof mit dem beschichteten Streifen kreuzt. Einige Studien berichten von einer hohen kategorischen Übereinstimmung (Einstufung anhand der MHKs in sensibel oder resistent) und keinen bis vereinzelt falsch als empfindlich eingestuften Isolaten, so dass der E-Test als gute Alternative zur MHK-Bestimmung bei Gram-negativen Bakterien eingesetzt werden könnte (Nhung et al. 2015; García-Meniño et al. 2020). Es gibt jedoch auch gegenteilige Berichte, die auf eine hohe Rate von 12 % bis 20 % von Isolaten hinweisen, die durch den E-Test fälschlicherweise als empfindlich eingestuft werden (Matuschek et al. 2018; Chew et al. 2017).

2.3.3.3 Plattendiffusionstest

Der klassische Agardiffusionstest unterscheidet sich vom E-Test durch das Aufbringen eines Papierplättchens anstelle eines Kunststoffstreifens, welches eine definierte Konzentration von Colistin enthält. Durch die Diffusion von Colistin in den Agar ergibt sich ein kreisrunder Hemmhof um das aufgetragene Plättchen, welcher nach 24 Stunden Inkubation bei 37 °C ausgemessen wird. Obwohl diese Art des Resistenztests keine Bestimmung der MHK erlaubt, ist eine Differenzierung in die Kategorien sensibel, intermediär oder resistent möglich, sofern entsprechende Grenzwerte verfügbar sind. Aufgrund elektrostatischer Wechselwirkungen von Colistin mit den Säure- und Sulfatgruppen von Agar diffundiert Colistin nur schlecht in den Nährboden, was zur Bildung kleinerer Hemmhöfe führen kann (Louvois 1982). Aufgrund der

Unzuverlässigkeit dieser Methode für die Resistenzbestimmung werden von der CLSI und EUCAST keine Grenzwerte zur Interpretation des Hemmhofdurchmessers zur Verfügung gestellt (CLSI 2022; EUCAST 2024). Trotzdem findet diese Art der Resistenztestung vereinzelt weiterhin Anwendung (Tran et al. 2023; Pallós et al. 2024), was an der vergleichsweise kostengünstigen sowie der schnellen Testung liegen könnte.

2.3.3.4 VITEK® 2

Eine halbautomatisierte Art der Resistenztestung, die häufig Einsatz in klinisch-mikrobiologischen Laboren findet, ist das VITEK® 2 (bioMérieux, Marcy l'Etoile, Frankreich). Mittels eines Transferrohrs wird eine standardisierte Bakteriensuspension des zu testenden Isolates automatisiert im Gerät in 64 Kavitäten einer Einweg-Testkarte befördert. Durch kontinuierliche Messungen des Bakterienwachstums in den Kavitäten, die unterschiedliche Konzentrationen verschiedener Antibiotika enthalten sowie einer Positivkontrolle ohne Antibiotikazusatz, wird schließlich ein MHK-Wert für jedes Antibiotikum errechnet. Dieses Verfahren basiert auf dem Vergleich des Wachstums des zu testenden Isolates mit dem Wachstum bereits analysierter Isolate mit bekannter MHK, die im VITEK® 2 hinterlegt sind. Da die Methode durch den automatisierten Prozess einfach durchzuführen ist und schnell eine MHK ermittelt werden kann, wurde es alternativ zur BMD eingesetzt. In mehreren Studien konnte jedoch gezeigt werden, dass sich diese Methode aufgrund einer hohen Rate an falsch-empfindlichen Isolaten und einer kategorischen Übereinstimmung von unter 90 % mit den Ergebnissen aus der BMD nicht zur Resistenztestung für Colistin eignet (Tan und Ng 2007; Chew et al. 2017; Khurana et al. 2020).

2.3.3.5 Polymerase-Kettenreaktion, Ganzgenomsequenzierung

Neben der phänotypischen Resistenztestung finden ebenfalls molekulargenetische Methoden Anwendung in der Mikrobiologie. Zum Nachweis von bekannten Resistenzgenen wie *mcr-1*- bis *mcr-10*-Genen kann die herkömmliche Polymerase-Kettenreaktion (Polymerase Chain Reaction = PCR) genutzt werden. Dabei werden bereits beschriebene spezifische Primer verwendet und in einem PCR-Ansatz als Multiplex-PCR kombiniert (Göpel et al. 2024a), um anschließend die amplifizierten Genabschnitte mittels Agarose-Gelelektrophorese nach Größe

aufzutrennen und die PCR-Produkte so als Bande sichtbar zu machen. Neben dieser qualitativen Analysemethode kann ebenso die sogenannte Real-Time PCR als quantitativer Ansatz zum Nachweis von Genen verwendet werden. Dabei wird durch den Einsatz fluoreszierender Sonden die Amplifikation der *mcr*-Gene in Echtzeit sichtbar gemacht (Mentasti et al. 2021). Resistenzvermittelnde Genmutationen können mit dieser Methode nicht identifiziert werden, wobei das Vorkommen von Resistenzgenen oder Genmutationen keinen direkten Rückschluss auf die phänotypische Resistenz von Isolaten zulässt. So waren z. B. von 48 *mcr*-positiven *E. coli*-Isolaten, die von sechs Masthähnchenbetrieben in Malaysia gewonnen wurden, nur 26 (54,2 %) Isolate sowohl geno- als auch phänotypisch resistent (MHK > 2 mg/L) gegen Colistin (Lemlem et al. 2023).

Die Sequenzierung und anschließende Analyse des bakteriellen Genoms ermöglicht wie die PCR den Nachweis von Resistenzgenen. Darüber hinaus können durch Ganzgenomanalysen resistenzvermittelnde Mutationen (z. B. im *pmrA*- oder *pmrB*-Gen) und *mcr*-Genvarianten identifiziert werden. Da die Ganzgenomsequenzierung von Bakterien immer kostengünstiger wird, etabliert sie sich zunehmend auch als Echtzeitdiagnostik, bis dato jedoch fast ausschließlich in der humanmedizinischen Mikrobiologie (Lakshmanan et al. 2023).

2.4 Verbreitung *mcr*-positiver *E. coli*

2.4.1 *mcr*-positive *E. coli* in der Umwelt

Zahlreiche Studien berichten über die Verbreitung von *mcr*-positiven Bakterien in der Umwelt. *E. coli*-Isolate, die überwiegend das *mcr-1*-Gen trugen, sind bereits im Abwasser, aus Bodenproben sowie in Gewässern (Flüsse und Seen) in Asien, Europa und Südamerika nachgewiesen worden (Anyanwu et al. 2020; Furlan et al. 2023). Lebensmittel, wie Gemüse oder tierische Produkte (Fleisch, Fisch, Meeresfrüchte) konnten ebenfalls als Quellen von *mcr*-positiven *E. coli* identifiziert werden (Slettemeås et al. 2017; Lv et al. 2018; Liu et al. 2019; Kassem et al. 2023).

Da lebensmittelliefernde Tiere, wie Schweine, Rinder und Geflügel, als Träger von *mcr*-tragenden Bakterien bekannt sind, wurde in mehreren Studien die Umgebung von nutztierhaltenden Betrieben beprobt. In Malaysia konnten dabei die Gene *mcr-1* (n = 7), *mcr-6* (n = 1)*, mcr-7* (n = 4) und *mcr-8* (n = 2) aus der Umwelt, unter anderem aus dem Trinkwasser, Futtermittel und der Einstreu eines Masthähnchenbetriebes isoliert werden (Lemlem et al.

2023). Erstmals 2021 berichteten Ali et al. über ein *mcr-1*-positives ST10-*E. coli*-Isolat, das aus dem Abwasser einer Milchviehhaltung in Pakistan isoliert werden konnte und dessen *mcr*-Gen auf einem IncI2-Plasmid lokalisiert war (Ali et al. 2021). Guenther et al. berichteten über den Nachweis von *mcr-1*-positiven *E. coli* aus der Umgebung von deutschen Schweinebetrieben (Guenther et al. 2017). Dabei wurden insgesamt sieben Isolate aus Dünger (n = 3), von Stiefelüberziehern (n = 2), Hundekot (n = 1) und einer Stallfliege (n = 1) gewonnen. Bei fünf Isolaten konnte das *mcr-1*-Gen auf einem IncX4-Plasmid lokalisiert werden. Der im Sinne eines One-Health-Ansatzes zu verhindernde Eintrag von Colistin-resistenten Bakterien in die Umwelt ist nicht nur für *E. coli* sondern auch für andere Gram-negative Bakterien, wie *Klebsiella* spp., von großer Bedeutung (Savin et al. 2022).

2.4.2 *mcr*-positive *E. coli* beim Menschen

Bei durch *E. coli*-bedingten Infektionskrankheiten, wie Durchfallerkrankungen, aber auch extraintestinalen Erkrankungen, wie Harnwegsinfektionen, Pneumonien oder Bakteriämien konnten bereits *mcr*-positive Stämme beim Menschen identifiziert werden (Wu et al. 2021; Xie et al. 2022; Sulian et al. 2020; Mariani et al. 2020). In einem Übersichtsartikel von Bastidas-Caldes et al. wurde unter Berücksichtigung von 59 klinischen Studien die höchste Prävalenz von *mcr*-positiven *E. coli*, die bei erkrankten Personen festgestellt wurde, in Afrika gefunden (53/699; 7,58 %), gefolgt von Nord- und Südamerika (436/12 128; 3,59 %), Asien (745/47 611; 1,56 %) und Europa (144/27 600; 0,52 %). Auf allen Kontinenten wurde primär das *mcr-1*-Gen nachgewiesen, vereinzelt aber auch *mcr-2* (Asien, Europa), *mcr-3* (Asien) und *mcr-5* (Amerika) (Bastidas-Caldes et al. 2022). In Deutschland gibt es (Stand August 2024) nur vereinzelt Berichte über den Nachweis von *mcr*-positiven *E. coli* bei klinisch erkrankten Menschen (siehe **Tabelle 2**).

Tabelle 2: Nachweise von *mcr*-positiven *E. coli* aus klinischem Untersuchungsgut von Menschen in Deutschland.

Jahr der Isolierung	Anzahl Isolate	Material	*mcr*-Gen	Plasmid	ST	Referenz
2012	1	-	*mcr-1**	-	-	(Informationsdienst Wissenschaft 2016)
2014	1	-	*mcr-1**	-	-	
2014	1	Fußwunde	*mcr-1**	IncHI2	ST362	(Falgenhauer et al. 2016)
2018	1	Blutkultur	*mcr-1.26*	IncX4	ST69	(Neumann et al. 2020)
	1	intraoperativer Abstrich	*mcr-1.27*	IncX4	ST155	

**mcr*-Gen-Variante nicht bekannt; - = nicht bekannt

Neben klinisch erkrankten Personen können auch gesunde Menschen Träger von *mcr*-positiven *E. coli* sein. Bastitdas-Caldes et al. konnten unter Berücksichtigung von 30 Studien zusammenfassend darstellen, dass die geschätzte Prävalenz von *mcr*-positiven *E. coli*-Isolaten von gesunden Menschen durchschnittlich 7,4 % (Konfidenzintervall: 3,9 % - 13,6 %) betrug (Bastidas-Caldes et al. 2022). Während in Nord- und Südamerika, Afrika und Europa ausschließlich das *mcr-1*-Gen bei gesunden Personen nachgewiesen werden konnte, wurden in Asien neben *mcr-1* ebenfalls *mcr-3* und *mcr-5* in einem geringen Prozentsatz beobachtet (3 % und 8 %).

Da *mcr*-Gene weltweit in Tierbeständen nachgewiesen werden konnten, wurden in einigen Studien Untersuchungen zum Vorkommen dieser Resistenzgene in gesunden Nutztierhaltern durchgeführt. Zwischen 2013 und 2015 sammelten Nakano et al. in Japan Kotproben von gesunden Rindern (n = 202), Schweinen (n = 93) und Landwirten (n = 62) von insgesamt 72 Betrieben und untersuchten diese auf *mcr*-positive *E. coli*. Dabei waren drei Landwirte (zwei Rinderhalter, ein Schweinehalter) Träger von *mcr-1*-positiven *E. coli* (ST10, ST746 und ST2929). In jeweils einem Rinder- und Schweinebetrieb konnten ebenfalls *mcr-1*-positive Isolate aus dem Kot der Tiere gewonnen werden (Nakano et al. 2021). Zheng et al. berichteten über das Vorkommen von zwei *mcr-1*-positiven *E. coli*-Stämmen (*mcr*-Gen lokalisiert auf IncI2- und IncHI2-Plasmiden), die 2016 aus dem Kot eines Nerzhalters in China isoliert werden konnten (Zheng et al. 2019b). Vergleichende Analysen von zwei *mcr*-tragenden IncHI2-Plasmiden, die vom Nerzhalter und aus dem Kot eines Nerzes gewonnen wurden, ergaben eine geringe Sequenzidentität, was auf eine hohe genetische Diversität der *mcr-1*-tragenden Plasmide von Nerz und Mensch hindeutet.

2.4.3 *mcr*-positive *E. coli* bei Tieren

Die Isolierung *mcr*-positiver *E. coli*-Stämme ist bereits aus zahlreichen tierischen Proben erfolgt. Zur besseren Übersicht werden diese Nachweise im Folgenden nach Tierarten geordnet dargestellt.

2.4.3.1 Kleintiere

Pathogene *E. coli* sind bei Kleintieren sowohl mit intestinalen als auch extraintestinalen Erkrankungen assoziiert. In jungen Hunden sind EPEC und ETEC als Auslöser von Darmerkrankungen bekannt. Bei gesunden sowie klinisch erkrankten Hunden mit Diarrhö konnten STEC aus dem Kot von Tieren isoliert werden (Beutin 1999). Extraintestinale Infektionen, die durch *E. coli* verursacht werden, sind bei Hunden und Katzen unter anderem Harnwegsinfektionen, Pneumonien und Wundinfektionen (Aurich et al. 2022; Soonthornsit et al. 2022; Yun et al. 2023).

Die meisten Berichte zum Vorkommen von *mcr*-positiven *E. coli*-Isolaten von Kleintieren stammen aus Asien, während ebenfalls vereinzelt Studien aus Südamerika von Nachweisen bei Hunden berichten (siehe **Tabelle 3**).

In Europa gibt es bislang wenige Berichte zur Isolierung *mcr*-positiver *E. coli*-Stämme von Kleintieren. Hamame et al. beprobten von 2019 bis 2020 klinisch gesunde Hunde (n = 52) und Katzen (n = 105) in einem Tierheim in Marseille, Frankreich (Hamame et al. 2022). Gewonnene Kotproben wurden auf einem Selektivnährboden, der 4 mg/L Colistin enthielt, ausgestrichen und gewachsene Kolonien anschließend identifiziert. Dabei konnten insgesamt 13 phänotypisch resistente *E. coli*-Stämme (von 10 Hunden und drei Katzen) isoliert werden, die alle das *mcr-1*-Gen trugen. In Deutschland berichteten Aurich et al. ein *mcr-4.6*-positives *E. coli*-Isolat, welches 2020 aus dem Urin eines Hundes mit Harnwegsinfektion gewonnen werden konnte (Aurich et al. 2023). Während das *mcr-1*-Gen weltweit prävalent bei *mcr*-positiven *E. coli*-Stämmen von Kleintieren identifiziert wurde, konnten neben dem *mcr-4.6*-Gen aus Deutschland ebenfalls das *mcr-3*-Gen in China und Thailand sowie das *mcr-9*-Gen in Japan bei Kleintieren nachgewiesen werden (Du et al. 2020; Nittayasut et al. 2021).

Tabelle 3: Nachweise von *mcr*-positiven *E. coli* von Kleintieren.

Land	Jahr der Isolierung	Wirt	Anzahl Isolate	Material	*mcr*-Gen	Plasmid	ST	Referenz
AE	-	Ht (2)	2	-	*mcr-1.1*	-	ST1011	(Habib et al. 2023)
AR	2014	Hd	1	Urin	*mcr-1.1*	IncI2	ST770	(Rumi et al. 2019)
CN	2012 - 2013, 2015, 2021	Hd (8), Ktz (1)	9	Kot	*mcr-1**	-	-	(Zhou et al. 2022)
CN	2019	Hd	1	Kot	*mcr-1.1, mcr-3.7*	IncX4, IncP1-Typ	ST132	(Du et al. 2020)
CN	2021	Hd (2), Ktz (5)	7	Kot (6), Wunde	*mcr-1**	IncI2	ST117	(Lin et al. 2022)
CN	-	Hd (7), Ktz (1)	8	Kot (6), Urin, Nase	*mcr-1**	IncN1-IncHI2/ST3 (4), IncI2 (1), n.a. (3)	ST93 (5), ST1011, ST3285, n.a.	(Wang et al. 2018a)
DE	2020	Hd	1	Urin	*mcr-4.6*	ColE10	ST73	(Aurich et al. 2023)
EC	2016	Hd (2)	2	Kot	*mcr-1.1*	IncI2	ST1630, ST2170	(Loayza-Villa et al. 2020)
FR	2019-2020	Hd (10), Ktz (3)	13	Kot	*mcr-1**	-	-	(Hamame et al. 2022)
JP	2018-2022	Hd	1	-	*mcr-1.1*	IncI2	-	(Yasugi et al. 2023)
		Ktz	1	-	*mcr-9**	IncHI2	-	
KR	2018/ 2019	Hd	1	Kot	*mcr-1**	IncI2	ST160	(Moon et al. 2020)
PT	2018-2020	Hd (8)	12	Kot	*mcr-1**	-	ST744 (3), ST117 (2), ST156 (2), ST162 (2), ST38, ST131, ST2179	(Menezes et al. 2022)
TH	2016	Hd	1	Urin	*mcr-3**	-	ST10	(Nittayasut et al. 2021)

mcr*-Gen-Variante nicht bekannt; **- = nicht bekannt

AE = Vereinigte Arabische Emirate, **AR** = Argentinien, **CN** = China, **DE** = Deutschland, **EC** = Ecuador, **FR** = Frankreich, **JP** = Japan, **KR** = Südkorea, **PT** = Portugal, **TH** = Thailand, **Hd** = Hund, **Ht** = Haustier, **Ktz** = Katze, **n.a.** = nicht angegeben, **-** = unbekannt

2.4.3.2 Schweine

Schweine stellen neben Nutzgeflügel eine wichtige Quelle für *mcr*-positive *E. coli* dar. Bastidas-Caldes et al. berichteten in ihrem systematischen Review von einer weltweiten Prävalenz von 8,8 % von *mcr*-Genen in *E. coli*-Isolaten (n = 7089) von gesunden Schweinen (n = 80 600) in insgesamt 85 Studien aus Afrika, Amerika, Asien und Europa (Bastidas-Caldes et al. 2022). Dabei war das *mcr-1*-Gen auf allen Kontinenten weit verbreitet (72,3 % in Asien bis 98 % in Amerika). Während in Amerika nur ein weiteres *mcr*-Gen (*mcr-3*) in 2 % aller *mcr*-positiven *E. coli* gefunden wurde, konnten in Afrika sowohl *mcr-5* und *mcr-8* (jeweils 2 %), in Europa *mcr-2*, *mcr-3*, *mcr-4*, *mcr-5* (0,9 %, 5,4 %, 6,6 % und 0,7 %) und in Asien die höchste Variation an *mcr*-Genen mit *mcr-2, mcr-3, mcr-5, mcr-6, mcr-7, mcr-8* und *mcr-9* (12,06 %, 0,96 %, 0,59 %, 0,05 %, 0,05 %, 0,02 % und 0,35 %) festgestellt werden. Dabei muss jedoch beachtet werden, dass in vielen Studien ausschließlich auf das *mcr-1*-Gen getestet wurde und deswegen die Verbreitung weiterer *mcr*-Gene unterschätzt sein könnte.

Einige Studien beschreiben die Verbreitung von *mcr*-Genen in *E. coli*-Isolaten, die von klinisch erkrankten Schweinen gewonnen wurden und/oder als *E. coli*-Pathovar typisiert werden konnten. Veröffentlichungen aus China, Japan, Korea und Thailand berichten neben dem Vorkommen von *mcr-1* (Prävalenzen von 1,1 % bis 100 %) ebenfalls von der Verbreitung von *mcr-3*-Genen (Prävalenzen von 2,2 % bis 70 %) in *E. coli*-Isolaten, die von an PWD erkrankten Schweinen isoliert wurden (Guo et al. 2020; Do et al. 2020; Kusumoto et al. 2016b; Trongjit et al. 2022). In Europa sind vor allem Studien aus Spanien zur Untersuchung zum Vorkommen von *mcr*-Genen in *E. coli* von erkrankten Schweinen veröffentlicht (Aguirre et al. 2020; García-Meniño et al. 2018; García-Meniño et al. 2019). García et al. beschrieben das Vorkommen von *mcr-4* (54,8 %), *mcr-1* (19,9 %) und *mcr-5* (2,7 %) in insgesamt 140 von 186 untersuchten *E. coli*-Isolaten, die von 2006 bis 2017 in Spanien von Schweinen mit PWD gewonnen werden konnten (García et al. 2018). Dabei war die Mehrzahl aller Isolate dem *E. coli*-Pathovar ETEC (86,5 %) zuzuordnen, sowie ETEC/STEC-Hybrid (8,1 %) und STEC (5,4 %).

Aus Deutschland gibt es einige Studien, die das Vorkommen von *mcr-1-*, *mcr-4*- und *mcr-5*-positiven *E. coli* bei Schweinen beschreiben (siehe **Tabelle 4**). Während die meisten Autoren eine geringe Anzahl an Proben untersuchten, ermittelten Irrgang et al. bei insgesamt 1589 *E. coli*-Isolaten (gewonnen aus Beprobungen in Stallungen oder auf dem Schlachthof), die

MHK von Colistin mittels Mikrodilution (Irrgang et al. 2016). Resistente Stämme (MHK > 2 mg/L) wurden dabei in 3,6 % (31/859) und 2,1 % (15/730) der Isolate von 2011 und 2015 festgestellt, wobei in jeweils 41,9 % und 73,3 % der resistenten Isolate das *mcr-1*-Gen mittels PCR nachgewiesen werden konnte. Drei der insgesamt sieben Studien bis 2021 untersuchten stichprobenmäßig die Lokalisierung der *mcr*-Gene in *E. coli*-Stämmen. Dabei wurden die Plasmidtypen IncX4, IncHI2 und IncI2 am häufigsten identifiziert. Nur eine Studie beschrieb die Probennahme an klinisch erkrankten Schweinen in Schweinebetrieben aus Bayern im Jahre 2016, wo das *mcr-1*-Gen in 11 von 16 nicht auf Colistin-Resistenz vorselektierten *E. coli*-Isolaten mittels PCR nachgewiesen werden konnte (Schirmeier et al. 2018).

Tabelle 4: Nachweise von *mcr*-positiven *E. coli* von Schweinen in Deutschland bis 2021.

Jahr der Isolierung	Anzahl Isolate**	Material, Klinik	*mcr*-Gen (n Isolate)	Plasmid (n Isolate)	ST (n Isolate)	Referenz
2011-2012	43/436 (9,9 %)	Kot/Stiefel-überzieher, n.a.	*mcr-1**	IncX4 (9), IncHI2 (3), IncX4/N, IncHI2/FIB/FII/X3, Chromosom, n.a. (28)	ST410 (2), ST2509 (2), ST34, ST57, ST448, ST540, ST1011, ST1196, ST1842, ST2040, ST2067, ST4398, n.i., n.a. (28)	(Roschanski et al. 2017)
2011	3/7 (42,9 %)	n.a.	*mcr-1**	-	ST1196 (3)	(Pietsch et al. 2018)
2012	2/2 (100 %)	n.a.	*mcr-1**	-	ST57, ST2040	
2011	13/**31** (41,9 %)	Kot, n.a.	*mcr-1**	-	-	(Irrgang et al. 2016)
2015	11/**15** (73,3 %)	Kot, n.a.	*mcr-1**	-	-	
2010-2015	3/**135** (2,2 %)	Kot, n.a.	*mcr-5.1* (2), *mcr-5.2*	ColE (2), n.i.	ST29, ST349, ST1494	(Hammerl et al. 2018)
2016	1/4 (25 %)	Kot, n.a.	*mcr-4.2*	-	ST410	(Rebelo et al. 2018)
2016	11/16 (68,8 %)	Kot, Diarrhö	*mcr-1**	-	-	(Schirmeier et al. 2018)
2018-2020	18/318 (5,7 %)	Kot, n.a.	*mcr-1**	IncX4 (5), IncI2 (2), n.a. (11)	-	(Effelsberg et al. 2021)

mcr*-Gen-Variante nicht bekannt; **n.a. = nicht angegeben, **n.i.** = nicht identifizierbar, **-** = nicht bekannt; **n** = Anzahl
**Fett markierte Zahlenwerte bezeichnen Isolate, die zuvor als Colistin-resistent identifiziert wurden (MHK > 2 mg/L) und daraufhin auf das Vorkommen von *mcr-1* getestet wurden.

2.4.3.3 Rinder, Ziegen, Schafe

Rinder und kleine Wiederkäuer, wie Ziegen und Schafe, können ebenfalls Träger von *mcr*-positiven *E. coli* sein. Viñes et al. sammelten 2021 in Spanien Kotproben von gesunden Mastkälbern (n = 152) und gesunden Mastschweinen (n = 57), deren Stallungen ungefähr 100 Meter entfernt voneinander auf dem Grundstück eines privaten Nutztierhalters lagen (Viñes et al. 2021). Der Halter gab ebenfalls eine Stuhlprobe ab. Alle gesammelten Proben wurden auf MacConkey Agar, der 2 mg/L Colistin enthielt, ausgestrichen. Bei Wachstum auf dem Agar wurde je ein Colistin-resistentes *E. coli*-Isolat pro Probe mittels PCR auf das Vorkommen von *mcr-1* bis *mcr-5* getestet. Insgesamt konnten so 18 Colistin-resistente Isolate (13 von Kälbern, vier von Schweinen, eins vom Nutztierhalter) gewonnen werden, die alle das *mcr-1*-Gen trugen und für weitere Analysen sequenziert wurden. Das *mcr-1*-Gen war überwiegend auf IncX4-Plasmiden lokalisiert (bei 14/18 Isolaten, 10 Kälber, drei Schweine, ein Nutztierhalter), konnte aber auch zweimal chromosomal lokalisiert identifiziert werden (ein Rind und ein Schwein) und je einmal auf einem IncHI2- und IncI2-Plasmid (beides Isolate von Rindern). Die Autoren vermuteten aufgrund der großen Ähnlichkeit von drei IncX4-Plasmiden von Kälbern und dem einen *mcr-1*-tragenden IncX4-Plasmid des Nutztierhalters (99 %-ige Sequenzabdeckung, 99,97 % Sequenzidentität), dass ein horizontaler Gentransfer von den Kälbern auf den Nutztierhalter stattgefunden haben könnte, wobei die *mcr*-positiven *E. coli* alle unterschiedlichen Sequenztypen angehörten (ST398 Nutztierhalter; ST43, ST224, ST1638 Kälber). Neben Spanien gibt es Studien aus Brasilien, China, Frankreich, Polen und Tunesien, die das Vorkommen von *mcr-1*-positiven *E. coli*-Isolaten bei Rindern beschreiben (Zheng et al. 2019a; Um et al. 2022; Saidani et al. 2019; Zając et al. 2019; Palmeira et al. 2018).

In Deutschland gibt es bislang nur einen Bericht zum Nachweis *mcr-1*-positiver *E. coli*-Isolate, die aus dem Kot von gesunden Rindern von 2010 bis 2015 isoliert werden konnten. Dabei wurden 45 Colistin-resistente *E. coli*-Stämme mittels PCR auf das Vorkommen von *mcr-1* getestet, wovon 27 (60 %) positiv für das Resistenzgen waren (Irrgang et al. 2016).

Neben Rindern sind auch Ziegen und Schafe als Träger von *mcr*-positiven *E. coli* in China, Portugal und Südafrika identifiziert worden (Zhao et al. 2022b; Ramatla et al. 2024; Dantas Palmeira et al. 2021). Treilles et al. sammelten 2018 und 2019 Kotproben von Ziegen aus 80

Ziegenzucht- und fünf Ziegenmastbetrieben in Frankreich (Treilles et al. 2022). Durch anschließende Untersuchungen konnte festgestellt werden, dass insgesamt 4,2 % (65/1561) der beprobten Tiere in Zuchtbetrieben und 60 % (84/140) der untersuchten Tiere in Mastbetrieben Träger von *mcr-1*-positiven *E. coli* waren. Dabei war das *mcr-1*-Gen entweder auf dem Chromosom (32,2 %) oder auf Plasmiden der Typen IncX4 (38,9 %) und IncHI2 (26,8 %) lokalisiert.

2.4.3.4 Geflügel

Neben Schweinen gilt Geflügel als weitere wichtige Quelle von *mcr*-positiven *E. coli*-Isolaten. Bastidas-Caldes beschrieben die höchste Prävalenz von *mcr*-Genen in *E. coli* bei gesundem Geflügel mit 10,4 % (7134/68 362) im Vergleich zu gesunden Schweinen (8,8 %; 7090/80 600) und Menschen (3,35 %; 789/23 585) unter Berücksichtigung von insgesamt 159 Studien aus Afrika, Amerika, Asien und Europa (Bastidas-Caldes et al. 2022). Während in Nord- und Südamerika sowie Europa ausschließlich *mcr-1* bei *E. coli* von gesundem Nutzgeflügel gefunden wurde, konnten neben *mcr-1* auch zu einem geringen Prozentsatz weitere *mcr*-Gene in Afrika (*mcr-5*, 1 %; *mcr-8*, 0,4 %) und Asien (*mcr-2,* 11,9 %; *mcr-3,* 0,1 %) festgestellt werden.
Lima Barbieri et al. untersuchten insgesamt 980 aviäre pathogene *E. coli* (APEC), die von an Kolibazillose erkranktem Nutzgeflügel (Masthähnchen, Legehennen, Puten, Enten und Gänsen) aus Amerika (n = 814), Europa (n = 105), Asien (n = 31) und Afrika (n = 30) von 1980 bis 2015 isoliert worden waren (Lima Barbieri et al. 2017). Mittels PCR wurden diese Isolate auf das Vorkommen von *mcr-1* und *mcr-2* getestet. Insgesamt waren 12 APEC (1,22 %), die von acht Hühnern in China (beprobt 2012-2014) und vier Hühnern in Ägypten (beprobt 2010) isoliert worden waren, positiv für das *mcr-1*-Gen. Zwei Studien aus China, die APEC von klinisch erkrankten Hühnern, Enten und Gänsen auf das Vorkommen von *mcr-1* testeten, berichteten von einer höheren Prävalenz des Resistenzgens in APEC von 4,2 % (3/72, *mcr-1* lokalisiert auf IncI2-Plasmiden) und 6,3 % (8/126) (Yin et al. 2021; Tan et al. 2023). Azam et al. untersuchten 75 APEC, die von verstorbenen Masthähnchen mit Kolibazillose auf einer Farm in Pakistan isoliert wurden. Dabei konnte in 38,7 % (n = 29) aller APEC das *mcr-1*-Gen nachgewiesen werden (Azam et al. 2020).
Eine Übersicht von *mcr*-positiven *E. coli*-Nachweisen von Geflügel aus Deutschland findet sich in **Tabelle 5**. Irrgang et al. untersuchten *E. coli*-Isolate von Nutztieren (beprobt in Stallungen oder bei Schlachtung), die im Rahmen des nationalen Zoonose-Monitorings von 2010 bis 2015

isoliert werden konnten (Irrgang et al. 2016). Die gewonnenen Isolate aus dem Kot von Legehennen (n = 1795), Masthähnchen (n = 1474) und Puten (n = 1459) wurden per Mikrodilution auf Colistin-Resistenz untersucht und Stämme mit erhöhter MHK (> 2 mg/L) wurden mittels PCR auf das Vorkommen von *mcr-1* getestet. Dabei konnten hohe Prävalenzen von Colistin-resistenten *E. coli* bei Puten (23,5 %) und Masthühnern (12,9 %) festgestellt werden, die zu einem hohen Prozentsatz (je 90,7 % und 91,1 %) das *mcr-1*-Gen trugen. Während die meisten Veröffentlichungen aus Deutschland keine Angaben zur Klinik des beprobten Nutzgeflügels dokumentierten, berichteten Ewers et al. über den Fund eines *mcr-1*-positiven ExPEC-Isolates, welches aus der Leber eines verstorbenen Masthuhnes mit Sepsis im Jahre 2010 isoliert werden konnte (Ewers et al. 2016). Während das *mcr*-Gen auf einem IncHI2-Plasmid lokalisiert war, konnte das ExPEC-Isolat dem Sequenztyp 131 zugeordnet werden. ST131 ist eine epidemiologisch wichtige klonale Linie, da sie ein breites Spektrum an Infektionen verursachen kann, sowie häufig multiresistent gegen zahlreiche Antibiotika (z. B. Beta-Laktam-Antibiotika und Fluorquinolone) ist (Nicolas-Chanoine et al. 2014).

Tabelle 5: Nachweise von *mcr*-positiven *E. coli* von Geflügel in Deutschland.

Jahr der Isolierung	Wirt	Anzahl Isolate**	Material, Klinik	*mcr*-Gen	Plasmid (n Isolate)	ST	Referenz
2002-2014	Hühner	38/**58** (65,5 %)	Kot, n.a. (Schlachtung)	*mcr-1**	-	-	(El Garch et al. 2018)
2010	Masthuhn	1/1 (100 %)	Leber, Septikämie	*mcr-1**	IncHI2	ST131	(Ewers et al. 2016)
2010-2014	Legehennen	3/**24** (12,5 %)	Kot, n.a.	*mcr-1**	-	-	(Irrgang et al. 2016)
2010-2014	Masthühner	173/**190** (91,1 %)	Kot, n.a.	*mcr-1**	-	-	
2010-2014	Puten	311/**343** (90,7 %)	Kot, n.a.	*mcr-1**	-	-	
2011-2012	Hühner	3/41 (7,3 %)	n.a.	*mcr-1**	-	ST57, ST354, ST1196	(Pietsch et al. 2018)
2012-2014	Puten	1/4 (25 %)	n.a.	*mcr-1**	-	ST1196	
2011-2012	Masthühner	1/2 (50 %)	Kot	*mcr-1**	IncX4 (2)	ST354	(Roschanski et al. 2018)
2016	Masthuhn	1/1 (100 %)	n.a., verstorben	*mcr-1**	IncHI2	ST69	(Hornsey et al. 2019)
2018-2019	Puten	123/**123** (100 %)	Kot, n.a.	*mcr-1**	IncHI2 (5), IncX4 (5), n.a. (113)	-	(Nordhoff et al. 2023)

mcr*-Gen-Variante nicht bekannt; **n.a. = nicht angegeben, **-** = nicht bekannt; **n** = Anzahl;
**Fett markierte Zahlenwerte bezeichnen Isolate, die zuvor als Colistin-resistent identifiziert wurden (MHK > 2mg/L) und daraufhin auf das Vorkommen von *mcr-1* getestet wurden.

2.4.3.5 Pferde

Im Gegensatz zu den bisher erwähnten Tierarten spielt *E. coli* als Auslöser von Durchfallerkrankungen bei Pferden eine untergeordnete Rolle (Feary und Hassel 2006). ExPEC spielen bei Pferden vor allem eine Rolle bei Fruchtbarkeitsstörungen, da sie asymptomatisch verlaufende Endometritiden verursachen können (Díaz-Bertrana et al. 2021). Eine Recherche der internationalen Literatur zeigt, dass es bis August 2024 lediglich zwei Studien gibt, die von Nachweisen *mcr*-positiver *E. coli* im Zusammenhang mit extraintestinalen *E. coli*-Infektionen bei Pferden berichten (siehe **Tabelle 6**). In Brasilien konnte aus einem im Jahre 2012 gewonnenen Isolat aus der Lunge eines toten Pferdes ein *mcr-5.3*-positives *E. coli*-Isolat gewonnen werden (Fernandes et al. 2018). In Schweden konnten Börjesson et al. aus sechs Uterustupfern, zwei Urinproben und zwei Wundabstrichen von zehn unterschiedlichen Pferden insgesamt zehn *mcr-9*-positive *E. coli*-Stämme isolieren (Börjesson et al. 2020). Die Isolate konnten mehrfach

den Sequenztypen ST1861 (n = 3), ST9329 (n = 2) und ST1423 (n = 2) zugewiesen werden. Zudem wurden die *mcr-9*-Gene auf IncHI2- und IncHI2A-Plasmiden lokalisiert.

Tabelle 6: Nachweise von *mcr*-positiven *E. coli* von Pferden.

Land	Jahr der Isolierung	Anzahl Isolate	Material	*mcr*-Gen	Plasmid	ST	Referenz
BR	2012	1	Lunge	*mcr-5.3*	-	ST1711	(Fernandes et al. 2018)
SE	2017 - 2018	10	Uterus, Urin, Wunde	*mcr-9**	IncHI2, IncHI2A	ST1252, ST1423, ST1861, ST2557, ST4398, ST9329	(Börjesson et al. 2020)

mcr*-Gen-Variante nicht bekannt; **BR = Brasilien, **SE** = Schweden, **-** = unbekannt

Während bei anderen Tierarten größtenteils das *mcr-1*-Gen nachgewiesen werden konnte, gibt es lediglich einen Bericht über ein *mcr-1*-positives ST405-*E. coli*-Isolat, welches im März 2017 aus Pferdedung, der im Nordwesten Algeriens auf landwirtschaftlicher Fläche aufgebracht war, isoliert werden konnte (Touati et al. 2020). Aufgrund der geringen Anzahl an Studien, die sich mit der Resistenz gegen Colistin von *E. coli* isoliert aus Pferden befassen, kann die mögliche Rolle von Pferden in der Verbreitung von *mcr*-Genen nicht abschließend beurteilt werden.

2.4.3.6 Weitere Tiere

Weitere Tierarten, die als Träger von *mcr*-positiven *E. coli*-Isolaten beschrieben wurden, sind in **Tabelle 7** zusammengefasst. In China wurden sechzig Kotproben von an Durchfall erkrankten Kaninchen aus großen Kaninchenfarmen (300 - 500 Muttertiere pro Farm) gesammelt und Polymyxin B-resistente *E. coli*-Isolate (MHK > 2 mg/L) mittels PCR auf das Vorkommen von *mcr-1* untersucht. Das Resistenzgen konnte dabei in acht von 55 getesteten Isolaten identifiziert werden (Wang et al. 2021). Studien aus China, Vietnam und dem Libanon berichteten über das Vorkommen von Plasmid-lokalisierten *mcr-1*-Genen in *E. coli*-Isolaten, die aus dem Darm von Fischen aus Aquakulturen gewonnen wurden (Lv et al. 2018; Hoa et al. 2020; Hassan et al. 2020).

Auch Wildtiere können Träger von *mcr*-positiven *E. coli*-Stämmen sein. Aus dem Kot von vier Damhirschen, die zwischen 2018 und 2020 südöstlich von Zentralportugal erlegt wurden, konnten *mcr-1*-positive *E. coli* nachgewiesen werden (Torres et al. 2021). Cilia et al. beprobten 200 Wildschweine, die 2018 und 2019 in der Toskana, Italien erlegt wurden (Cilia et al. 2021).

Aus den Kotproben konnten 168 *E. coli*-Isolate gewonnen werden, die mittels PCR auf *mcr-1* und *mcr-2* untersucht wurden. Dabei erwiesen sich 44,6 % (75/168) der untersuchten Isolate als positiv für mindestens eines der Resistenzgene. Die Autoren vermuteten, dass die hohe Prävalenz durch den Kontakt mit antimikrobiellen Rückständen oder antibiotikaresistenten Bakterien aus der Umwelt, beispielsweise über Kontakt mit Abwasser oder landwirtschaftlichem Dünger, sowie dem Fressverhalten der Tiere (Allesfresser/Aasfresser) zusammenhängen könnte. Zugvögel, wie zum Beispiel der Weißstorch, konnten ebenfalls als Träger von *mcr-1*-positiven *E. coli* identifiziert werden (Mohsin et al. 2016; Migura-Garcia et al. 2019).
In einer Studie von 2016 untersuchten Unger et al. 142 *E. coli*-Isolate, die aus 150 Kotproben von Transportboxen, in denen Reptilien transportiert worden waren, stammten (Unger et al. 2017). Die Stämme wurden auf Müller Hinton Agar mit 4 mg/L Colistin ausgestrichen und von gewachsenen Isolaten wurde mittels Mikrodilution die MHK festgestellt. Zwei *E. coli*-Stämme mit erhöhter MHK (> 4 mg/L) konnten mittels PCR positiv für das *mcr-1*-Gen getestet werden. Das Resistenzgen eines der beiden Isolate wurde auf dem IncHI2-Plasmid lokalisiert. Die Isolate stammten von zwei Sechsstreifen-Langschwanzeidechsen, die 2013 und 2014 in Vietnam beprobt worden waren. In einer Studie aus Thailand konnten 31 *mcr-1*-positive *E. coli*-Stämme aus 300 untersuchten Schmeißfliegen isoliert werden, das Resistenzgen war dabei primär auf IncX4-Plasmiden lokalisiert (bei 12/18 untersuchten Isolaten) (Yang et al. 2019).

Tabelle 7: Nachweise von *mcr*-positiven *E. coli* bei weiteren Tierarten.

Wirt	Land	Anzahl Isolate	Material	*mcr*-Gen	Plasmid (n Isolate)	ST (n Isolate)	Referenz
Damhirsch	PT	4	Kot	*mcr-1**	-	ST345 (2), ST155, ST533	(Torres et al. 2021)
Kamel	EG	75	Kot	*mcr-1** (3) *mcr-2** (2) *mcr-3** (21) *mcr-4** (3)	-	-	(Youseef et al. 2024)
Kaninchen	CN	8	Kot	*mcr-1**	-	ST88 (5), ST2, ST24, ST353	(Wang et al. 2021)
Regenbogen-forelle	LB	5	Darm	*mcr-1.1*	IncX4 (5)	ST101 (3), ST48 (2)	(Hassan et al. 2020)
Schmeißfliege	TH	31	-	*mcr-1**	IncX4 (12), IncHI1B (3), IncHI1A (2), IncHI1A-IncHI1B (1), n.a. (13)	ST10 (7), ST648 (5), ST549 (4), ST58 (3), ST181 (3), ST218 (2), ST162, ST201, ST457, ST1244, ST2345, ST2705, ST5487	(Yang et al. 2019)
Sechsstreifen-Langschwanz-eidechse	VN	2	Darm	*mcr-1.1*	IncHI2, Chromosom	ST117, ST1011	(Unger et al. 2017)
Weißstorch	ES	5	Kot	*mcr-1.1* (4), *mcr-1.2*	IncX4 (3), IncHI2 (2)	ST93 (2), ST10, ST351, ST1011	(Migura-Garcia et al. 2019)
Wildschwein	IT	75	Kot	*mcr-1** (26), *mcr-2** (26), *mcr-1** & *mcr-2** (23)	-	-	(Cilia et al. 2021)

mcr*-Gen-Variante nicht bekannt; **CN = China, **EG** = Ägypten, **ES** = Spanien, **IT** = Italien, **LB** = Libanon, **PT** = Portugal, **TH** = Thailand, **VN** = Vietnam, **n.a.** = nicht angegeben, **-** = unbekannt, **n** = Anzahl;

3 Forschungsfragen

Zu Beginn des Dissertationsprojektes lagen keine umfassenden Daten zum Vorkommen und zur Verbreitung von *mcr*-Genen und deren Lokalisation in *E. coli*-Pathovaren, die beim Schwein Durchfallerkrankungen und die Ödemkrankheit verursachen können, vor. In der institutseigenen Routinediagnostik wurden seit dem Jahr 2000 porcine pathogene *E. coli*-Isolate aus Einsendungen von Kotproben und Darmgewebe mit Darminhalt von Schweinen aus Betrieben in Deutschland gewonnen, mittels PCR auf ausgewählte Virulenzgene untersucht und anschließend asserviert. Diese Isolate wurden in unterschiedlicher Weise in die Untersuchungen einbezogen. Folgende Forschungsfragen lagen dieser Arbeit zu Grunde:

3.1 Vorkommen *mcr*-Gene

- Wie ist die Verbreitung der bisher bekannten *mcr*-Gene (*mcr-1* bis *mcr-10*) in pathogenen *E. coli*-Isolaten von Schweinen?
- Lässt sich eine Veränderung der Häufigkeit von *mcr*-Genen über die Jahre beobachten?
- Sind bisher unbekannte *mcr*-Genvarianten in porcinen pathogenen *E. coli*-Isolaten aus deutschen Schweinebetrieben nachweisbar?
- Sind *mcr*-positive *E. coli*-Isolate auch Träger anderer Resistenzgene?

3.2 Lokalisation *mcr*-Gene

- Sind *mcr*-Gene chromosomal oder plasmidlokalisiert in porcinen pathogenen *E. coli*-Isolaten nachzuweisen?
- Welche Plasmidtypen können als *mcr*-Gen-Träger identifiziert werden?
- Können die gleichen *mcr*-Gen-tragenden Plasmide über Jahre hinweg in deutschen Schweinebetrieben nachgewiesen werden?

3.3 Verteilung intestinal pathogener *E. coli*-Pathovare

- Wie ist die Prävalenz der sich aus den nachgewiesenen InPEC-assoziierten Virulenzgenprofilen ergebenden porcinen *E. coli*-Pathovare in Deutschland?
- Lassen sich bestimmte porcine *E. coli*-Pathovare überdurchschnittlich häufig als Träger von *mcr*-Genen identifizieren?

3.4 Phylogenie

- Kommen bei Schweinen bestimmte (klonale) *E. coli*-Linien vor, die bei Menschen oder anderen Tierarten ebenfalls zu Infektionen und Krankheitsausbrüchen führen?
- Lassen sich klonale *E. coli*-Linien, die identische Sequenztypen sowie Virulenzgen- und Resistenzgenprofile aufweisen, in einzelnen Betrieben über die Jahre hinweg feststellen?

4 Vorstellung der Publikationen

4.1 Publikation 1

"Occurrence of *mcr-1* and *mcr-2* colistin resistance genes in porcine *Escherichia coli* isolates (2010-2020) and genomic characterization of *mcr-2*-positive *E. coli."*

Vorkommen der Colistin-Resistenzgene *mcr-1* und *mcr-2* in *Escherichia coli*-Isolaten von Schweinen (2010-2020) und genomische Charakterisierung von *mcr-2*-positiven *E. coli.*

Publiziert in: Frontiers in Microbiology

doi: 10.3389/fmicb.2022.1076315

Eingegangen: 21.10.2022

Angenommen: 22.11.2022

Zu Beginn der Dissertation wurde das Vorkommen der mobilen Colistin-Resistenzgene *mcr-1* und *mcr-2* in porcinen *E. coli*-Isolaten, die von 2010 bis 2020 im Institut für Hygiene und Infektionskrankheiten der Tiere in Gießen isoliert werden konnten, untersucht. Zudem wurden *mcr-2*-positive *E. coli*-Isolate genomisch charakterisiert. Die Ergebnisse dieser Untersuchungen finden sich in **Publikation 1**. Die vollständige Originalpublikation befindet sich im Anhang.

Zusammenfassung (übernommen aus der Originalpublikation)

Das weltweite Auftreten Plasmid-vermittelter Colistin-Resistenzen bedroht die Wirksamkeit von Colistin als eine der letzten Therapieoptionen bei Infektionen mit multiresistenten Gram-negativen Bakterien. Bisher sind zehn *mcr*-Gene (*mcr-1* bis *mcr-10*) bekannt. Während *mcr-1* weltweit verbreitet ist, gibt es kaum Berichte über das Vorkommen von *mcr-2*. Wir haben das Vorkommen von *mcr-1*- und *mcr-2*-Genen in *Escherichia coli*-Isolaten von Schweinen untersucht und eine detaillierte genomische Charakterisierung von *mcr-2*-positiven Stämmen durchgeführt. Zwischen 2010 und 2017 wurden 7614 *E. coli*-Stämme von Schweinekotproben aus Europa gewonnen und Isolate asserviert, die mindestens eines der virulenzassoziierten Gene tragen, die Shigatoxin-bildende *E. coli* (STEC), enterotoxische *E. coli* (ETEC) oder enteropathogene *E. coli* (EPEC) charakterisieren. Insgesamt trugen 793 (10,4 %) dieser Isolate das *mcr-1* Gen. Die Untersuchung von weiteren 1477 *E. coli*-Isolaten, die zwischen 2018 und 2020 mittels Anzucht auf 4 mg/L colistinhaltigen Schafblutagarplatten gewonnen wurden, waren 36 (2,4 %) *mcr-1*-positiv. Im Gegensatz zu *mcr-1* war die Häufigkeit von *mcr-2* mit 0,13 % bei insgesamt 9091 Isolaten sehr gering. Die meisten *mcr-2*-positiven Isolate stammten aus Belgien (n = 9), eines aus Spanien und zwei aus Deutschland. Sie stammten aus sechs verschiedenen Betrieben und wiesen die Multilokus-Sequenztypen ST10, ST29, ST93, ST100, ST3057 und ST5786 auf. Während die ursprünglich beschriebene *mcr-2.1*-Variante vorherrschte, fanden wir bei zwei Isolaten aus Belgien eine neue *mcr-2*-Variante, die wir als *mcr-2.8* bezeichnen. MCR-2-positive Isolate wurden meist als ETEC oder ETEC-ähnlich klassifiziert, während ein Isolat aus Spanien einen atypischen enteropathogenen *E. coli* (aEPEC; *eae*+) darstellte. Das ST29-aEPEC-Isolat trug *mcr-2* auf dem Chromosom. Weitere acht Isolate trugen ihr *mcr-2*-Gen auf IncX4-Plasmiden, die dem ursprünglich 2015 in Belgien beschriebenen MCR-2-Plasmid pKP37-BE ähnelten. Drei ST100 *E. coli*-Isolate aus einem einzigen Betrieb in Belgien trugen das *mcr-2.1*-Gen auf einem 47 kb selbstübertragbaren IncP-Typ-Plasmid einer neuen IncP-1-Klade. Dies ist der erste Bericht über *mcr-2*-Gene in *E. coli*-Isolaten aus Deutschland. Der Nachweis eines neuen *mcr-2*-Allels und eines neuen Plasmids deutet auf die Existenz bisher unentdeckter *mcr-2*-Varianten und mobilisierbarer Vehikel hin.

4.2 Publikation 2

“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.”

Vorkommen der mobilen Colistin-Resistenzgene *mcr-1 – mcr-10* einschließlich neuer *mcr*-Genvarianten in verschiedenen Pathovaren von *Escherichia coli*-Isolaten von Schweinen, die von 2000 bis 2021 in Deutschland gesammelt wurden.

Publiziert in: Applied Microbiology
doi: 10.3390/applmicrobiol4010005
Eingegangen: 13.12.2023
Angenommen: 26.12.2023

Publikation 2 beinhaltet die Untersuchung von insgesamt 10 573 porcinen *E. coli*-Isolaten auf neun Virulenzfaktoren intestinaler *E. coli*-Pathovare, die zuvor im Institut für Hygiene und Infektionskrankheiten der Tiere in Gießen erfolgt war. Ergänzend wurde für diese Publikation das Vorkommen der Colistin-Resistenzgene *mcr-1* bis *mcr-10* untersucht und eine genomische Analyse von 220 repräsentativen *mcr*-positiven Isolaten durchgeführt. Die vollständige Originalpublikation befindet sich im Anhang.

Zusammenfassung (übernommen aus der Originalpublikation)

In der Europäischen Union sind intestinale Infektionskrankheiten bei Schweinen die Hauptindikation für den Einsatz von Colistin. Es fehlen jedoch groß angelegte epidemiologische Daten über die Häufigkeit mobiler Colistin-Resistenzgene (*mcr*) in schweineassoziierten Pathovaren von *Escherichia coli* (*E. coli*). In dieser Studie wurden insgesamt 10 573 *E. coli*-Isolate von Schweinen aus Deutschland zwischen Juli 2000 und Dezember 2021 isoliert. Mit Hilfe der Multiplex-Polymerase-Kettenreaktion wurden Virulenz-assoziierte Gene (VAGs) sowie *mcr-1*- bis *mcr-10*-Gene nachgewiesen. Eine Ganzgenomsequenzierung wurde von 220 repräsentativen *mcr*-positiven *E. coli*-Stämmen durchgeführt. Die Gesamtprävalenz der *mcr*-Gene betrug 10,2 %, wobei *mcr-1* (8,4 %) und *mcr-4* (1,6 %) am häufigsten nachgewiesen wurden. Alle anderen *mcr*-Gene wurden nur selten identifiziert (*mcr-2*, *mcr-3*, *mcr-5*) oder waren nicht vorhanden (*mcr-6* bis *mcr-10*). Die höchste Häufigkeit von *mcr*-Genen wurde bei enterotoxischen

und Shigatoxin-bildenden *E. coli* (ETEC/STEC-Hybrid) und bei Ödemkrankheit verursachenden *E. coli* (EDEC) festgestellt (21,9 % und 17,6 %). Wir berichten über drei neue *mcr*-Varianten, *mcr-1.36*, *mcr-4.8* und *mcr-5.5*. Bei 39 analysierten „attaching and effacing" *E. coli*-Isolaten (AEEC) war der *eae*-Subtyp β1 am weitesten verbreitet (71,8 %). Bei der Überwachung des Vorkommens von *mcr*-Genen in verschiedenen Bereichen sollte die unterschiedliche Häufigkeit von *mcr*-positiven Isolaten bei pathogenen *E. coli* berücksichtigt werden.

4.3 Publikation 3

„Repeated occurrence of mobile colistin resistance gene-carrying plasmids in pathogenic *Escherichia coli* from German pig farms."

Wiederholtes Auftreten von mobilen Colistin-Resistenzgen-tragenden Plasmiden in pathogenen *Escherichia coli* aus Schweinebetrieben in Deutschland.

Publiziert in: Microorganisms
doi: 10.3390/microorganisms12040729
Eingegangen: 06.03.2024
Angenommen: 28.03.2024

Aus dem Gesamtpool von 10 573 porcinen *E. coli*-Isolaten, die in Publikation 2 Eingang fanden, wurden anhand definierter Kriterien drei Schweinebetriebe ausgewählt, die in mindestens vier aufeinander folgenden Jahren *mcr*-positive *E. coli*-Isolate aufwiesen. Für die **Publikation 3** wurde eine Ganzgenomsequenzierung repräsentativer Isolate der drei Farmen durchgeführt. Besonderes Augenmerk wurde in den anschließenden Analysen auf die Lokalisierung der nachgewiesenen *mcr*-Gene gelegt. Die vollständige Originalpublikation befindet sich im Anhang.

Zusammenfassung (übernommen aus der Originalpublikation)

Die weltweite Verbreitung Plasmid-vermittelter mobiler Colistin-Resistenzgene (*mcr*) bedroht die lebenswichtige Rolle von Colistin als Medikament der letzten Wahl. Es wurde untersucht, ob das wiederholte Auftreten spezifischer *E. coli*-Pathovare und Plasmide in einzelnen Schweinebetrieben auf den Verbleib oder die wiederholte Einschleppung verschiedener *E. coli*-

Stämme zurückzuführen ist. Es wurden 154 pathogene *E. coli*-Isolate von drei Schweinebetrieben analysiert, in denen das *mcr*-Gen in mindestens vier aufeinanderfolgenden Jahren nachgewiesen werden konnte. Alle Isolate waren positiv für mindestens ein virulenzassoziiertes Gen (VAG) mittels Polymerasekettenreaktion getestet worden. Bei 87 ausgewählten Isolaten wurden VAGs, antimikrobielle Resistenzgene und Plasmid-Inc-Typen durch Ganzgenomanalysen bestimmt. Sechzig Isolate waren positiv für *mcr-1* und ein Isolat für *mcr-4*. In Betrieb 1 handelte es sich bei den *mcr*-positiven Isolaten um Ödemkrankheit auslösende *E. coli* (EDEC; 77,3 %) oder um enterotoxinbildende *E. coli* (ETEC; 22,7 %). Bei Betrieb 2 waren alle *mcr*-positiven Stämme ETEC, während *mcr*-positive Isolate von Betrieb 3 ein breiteres Spektrum an Pathovaren aufwiesen. Das *mcr-1.1*-Gen befand sich entweder auf IncHI2 (Betrieb 1), IncX4 (Betrieb 2) oder IncX4 und IncI2 Plasmiden (Betrieb 3). Diese Ergebnisse deuten darauf hin, dass verschiedene pathogene *E. coli*-Stämme eine wichtige Rolle bei der Aufrechterhaltung der Plasmid-kodierten Colistin-Resistenz in der Umwelt von Schweinen im Laufe der Zeit spielen könnten.

5 Diskussion

Ziel dieser Arbeit war es, einen Überblick über die Verbreitung von *mcr*-Genen in pathogenen *E. coli*-Isolaten von erkrankten Schweinen aus deutschen Schweinebetrieben zu gewinnen. Dabei lag der Fokus auf i) dem Vorkommen aller zum Zeitpunkt der Arbeit bekannten *mcr*-Gene (*mcr-1* bis *mcr-10*) in *E. coli*-Isolaten von erkrankten Schweinen, ii) der Lokalisierung von *mcr*-Genen ausgewählter Pathovare auf dem bakteriellen Chromosom oder Plasmiden sowie der Charakterisierung von Plasmiden und iii) Untersuchungen von *mcr*-positiven und *mcr*-negativen *E. coli*-Isolaten aus drei deutschen Schweinebetrieben über mehr als vier aufeinander folgende Jahre. Grundlage für diese Untersuchungen bildete die hausinterne Sammlung von porcinen pathogenen *E. coli*-Isolaten, welche im Zeitraum von Juli 2000 bis Dezember 2021 aus eingesandtem Probenmaterial von erkrankten Schweinen isoliert, als *E. coli*-Pathovar bzw. als positiv für mindestens ein InPEC-assoziiertes VAG identifiziert und asserviert wurden.

5.1 Vorkommen von *mcr*-Genen in porcinen pathogenen *E. coli*

Das horizontal übertragbare und Plasmid-kodierte Resistenzgen *mcr-1* wurde erstmals 2015 in *E. coli*-Isolaten von Schweinen nachgewiesen (Liu et al. 2016). Seitdem konnten neun weitere *mcr*-Gene (*mcr-2* bis *mcr-10*) identifiziert werden. Bis auf das *mcr-6*-Gen, welches vor allem in *Moraxella* spp. nachgewiesen wurde (AbuOun et al. 2018), sind die übrigen bekannten *mcr*-Gene hauptsächlich in Enterobacterales lokalisiert (Liu et al. 2024). Phylogenetische Analysen zeigten, dass MCR-1 eine hohe Ähnlichkeit mit MCR-2 (81 % Aminosäuresequenz-Identität mit MCR-1) und MCR-6 (83 %) aufweist und eine Untergruppe der Colistin-Resistenzgene darstellt (Shen et al. 2020b), deren Ursprung chromosomal-lokalisierte *mcr*-ähnliche Gene von *Moraxella* spp. sein könnten (Kieffer et al. 2017; Khedher et al. 2020). Während MCR-5 (36 %) eine weitere separate Untergruppe darstellt, bilden MCR-3 (32 %), MCR-4 (34 %), MCR-7 (35 %), MCR-8 (31 %), MCR-9 (36 %) und MCR-10 (36 %) phylogenetisch die größte Untergruppe. Ein Teil dieser MCR-Varianten könnte dabei ursprünglich in Bakterien wie *Aeromonas* spp. (MCR-3 und MCR-7) oder *Shewanella* spp. (MCR-4) entstanden sein, die in aquatischer Umgebung vorkommen (Shen et al. 2020b). Trotz der unterschiedlichen potentiellen Entstehungsursprünge resultiert das Vorhandensein von

mcr-Genen typischerweise in einer phänotypischen Resistenz von Bakterien gegenüber Colistin (Liu et al. 2024). Eine Ausnahme bildet das Gen *mcr-9*, dessen Expression erst durch subinhibitorische Colistin-Konzentrationen induziert wird, was in der Folge zu einer erhöhten MHK führt (Kieffer et al. 2019). Im Rahmen einer weiteren Studie wurde über die Identifizierung einer *mcr-3*- und *mcr-4*-ähnlichen Genvariante berichtet, deren Vorkommen in Carbapenemase-produzierenden *Enterobacteriaceae* keine phänotypische Colistin-Resistenz vermittelte (Teo et al. 2018).

Die Verbreitung aller bislang identifizierten *mcr*-Gene wurde kürzlich in einem umfangreichen Review zusammengefasst (Liu et al. 2024). Während *mcr-1* in mehr als 60 Ländern und auf allen Kontinenten verbreitet ist, sind andere *mcr*-Gene, wie z. B. *mcr-6* und *mcr-7* nur vereinzelt festgestellt worden (Liu et al. 2024). Vor allem Nutztiere, wie Geflügel und Schweine, konnten als Träger von *mcr-1*-positiven *E. coli*-Isolaten identifiziert werden. Daten zum Vorkommen von *mcr*-Genen in porcinen pathogenen *E. coli*, die von klinisch erkrankten Schweinen isoliert wurden, liegen bisher kaum vor. **Publikation 1** und **2** dieser Dissertation stellen die ersten umfangreichen Berichte zur Verbreitung von *mcr-1* bis *mcr-10* bei pathogenen *E. coli*-Isolaten von Schweinen aus Deutschland dar (Ewers et al. 2022; Göpel et al. 2024a). Ergänzend dazu wurden im Rahmen von **Publikation 1** 1936 porcine pathogene *E. coli*-Isolate aus insgesamt 17 europäischen Ländern von 2010 bis 2020 auf das Vorkommen von *mcr-1* und *mcr-2* untersucht. Das *mcr-1*-Gen konnte in geringer Häufigkeit (0 – 0,7 %) in Isolaten aus der Schweiz (0 von 129), Österreich (0/73), den Niederlanden (3/757) und Dänemark (1/140), sowie in einem Großteil (33,3 – 60,7 %) der Isolate aus Ungarn (4/12), Spanien (16/28), Italien (25/42) und Portugal (17/28) von 2010 bis 2017 nachgewiesen werden. Dabei ist zu beachten, dass aufgrund der geringen Anzahl von Isolaten aus den meisten Ländern kein Rückschluss auf die wahre Prävalenz von *mcr-1* in porcinen pathogenen *E. coli*-Isolaten gezogen werden kann. Das Vorkommen von *mcr-1* unter insgesamt 6158 untersuchten *E. coli*-Isolaten von 2010 bis 2017 aus Deutschland betrug insgesamt 11,5 %, mit steigender Nachweisrate ab 2010 (8,9 %) bis 2015 (15,2 %) und anschließender Abnahme des Nachweises von *mcr-1* über die Jahre 2016 und 2017 (je 11,4 % und 6,3 %; **Publikation 1**). In **Publikation 2** konnte das Vorkommen des *mcr-1*-Gens in 8,4 % von insgesamt 10 573 untersuchten *E. coli*-Isolaten festgestellt werden, welche von Juli 2000 bis Dezember 2021 von Schweinen aus Deutschland gewonnen wurden. Dabei wiesen die Pathovare ETEC/STEC-Hybrid mit 16,9 % und EDEC mit 13,2 % die höchsten

Prozentsätze für *mcr-1*-Gene auf. Veröffentlichte Studien zum Vorkommen von *mcr-1* in *E. coli* von klinisch gesunden Schweinen aus Deutschland von 2011 bis 2012 (Roschanski et al. 2017) und von 2018 bis 2020 (Effelsberg et al. 2021) wiesen mit 9,9 % und 5,8 % eine ähnliche Häufigkeitsverteilung des *mcr-1*-Gens auf. Miguela-Villoldo et al. untersuchten insgesamt 700 *E. coli*-Isolate, die von 1999 bis 2021 aus Zäkumproben von klinisch gesunden Mastschweinen bei der Schlachtung in Spanien isoliert wurden, auf das Vorkommen von *mcr-1* (Miguela-Villoldo et al. 2022). Von 2004 bis 2015 konnte dabei eine Zunahme von *mcr-1*-positiven Isolaten festgestellt werden (2015 höchste Prävalenz von 66 %), mit einem abnehmenden Trend des Nachweises von 2017 (54 %) bis 2021 (18 %). Diese veränderten Nachweisraten stimmen mit den aus Deutschland in der vorgelegten Arbeit gewonnenen Daten von klinisch erkrankten Schweinen über die Jahre überein. Ob die Abnahme resistenter Isolate über die vergangenen Jahre mit den Empfehlungen der WHO von 2017 zur Reduzierung des Colistineinsatzes bei Nutztieren zusammenhängt, kann aufgrund fehlender Metadaten von Schweinebetrieben zur Behandlung mit Colistin in unserer Studie nicht belegt werden. Infolge des in China ausgesprochenen Verbots der Verwendung von Colistin als Leistungsförderer in der Nutztierhaltung konnte ein signifikanter Rückgang des Nachweises von *mcr-1* in Schweinekotproben beobachtet werden. Während im Jahr 2016 noch eine Verbreitungsrate von 45 % (308/684) festgestellt wurde, lag diese nach dem Verbot Ende 2017 und 2018 bei jeweils 31 % (486/1575) sowie 19 % (274/1416) (Shen et al. 2020a). In einer Langzeitstudie aus China konnte seit 2017 ebenfalls ein Rückgang von Colistin-resistenten *Enterobacteriaceae*, die aus menschlichem Probenmaterial gewonnen wurden, festgestellt werden. Die Autoren führen diese Beobachtung auf das seit dem 1. Mai 2017 bestehende nationale Verbot für den Einsatz von Colistin als Leistungsförderer bei Nutztieren in China zurück (Zhao et al. 2022a).

Die Genome von 132 *mcr-1*-positiven Isolaten wurden im Zuge von **Publikation 2** sequenziert und es konnte zum Großteil die *mcr-1.1*-Variante (96,2 %) identifiziert werden. Neben dem Nachweis von drei weiteren *mcr-1.1*-ähnlichen Genen und einem *mcr-1.26*-positiven Isolat, konnte in einem ST48-AEEC aus dem Jahre 2016 die bisher unbekannte Variante *mcr-1.36* festgestellt werden. In 53 Isolaten konnte zudem die Lokalisierung von *mcr-1* auf Plasmiden IncX4 (44/53; 83 %), IncHI2 (7/53; 13,2 %) und IncI2 (2/53; 3,8 %) nachgewiesen werden. Diese Plasmidtypen sind sowohl in Deutschland als *mcr-1*-tragende Plasmide in Schweinen (Roschanski et al. 2017; Effelsberg et al. 2021) sowie international in Tieren, Menschen und

der Umwelt identifiziert worden (Matamoros et al. 2017). Die Lokalisierung von *mcr-1*-Genen auf IncX4, IncHI2 und IncI2 spielt eine entscheidende Rolle sowohl für die weltweite Verbreitung als auch für das anhaltende Vorkommen in Nutztierbeständen. Eine verstärkte Expression von *mcr-1* führt zu einer Beeinträchtigung des Wirtsbakteriums, was sich in einer Verringerung der bakteriellen Wachstumsrate und einem Abbau der Zellmembran äußern kann (Yang et al. 2017; Liu et al. 2020). In ihrer Studie aus dem Jahr 2021 konnten Yang et al. nachweisen, dass das untersuchte *mcr-1*-tragende IncI2-Plasmid gleichzeitig für ein Protein kodiert, welches die Plasmidkopienzahl pro Zelle verringert und somit die *mcr-1*-Expression reguliert (Yang et al. 2021). Des Weiteren wird die Verbreitung von *mcr-1*-tragenden IncX4-Plasmiden durch die Konjugation mittels Plasmid-kodierter Transfer-Aktivierer gefördert (Yi et al. 2022). Die beschriebenen Mechanismen reduzieren die mit dem Auftreten und der Expression von *mcr-1* in der Wirtszelle einhergehenden Fitnessverluste. Sie können als wesentliche Einflussfaktoren für das trotz einer Reduzierung des Colistin-Einsatzes weiterhin bestehende Vorkommen von *mcr-1* in Nutztierbeständen betrachtet werden (Usui et al. 2021).

Publikation 1 beinhaltet den Nachweis von 12 *mcr-2*-positiven *E. coli*-Stämmen, die von insgesamt sechs Schweinebetrieben aus Belgien (drei Farmen, neun Isolate), Deutschland (zwei Farmen, zwei Isolate) und Spanien (eine Farm, ein Isolat) isoliert werden konnten. Die sehr niedrige Nachweisrate von *mcr-2* mit 0,13 % im Gegensatz zum häufigen Vorkommen von *mcr-1* in porcinen *E. coli*-Isolaten von 2010 bis 2020 ist vergleichbar mit ähnlichen Studienergebnissen aus Spanien (García et al. 2018; Miguela-Villoldo et al. 2020). Die Erstbeschreibung des *mcr-2*-Gens in Colistin-resistenten *E. coli*-Isolaten von Schweinen und Rindern im Jahr 2016 identifizierte *mcr-2* in 20,8 % (11/53) der getesteten *E. coli*, die zwischen 2011 und 2012 von Schweinen mit Diarrhö in Belgien gewonnen wurden (Xavier et al. 2016). In China und Thailand konnten in Untersuchungen zum Vorkommen von *mcr-2* in *E. coli* von gesunden Schweinen hohe Prävalenzen von 38 % bis 46,8 % nachgewiesen werden (Zhang et al. 2019; Ketkhao et al. 2021). Die geringere Verbreitung von *mcr-2* im Vergleich zu *mcr-1* wird in der Literatur auf lokale Faktoren, wie geographische Unterschiede und veterinärmedizinische Praktiken, sowie auf Unterschiede in der plasmidspezifischen Übertragungshäufigkeiten zurückgeführt (Xavier et al. 2016; Kawanishi et al. 2017).

Neben dem bislang ersten Nachweis des *mcr-2*-Gens von Schweinen in Deutschland konnte eine neue *mcr-2*-Genvariante (*mcr-2.8*) in zwei ETEC-Isolaten, die im Juni 2015 von zwei

Schweinen aus einer Farm in Belgien gewonnen wurden, von uns identifiziert werden. Diese neue Variante, sowie sechs weitere *mcr-2.1*-positive Isolate in unserer Studie trugen das *mcr*-Gen auf IncX4-Plasmiden, die eine hohe Ähnlichkeit von über 99,8 % verglichen mit dem ursprünglich beschriebenen Plasmid pKP37-BE aus Belgien aufwiesen (Xavier et al. 2016). Nur zwei Plasmidtypen (IncX4 und IncHI1B/IncFIB) waren bis 2021 als Träger von *mcr-2*-Genen identifiziert worden (Stosic et al. 2021). In **Publikation 1** konnten wir in drei ST100-*E. coli* von drei Schweinen aus einer Farm in Belgien *mcr-2.1* auf einem bis dahin unbekannten übertragbaren IncP-ähnlichen Plasmid lokalisieren. Zudem wurde das *mcr-2.1*-Gen in einem aEPEC-Isolat von 2013 aus Spanien in unserer Studie chromosomal lokalisiert. Boonyasiri et al. berichteten ebenfalls von einem chromosomal lokalisierten *mcr-2.3*-Gen in einem *E. coli*-Isolat, welches 2016 aus dem Kot von einem Schwein in Thailand isoliert werden konnte (Boonyasiri et al. 2023). Die meisten Studien, die *mcr-2*-positive *E. coli* aus Probenmaterial von Tieren und Menschen isolieren konnten, haben keine weiteren Analysen zur Lokalisierung von *mcr-2* durchgeführt. Die Rolle des IncP-ähnlichen Plasmides in der Verbreitung von *mcr-2*, sowie die Häufigkeit von *mcr-2* integriert im Bakterienchromosom ist deswegen schwer zu beurteilen (Ewers et al. 2022).

Das Vorkommen von *mcr-3* bis *mcr-10* in 10 573 porcinen pathogenen *E. coli*-Isolaten aus Deutschland wurde in **Publikation 2** behandelt. Dabei konnte *mcr-4* in 1,6 % aller Isolate festgestellt werden, während *mcr-5* und *mcr-3* in jeweils 0,3 % und 0,03 % der getesteten Stämme nachgewiesen wurde. ETEC/STEC-Hybrid-Isolate wiesen, wie schon im Fall von *mcr-1*, die höchste Rate an *mcr-4* (3,9 %) und *mcr-5* (1,6 %) im Vergleich zu anderen Pathovaren auf. Die drei *mcr-3*-positiven EDEC-Isolate wurden im Jahre 2014 von drei Schweinen aus einer Farm gewonnen. Sie stellen unseres Wissens nach den ersten Nachweis von *mcr-3* in *E. coli* von Schweinen aus Deutschland dar. Im Gegensatz zu dem geringen Vorkommen von *mcr-3* in porcinen pathogenen *E. coli*-Isolaten aus Europa berichteten Studien vom häufigen Nachweis (8,3 % bis 70 % der untersuchten Isolate) in Japan, Südkorea und Thailand in erkrankten Schweinen (Fukuda et al. 2018; Mechesso et al. 2020; Trongjit et al. 2022). Die regionalen Differenzen im Vorkommen von *mcr-3*-Genen sind, ähnlich wie bei *mcr-2*, letztlich nicht geklärt. Neben den oben aufgeführten möglichen Faktoren wird in der Literatur zudem die seltenere Untersuchung auf *mcr-2* bis *mcr-10* genannt (Bastidas-Caldes et al. 2022).

Unter 70 repräsentativen sequenzierten *mcr-4*-positiven Isolaten konnten wir sowohl *mcr-4.6* (61,4 %), *mcr-4.2* (30 %), *mcr-4.1* (1,4 %), *mcr-4.3* (1,4 %) und die neue *mcr-4.8*-Variante in drei ETEC und einem ETEC-ähnlichen Isolat identifizieren (5,7 %). Von 17 sequenzierten *mcr-5*-positiven Isolaten war *mcr-5.1* überwiegend (94,1 %) vertreten, die bis dato unbekannte *mcr-5.5*-Variante konnte in einem ST29-AEEC Isolat von einem acht Wochen alten Schwein mit Diarrhö festgestellt werden. Bisher war nur ein Nachweis in Deutschland von einem *mcr-4.2*-positiven *E. coli*-Isolat aus Kotmaterial eines Schweines in 2016 erfolgt (Rebelo et al. 2018), sowie ein weiterer Bericht zum Nachweis von drei *mcr-5*-positiven *E. coli*-Isolaten aus Deutschland vorhanden (Hammerl et al. 2018). Während in unserer Studie in 97,1 % von *mcr-4*-positiven Isolaten ColE10 als *mcr-4*-tragendes Plasmid nachgewiesen werden konnte, ergaben unsere Analysen keine Identifizierung von *mcr-5*-tragenden Plasmiden. Bislang wurden nur zwei Plasmide, ColE und ColE10, als *mcr-4*-tragende Plasmide identifiziert (Liu et al. 2024). Diese kleinen (häufig um die 8000 bp) Plasmide können sich zwar in verschiedenen Bakterienarten replizieren, jedoch ist für die Übertragung dieser Plasmidtypen auf andere Bakterien ein Helferplasmid erforderlich, welches die Konjugation fördert (Carattoli et al. 2017). Im Gegensatz dazu können die selbstübertragbaren Plasmide des IncX4-Typs, welche das *mcr-1*-Gen tragen, eine entscheidende Rolle in Hinblick auf die schnelle internationale Ausbreitung dieses Gens gespielt haben (Fernandes et al. 2016).

Keines der untersuchten Isolate in **Publikation 2** erwies sich als positiv für die weiteren aktuell bekannten *mcr*-Gene *mcr-6* bis *mcr-10*. Diese Erkenntnis deckt sich mit bisher veröffentlichten Ergebnissen zum Vorkommen dieser Resistenzgene in porcinen pathogenen *E. coli*-Isolaten aus China, Spanien und Thailand (Flament-Simon et al. 2020; Khine et al. 2022; Nguyet et al. 2022; Hu et al. 2023). Bislang berichten nur zwei Studien von vereinzelten Nachweisen des *mcr-9*-Gens in *E. coli*-Isolaten, die von klinisch erkrankten Schweinen gewonnen wurden. Die Berichte stammen von einem an Enteritis erkrankten Schwein aus Italien (Guarneri et al. 2023) sowie von einem an PWD erkrankten Schwein aus Japan aus dem Jahr 2012. Von diesem Tier konnte ein *mcr-9*-positives ST1196-*E. coli*-Isolat gewonnen werden, in dem das Colistin Resistenzgen auf einem IncHI2/HI2A-Plasmid lokalisiert wurde (Fukuda et al. 2022). Die Mehrzahl der Studien, die das Vorkommen von *mcr*-Genen in Probenmaterial von Tieren, Menschen und der Umwelt untersuchten, testeten nicht auf *mcr-6* bis *mcr-10* (siehe Anhang von **Publikation**

1 und **2**, Kapitel 12). Deshalb ist die Datenlage zur Verbreitung dieser *mcr*-Gene nicht so umfangreich wie von *mcr-1* bis *mcr-5*. Die Ergebnisse aus **Publikation 2** können jedoch darlegen, dass zumindest in deutschen Schweinebetrieben die Verbreitung von *mcr-6* bis *mcr-10* in pathogenen *E. coli*-Isolaten bis Ende 2021 keine Rolle spielte. Bis auf das *mcr-6*-Gen, welches bislang primär in *Moraxella* spp. identifiziert wurde, konnten die übrigen *mcr*-Gene bereits häufiger in *Enterobacteriaceae* wie *Klebsiella pneumoniae* (*mcr-7* und *mcr-8*), *Enterobacter cloacae* complex (*mcr-9*) sowie *Enterobacter roggenkampii* (*mcr-10*) lokalisiert werden (Liu et al. 2024). Dagegen gibt es nur vereinzelte Berichte über das Vorkommen von *mcr-6* bis *mcr-10* in *E. coli*-Isolaten, z. B. von geschlachteten Schweinen aus Thailand (*mcr-6* bis *mcr-9*) (Khanawapee et al. 2021) und von desinfiziertem Geschirr aus China (*mcr-10*) (Zhang et al. 2022). Ein möglicher Grund für das geringe Vorkommen dieser *mcr*-Gene in *E. coli* könnten die *mcr*-tragenden Plasmide spielen. In einer Studie konnten Xu et al. nachweisen, dass sich *mcr-9*- und *mcr-10*-kodierende Plasmide zwar von *Enterobacter cloacae* complex auf einen *E. coli*-Stamm übertrugen, diese Plasmide jedoch nicht dauerhaft im Stamm verblieben. Die Autoren vermuteten, dass eine Inkompatibilität zwischen den *mcr*-tragenden Plasmiden und *E. coli* sowie eine Wirtspräferenz für *Enterobacter cloacae* complex für die geringe Verbreitung von *mcr-9* und *mcr-10* in *E. coli* verantwortlich sein könnte (Xu et al. 2022).

5.2 Nachweis von *mcr*-positiven *E. coli* über mehr als vier Jahre in Schweinebetrieben in Deutschland

Die umfangreiche Daten- und Biobank an asservierten porcinen *E. coli*-Isolaten am Institut für Hygiene und Infektionskrankheiten der Tiere, die bereits die Grundlage für **Publikation 1** und **2** bildete, ermöglichte die Identifizierung von drei deutschen Schweinebetrieben, in denen für mindestens sechs aufeinanderfolgende Jahre in der Routinediagnostik pathogene *E. coli*-Stämme nachgewiesen wurden. Die Bestimmung von *E. coli*-Pathovaren und das Vorkommen von *mcr*-Genen über die Jahre sowie Genomanalysen von ausgewählten Isolaten aller drei Betriebe fand Eingang in **Publikation 3**.

Während die Verbreitung von *mcr*-Genen bei Tieren (vor allem in Nutztierbeständen, wie Geflügel und Schweinen), Menschen und in der Umwelt durch zahlreiche Studien belegt ist (Anyanwu et al. 2020; Bastidas-Caldes et al. 2022), gibt es wenige Berichte zum Vorkommen von *mcr*-Genen über aufeinanderfolgende Jahre in landwirtschaftlichen Betrieben mit

Tierhaltung (Randall et al. 2018; Khine et al. 2022). Unter Anwendung von spezifischen Kriterien konnten wir drei deutsche Schweinebetriebe in der institutseigenen Datenbank identifizieren, von denen für mindestens vier aufeinander folgende Jahre *mcr*-positive *E. coli* nachgewiesen werden konnten (Göpel et al. 2024b). Die am häufigsten festgestellten *E. coli*-Pathovare unterschieden sich je nach Betrieb (Betrieb 1: EDEC, 23/50; Betrieb 2: ETEC, 53/78; Betrieb 3: ETEC/STEC-Hybrid, 14/26) und stimmten mit den vorherrschenden Pathovaren der *mcr*-positiven *E. coli*-Isolate pro Betrieb überein. Die Pathovare ETEC (50,7 %) und EDEC (20,8 %) wurden sowohl am häufigsten in den gesamten 154 asservierten Isolaten identifiziert als auch in *mcr*-positiven Isolaten (ETEC: 48,3 %; EDEC: 30 %). García-Meniño et al. untersuchten 481 *E. coli*-Isolate, die von 2006 bis 2016 in Spanien von Absatzferkeln (73 %) und Saugferkeln (27 %) mit Diarrhö gewonnen wurden (García-Meniño et al. 2018). Dabei konnte das Vorkommen von ETEC (67 %) signifikant mit dem Auftreten von PWD assoziiert werden. Zudem wurden von 123 *mcr-1*-positiven *E. coli*-Isolaten 57,7 % als ETEC, sowie aEPEC (29,3 %), ETEC/STEC-Hybrid (8,1 %) und STEC (4,9 %) identifiziert.

In unserer Studie konnten von 2009 bis 2012 (42 %, Betrieb 1), 2013 bis 2018 (30,8 %, Betrieb 2) und 2014 bis 2019 (61,5 %, Betrieb 3) hohe Prävalenzen von *mcr*-positiven pathogenen *E. coli*-Isolaten festgestellt werden. Dabei handelte es sich überwiegend um *mcr-1.1*, mit der Ausnahme eines *mcr-4.8*-positiven ETEC-ähnlichen Isolates, welches im April 2019 auf dem Betrieb 3 isoliert wurde. Bislang konnten nur in einer weiteren Studie aus Deutschland in zwei Schweinebetrieben zu zwei Zeitpunkten einer Mastperiode wiederholt *mcr-1*-positive *E. coli* nachgewiesen werden (Effelsberg et al. 2021). Auch weltweit wurde über ein jahrelanges kontinuierliches Auftreten von *mcr*-positiven *E. coli* in Schweinebetrieben bislang lediglich in einigen wenigen Fällen berichtet, wie beispielsweise in einer Studie aus Thailand (Khine et al. 2022). Die Ergebnisse unserer Studie legen nahe, dass das langjährige Vorkommen von *mcr*-positiven Stämmen in Schweinebeständen möglicherweise unterschätzt wird. Dies könnte auf eine unzureichende kontinuierliche Probennahme und Datenerhebung zurückzuführen sein.

Die Überwachung der Resistenzsituation bei Zoonoseerregern erfolgt seit 2009 im Rahmen des nationalen Zoonose-Monitorings. Dieses beinhaltet die Erfassung, Auswertung und Veröffentlichung von Daten über Zoonosen und Zoonoseerregern sowie deren Antibiotikaresistenzen in Lebensmitteln, Futtermitteln und lebenden Tieren (BVL 2024). Im Rahmen dieses Monitorings werden jährlich alternierend entweder von Masthähnchen und Mastputen oder

Mastkälbern/Jungrindern und Mastschweinen am Schlachthof sowie deren Fleisch im Einzelhandel und an Grenzkontrollstellen kommensale *E. coli* sowie ESBL/AmpC-bildende und Carbapenemase-bildende *E. coli* gewonnen. Im Jahr 2021 wurden lediglich 190 kommensale *E. coli* aus dem Blinddarminhalt von Mastschweinen am Schlachthof isoliert und auf Antibiotikaresistenzen getestet (BVL 2021). Das EU-weite Monitoring zur Antibiotikaresistenz von Zoonose- und Indikatorbakterien bei Menschen, Tieren und Lebensmitteln von 2021/2022 beinhaltete die Untersuchung von 4586 kommensalen *E. coli* von geschlachteten Schweinen aus insgesamt 31 Ländern, von denen 0,2 % Colistin-resistent (MHK > 2 mg/L) waren (EFSA 2024). Obgleich diese Monitoring-Programme einen Beitrag zur Datenlage der Resistenzen in *E. coli* leisten, ist die Stichprobenanzahl, sowohl in Deutschland als auch EU-weit gesehen, relativ gering. Zudem beschränkt sich die Datenerhebung auf gesunde Schweine, welche bei der Schlachtung untersucht werden. Eine Ausweitung der Probengewinnung beispielsweise in ausgewählten Schweinebetrieben über mehrere Jahre, in Kombination mit einer umfassenden Datenerhebung zu Haltung, Import und Export von Schweinen, Antibiotikatherapie und Hygienemaßnahmen, könnte eine sinnvolle Ergänzung zu den bestehenden Monitoring-Programmen darstellen.

Im Rahmen von **Publikation 3** wurden 87 repräsentative Isolate für detaillierte Untersuchungen genomsequenziert und auf das Vorkommen weiterer, nicht zuvor mittels PCR nachgewiesener VAGs sowie das Vorhandensein von antimikrobiellen Resistenzgenen und Plasmid-Inc-Typen geprüft. Obwohl in phylogenetischen Vergleichsanalysen Clusterbildungen bei der Mehrzahl von *E. coli*-Isolaten aus den einzelnen Betrieben unabhängig vom Jahr der Isolierung beobachtet wurden, konnten wir keine klonalen *E. coli*-Linien über die Jahre in den drei ausgewählten Betrieben identifizieren. Die am häufigsten festgestellten Sequenztypen in unserer Studie waren ST100 (27/87; alle ETEC), ST1 (20/87; 19/20 EDEC) und ST10 (10/87; 5/10 ETEC). In der Literatur werden ST10 und ST100 als mit ETEC assoziierte klonale Linien beschrieben, während ST1 als mit EDEC assoziierte klonale Linie bei erkrankten Schweinen beschrieben wird (Kusumoto et al. 2016a; García-Meniño et al. 2018). Die Möglichkeit eines wiederkehrenden Neueintrags von *mcr*-positiven pathogenen *E. coli*-Isolaten in die Betriebe kann in unserer Studie nicht ausgeschlossen werden. Das Vorkommen der gleichen *mcr*-tragenden Plasmide über

die Jahre in nicht-klonalen Isolaten könnte jedoch darauf hindeuten, dass die gleichen Plasmide über die Jahre in den Betrieben verbleiben, u. a. aufgrund der selbstinitiierten Übertragung von Plasmiden des Inc-Typs zwischen *E. coli*.

Besonders das Vorkommen von *mcr*-positiven Isolaten in **Publikation 3**, die den Sequenztypen ST10, ST100 und ST131 angehören, ist bemerkenswert, da diese als *E. coli* mit zoonotischem Potential gelten (Silva et al. 2023). Im Jahr 2018 berichteten García-Meniño et al. über die Identifizierung von *mcr-1*-positiven *E. coli*-Isolaten des Sequenztyps 131 bei an Durchfall erkrankten Schweinen aus Spanien. Zwei der Isolate wiesen eine hohe genetische Identität mit ST131-Isolaten von Menschen auf, was auf eine potenzielle Humanpathogenität von ST131 von Schweinen hinweisen könnte (García-Meniño et al. 2018). Eine Studie aus Thailand konnte zudem identische *mcr*-tragende IncX4-Plasmide in *E. coli*-Isolaten von gesunden Schweinen und Schweinehaltern von unterschiedlichen Farmen identifizieren. Dieses Plasmid war zudem sowohl in einem *E. coli*-Isolat als auch in einem *Klebsiella pneumoniae*-Stamm aus der gleichen Farm gefunden worden (Leangapichart et al. 2023). So besteht die Möglichkeit, dass sowohl ein zoonotisches Risiko in der Übertragung von Isolaten bestimmter klonaler Linien (ST10, ST131) als auch in der Übertragung von *mcr*-tragenden Plasmiden gegeben ist.

Weitere Analysen konnten zeigen, dass Virulenzgene porciner *E. coli*-Isolate über teilweise mehrere Jahre in den drei Betrieben auf ähnlichen Plasmiden lokalisiert waren. Die Rolle dieser Plasmide in Bezug auf die Übertragbarkeit zwischen verschiedenen *E. coli*-Isolaten und damit die Entstehung von *E. coli*-Pathovaren in Schweinen, welche zu Krankheitsausbrüchen in Betrieben führen können, ist bislang kaum erforscht. VAGs, die klassisch für ETEC-Isolate sind, konnten bereits vor über 25 Jahren auf Virulenzplasmiden des IncF-Typs lokalisiert werden (Mainil et al. 1998). Eine aktuellere Studie berichtete zudem von einem IncFII/IncX1-Plasmid, welches sowohl VAGs für ETEC als auch STEC trug (Brilhante et al. 2019). Das Vorkommen von Virulenzplasmiden in porcinen *E. coli*-Isolaten über mehrere Jahre in einzelnen Betrieben ist bislang jedoch nicht Gegenstand umfassender Untersuchungen gewesen.

In **Publikation 3** wird zudem die Lokalisierung von *mcr-1*-Genen auf den gleichen IncHI2 (Betrieb 1), IncX4 (Betrieb 2) und sowohl IncX4 als auch IncI2 Plasmidtypen (Betrieb 3) über die Jahre beschrieben. Im Rahmen einer groß angelegten Analyse von über 14 000 Plasmidgenomen konnte gezeigt werden, dass die Wahrscheinlichkeit einer Ko-Lokalisierung von Colistin-Resistenzdeterminanten mit weiteren Resistenzgenen oder VAGs auf demselben

Plasmid im Vergleich zu anderen Resistenzgenen geringer ist (Orlek et al. 2023). Diese Feststellung wird durch die Ergebnisse der hier vorliegenden Arbeit gestützt, da keine zusätzlichen Resistenzdeterminanten auf *mcr*-tragenden Plasmiden identifiziert werden konnten. Obwohl aufgrund von unterschiedlichen Virulenz- als auch Resistenzgenprofilen in *E. coli* des gleichen ST nicht von klonalen Linien gesprochen werden darf, ist es andererseits äußerst unwahrscheinlich, dass es sich um wiederholte Einträge von pathogenen *E. coli* mit denselben *mcr*-tragenden Plasmiden über die Jahre handelt. Dies hat bedeutende Konsequenzen für das Ausbruchsmanagement in einzelnen Betrieben, da wir zumindest den Verbleib von identischen *mcr*-tragenden Plasmiden über die Jahre nachweisen konnten. Das Einführen von verschärften Hygienemaßnahmen in betroffenen Betrieben könnte dazu beitragen, die Verbreitung von resistenzvermittelnden Plasmiden innerhalb eines Betriebes sowie deren längerfristigen Verbleib zu verhindern.

5.3 Limitationen und Stärken

Diese Dissertation hat als wissenschaftliche Arbeit einige Limitationen, die bereits in den Originaltexten von **Publikation 1**, **Publikation 2** und **Publikation 3** diskutiert wurden. Der retrospektive Untersuchungsansatz, die Beschränkung auf pathogene *E. coli* und die begrenzte Zahl von Ganzgenom-sequenzierten Isolaten sind Limitationen, die für alle drei Publikationen relevant sein könnten.

Die hohe Fallzahl, die Verfügbarkeit von Isolaten und Metadaten aus einem retrospektiven Zeitraum von mehr als 20 Jahren, die erstmalige Untersuchung der Verbreitung von *mcr*-Genen bei über 10 000 als intestinal pathogen definierten *E. coli*-Isolaten sowie die Vielzahl der Einsender*innen von Proben, die einen gewissen Grad an Repräsentanz gewähren, zählen zu den Stärken dieser Arbeit. Diese Aspekte werden detailliert in den jeweiligen Originalpublikationen diskutiert.

5.4 Ausblick

Schweine gelten als wichtige Träger mobiler Colistin-Resistenzdeterminanten. Die vorliegende Arbeit erhebt erstmals umfassende Daten zum Vorkommen aller bisher beschriebenen *mcr*-Gene in pathogenen *E. coli*-Isolaten, die von Schweinen aus Deutschland über einen Zeitraum von mehr als 20 Jahren gewonnen wurden. Sowohl die Differenzierung der *E. coli*-Pathovare als auch die exemplarische longitudinale Betrachtung der Verbreitung von *mcr*-Genen und Pathovaren in bestimmten Betrieben liefern hier wesentliche neue Erkenntnisse zur molekularen Epidemiologie von porcinen Colistin-resistenten *E. coli*. Im Rahmen eines EU-weiten Überwachungsprogrammes werden bereits ausgewählte Mikroorganismen, wie kommensale *E. coli* von gesunden Schlachtschweinen aus 31 Ländern alle zwei Jahre auf phänotypische Colistin-Resistenz untersucht. Eine Ausweitung der Beprobung sowie eine ergänzende Erhebung von innerbetrieblichen Datensätzen könnte dazu beitragen, mögliche Risikofaktoren für die Verbreitung von Colistin-resistenten Mikroorganismen frühzeitig zu erkennen und durch gezielte Maßnahmen eine mögliche Ausbreitung von Colistin-Resistenzdeterminanten zu verhindern. Eine diesbezügliche Ergänzung bereits bestehender nationaler und internationaler Systeme zur Überwachung von lebensmittelliefernden Tieren auf Resistenzdeterminanten wäre wünschenswert, um ausgewählte horizontal übertragbare Gene festzustellen, die Resistenzen gegen wichtige Reserveantibiotika vermitteln und ein systematisches longitudinales Screening zu gewährleisten. Dies wäre eine wesentliche Maßnahme, um die Gefährdung der Öffentlichkeit durch *mcr*-Gene besser einschätzen zu können. Dazu wäre die Erfassung von weiteren Datensätzen, insbesondere zur Haltung, Fütterung und Import/Export von Tieren, Antibiotikatherapie und Hygienemaßnahmen von Nutztierbetrieben wünschenswert, um auf Grundlage dieser Informationen wirksame Maßnahmen gegen die Verbreitung von *mcr*-positiven Isolaten innerhalb der Betriebe treffen zu können. Im Sinne des One-Health-Ansatzes wäre es erstrebenswert, eine zusätzliche, gezielte Beprobung von Abwasser und Gülle, die in der Literatur bereits als Reservoire von Antibiotikaresistenzgenen wie *mcr-1* in Nutztierbeständen beschrieben wurden, einzuführen. Die Gesamtsumme der Daten könnte als Basis für die Entwicklung betriebsspezifischer Strategien gegen die Austragung von Resistenzgenen aus Nutztierbetrieben verwendet werden.

6 Zusammenfassung

Colistin stellt in der Humanmedizin als Reserveantibiotikum eine der letzten Therapieoptionen bei der Behandlung von Infektionen mit multiresistenten Gram-negativen Bakterien dar. Die weltweite Ausbreitung von mobilen Colistin-Resistenzgenen (*mcr*), unter anderem in lebensmittelliefernden Tieren, gefährdet diese wichtige Rolle von Colistin und stellt eine erhebliche Bedrohung für die öffentliche Gesundheit dar. Bei Schweinen wurde das Vorkommen von *mcr*-Genen in kommensalen *E. coli* bereits in zahlreichen Studien untersucht. Dagegen ist zur Ausbreitung von *mcr*-Genen in *E. coli*-Pathovaren, die als Krankheitsauslöser beim Schwein eine Rolle spielen, bislang wenig bekannt. In dieser Arbeit wurden 10 573 porcine *E. coli*-Isolate, die von Juli 2000 bis Dezember 2021 von Schweinen aus Deutschland gewonnen wurden, mittels Multiplex-PCRs auf das Vorhandensein von Virulenz-assoziierten Genen (VAGs) sowie der Resistenzgene *mcr-1* bis *mcr-10* untersucht. Die Mehrzahl der Isolate konnte als eines von verschiedenen *E. coli*-Pathovaren identifiziert werden, die im Zusammenhang mit Krankheitsgeschehen bei Schweinen bekannt sind, wie enterotoxische *E. coli* (ETEC, 31,9 %), Ödemkrankheit auslösende *E. coli* (EDEC, 12,8 %), attaching and effacing *E. coli* (AEEC, 12,4 %) und Shigatoxin-bildende *E. coli* (STEC, 3,5 %). In 10,2 % der untersuchten Isolate konnte ein *mcr*-Gen nachgewiesen werden, wobei *mcr-1* (8,4 %) und *mcr-4* (1,6 %) am häufigsten vorkamen. Die Gene *mcr-2* (0,02 %), *mcr-3* (0,03 %) und *mcr-5* (0,3 %) konnten ebenfalls vereinzelt nachgewiesen werden, wohingegen die *mcr*-Gene *mcr-6* bis *mcr-10* in keinem der untersuchten Isolate identifizierbar waren. Die *mcr-1* Nachweisraten stiegen kontinuierlich bis zum Jahr 2015 (15,2 %) an und gingen im Jahr 2016 auf 11,4 % und im Jahr 2017 auf 6,3 % zurück. Enterotoxische und Shigatoxin-bildende *E. coli* (ETEC/STEC-Hybrid) sowie EDEC waren am häufigsten positiv für *mcr*-Gene (je 21,9 % und 17,6 %). Mittels Ganzgenomsequenzanalysen von 220 repräsentativen *mcr*-positiven *E. coli*-Isolaten konnten sowohl bereits zuvor nachgewiesene *mcr*-Varianten (*mcr-1.1*, *mcr-1.26*, *mcr-3.12*, *mcr-4.1*, *mcr-4.2*, *mcr-4.3*, *mcr-4.6* und *mcr-5.1*) sowie drei neue Varianten (*mcr-1.36*, *mcr-4.8* und *mcr-5.5*) identifiziert werden. Das am meisten verbreitete *mcr-1*-Gen konnte auf IncX4- (83 %), IncHI2- (13 %) und IncI2-Plasmiden (4 %) lokalisiert werden, während der Großteil der *mcr-4*-Gene (97,1 %) auf einem ColE10-Plasmid vorkam.

Neben der Untersuchung von *E. coli*-Isolaten aus Deutschland wurden ebenfalls 1936 pathogene Isolate aus 17 europäischen Ländern, die von 2010 bis 2020 isoliert wurden, auf das Vorkommen von *mcr-1* und *mcr-2* untersucht. Dabei konnte in einem Großteil der *E. coli*-Isolate aus Ungarn, Spanien, Italien und Portugal das *mcr-1*-Gen nachgewiesen werden (33,3 - 60,7 %). In zwei ETEC-Isolaten, die von zwei Schweinen aus einem Betrieb in Belgien gewonnen wurden, wurde eine bislang unbekannte *mcr-2*-Genvariante (*mcr-2.8*) gefunden. Neben der Lokalisierung von *mcr-2* auf IncX4-Plasmiden (n = 8) und dem Chromosom (n = 1), konnte ein bislang unbekanntes IncP-ähnliches Plasmid als Träger von *mcr-2.1* in drei Isolaten aus einem belgischen Betrieb identifiziert werden.

Unter Anwendung definierter Kriterien wurden retrospektiv drei Schweinebetriebe in Deutschland identifiziert, bei denen über mindestens vier aufeinander folgende Jahre *mcr*-positive *E. coli*-Isolate nachgewiesen wurden. Um Rückschlüsse auf den möglichen Verbleib von bestimmten *E. coli*-Pathovaren und *mcr*-tragenden Plasmiden oder einen wiederholten Eintrag unterschiedlicher *mcr*-positiver *E. coli*-Isolate ziehen zu können, wurden 87 repräsentative Isolate von insgesamt 154 vorliegenden *E. coli*-Isolaten Ganzgenomsequenziert und mittels bioinformatischer Methoden auf das Vorkommen von VAGs, Resistenzgenen und Plasmidtypen untersucht. Je 42 %, 30,8 % und 57,7 % dieser Isolate aus den Betrieben 1 bis 3 waren *mcr-1.1*-positiv und ein ETEC-ähnliches Isolat aus Betrieb 3 war positiv für *mcr-4.8*. Basierend auf der Bestimmung von Sequenztypen sowie Virulenzgen- als auch Resistenzgenprofilen konnten keine klonalen *E. coli*-Linien in den Betrieben festgestellt werden. *E. coli*-Isolate der Sequenztypen ST10 und ST131, die als *E. coli* mit zoonotischem Potenzial gelten, wurden in den drei untersuchten Betrieben als Träger von *mcr*-Genen identifiziert. Über mehrere Jahre hinweg konnten *mcr-1.1*-Gene auf identischen Plasmiden in Betrieb 1 (IncHI2), Betrieb 2 (IncX4) und Betrieb 3 (IncX4, IncI2) nachgewiesen werden.

Die vorliegende Arbeit betont die Relevanz der Weiterentwicklung von bestehenden Überwachungskonzepten, welche durch eine umfassende und kontinuierliche Datenerhebung in Nutztierbetrieben mögliche Risikofaktoren zum Eintrag, Verbleib und der Verbreitung von Colistin-resistenten Mikroorganismen identifizieren und mit gezielten Maßnahmen reduzieren können.

7 Summary

Colistin is a reserve antibiotic, representing one of the final therapeutic options in human medicine for the treatment of infections caused by multidrug-resistant Gram-negative bacteria. The global spread of mobile colistin resistance (*mcr*) genes, including in food-producing animals, endangers the crucial function of colistin and represents a substantial risk to public health. The presence of *mcr* genes in commensal porcine *E. coli* has been subject of numerous studies. In contrast, there is a paucity of knowledge regarding the distribution of these genes in pig-associated pathotypes of *E. coli*, which are responsible for diseases in pigs. A total of 10,573 porcine *E. coli* isolates obtained from pigs in Germany between July 2000 and December 2021 were analysed for the presence of virulence-associated genes (VAGs) and resistance genes *mcr-1* to *mcr-10* using multiplex PCR. The majority of isolates were identified as one of several *E. coli* pathovars that have been previously associated with disease in pigs. These included enterotoxigenic *E. coli* (ETEC, 31.9 %), edema disease *E. coli* (EDEC, 12.8 %), attaching and effacing *E. coli* (AEEC, 12.4 %), and shigatoxin-encoding *E. coli* (STEC, 3.5 %). The *mcr* gene was detected in 10.2 % of the isolates examined, with *mcr-1* (8.4 %) and *mcr-4* (1.6 %) being most frequently detected. Resistance genes *mcr-2* (0.02 %), *mcr-3* (0.03 %), and *mcr-5* (0.3 %) were also identified sporadically in isolates, in contrast to *mcr-6* to *mcr-10*, which were not detected. There was a steady increase in the *mcr-1* detection rates until 2015 (15.2 %), followed by a decline to 11.4 % in 2016 and 6.3 % in 2017. Enterotoxigenic and shigatoxin-encoding *E. coli* (ETEC/STEC hybrid) and EDEC were most frequently positive for *mcr* genes (21.9 % and 17.6 %, respectively). Whole genome sequencing of 220 representative *mcr*-positive *E. coli* strains revealed the presence of several known *mcr* variants, including *mcr-1.1*, *mcr-1.26*, *mcr-3.12*, *mcr-4.1*, *mcr-4.2*, *mcr-4.3*, *mcr-4.6,* and *mcr-5.1*, as well as three new *mcr* variants, *mcr-1.36*, *mcr-4.8,* and *mcr-5.5*. The most frequently identified *mcr-1* gene was found to be localized on plasmid types IncX4 (83 %), IncHI2 (13 %) and IncI2 (4 %), while the majority of *mcr-4* genes (97.1 %) were observed to be present on ColE10 plasmids.
In addition to inspected isolates from Germany, 1,936 porcine pathogenic *E. coli* strains from 17 European countries, which were isolated between 2010 and 2020, were also examined for the presence of *mcr-1* and *mcr-2*. The *mcr-1* gene was detected in the majority of *E. coli* strains from Hungary, Spain, Italy, and Portugal (33.3 - 60.7 %). A previously unknown *mcr-2* gene

variant (*mcr-2.8*) was detected in two ETEC isolates obtained from two pigs from a farm in Belgium. In addition to the localization of *mcr-2* on IncX4 plasmids (n = 8) and the chromosome (n = 1), a previously unknown IncP-like plasmid was identified as harbouring *mcr-2.1* in three isolates from one farm in Belgium.

Three pig farms in Germany were identified retrospectively on the basis of defined criteria where *mcr*-positive *E. coli* isolates had been detected for at least four consecutive years. To investigate whether the recurrent occurrence of certain *E. coli* pathotypes and plasmids in individual pig farms was due to the continuous presence or repeated reintroduction of *E. coli* strains, 87 representative isolates from a total of 154 *E. coli* were whole genome sequenced. The occurrence of VAGs, resistance genes, and plasmid types were analysed using bioinformatic methods. A high prevalence of 42 %, 30.8 %, and 57.7 % was observed for *mcr-1.1*-positive isolates obtained from farms 1, 2, and 3, respectively. Additionally, one ETEC-like isolate from farm 3 was identified as positive for *mcr-4.8*. Based on the determination of sequence types, virulence and resistance gene profiles, no clonal *E. coli* lineages were identified on any of the farms. The analysis revealed the presence of *E. coli* isolates of sequence types ST10 and ST131, which are considered to be *E. coli* with zoonotic potential, as carriers of *mcr* genes on the three farms under investigation. The *mcr-1.1* genes were identified on identical plasmids in farm 1 (IncHI2), farm 2 (IncX4) and farm 3 (IncX4, IncI2) over a period of several years.

This study highlights the need for further development of existing surveillance approaches that can identify potential risk factors for the introduction, maintenance and dissemination of colistin-resistant microorganisms through comprehensive and continuous data collection in livestock farms. This allows targeted measures to be taken in order to mitigate these risks.

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10 Selbstständigkeitserklärung

Ich erkläre: Ich habe die vorgelegte Dissertation selbständig und ohne unerlaubte fremde Hilfe und nur mit den Hilfen angefertigt, die ich in der Dissertation angegeben habe. Alle Textstellen, die wörtlich oder sinngemäß aus veröffentlichten oder nicht veröffentlichten Schriften entnommen sind, und alle Angaben, die auf mündlichen Auskünften beruhen, sind als solche kenntlich gemacht. Bei den von mir durchgeführten und in der Dissertation erwähnten Untersuchungen habe ich die Grundsätze guter wissenschaftlicher Praxis, wie sie in der "Satzung der Justus-Liebig-Universität Gießen zur Sicherung guter wissenschaftlicher Praxis" niedergelegt sind, eingehalten.

Lübeck, 02.01.2025 Lisa Göpel

11 Eigenanteil in den Publikationen

Der Eigenanteil ist den Publikationen direkt zu entnehmen.

12 Anhang

Volltexte Publikation 1, Publikation 2 und Publikation 3

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Occurrence of *mcr-1* and *mcr-2* colistin resistance genes in porcine *Escherichia coli* isolates (2010–2020) and genomic characterization of *mcr-2*-positive *E. coli*

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Introduction: The global emergence of plasmid-mediated colistin resistance is threatening the efficacy of colistin as one of the last treatment options against multi-drug resistant Gram-negative bacteria. To date, ten *mcr*-genes (*mcr-1* to *mcr-10*) were reported. While *mcr-1* has disseminated globally, the occurrence of *mcr-2* was reported scarcely.

Methods and results: We determined the occurrence of *mcr-1* and *mcr-2* genes among *Escherichia coli* isolates from swine and performed detailed genomic characterization of *mcr-2*-positive strains. In the years 2010-2017, 7,614 porcine *E. coli* isolates were obtained from fecal swine samples in Europe and isolates carrying at least one of the virulence associated genes predicting Shiga toxin producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC) or enteropathogenic *E. coli* (EPEC) were stored. 793 (10.4%) of these isolates carried the *mcr-1* gene. Of 1,477 additional *E. coli* isolates obtained from sheep blood agar containing 4 mg/L colistin between 2018 and 2020, 36 (2.4%) isolates were *mcr-1*-positive. In contrast to *mcr-1*, the *mcr-2* gene occurred at a very low frequency (0.13%) among the overall 9,091 isolates. Most *mcr-2*-positive isolates originated from Belgium (n = 9), one from Spain and two from Germany. They were obtained from six different farms and revealed multilocus sequence types ST10, ST29, ST93, ST100, ST3057 and ST5786. While the originally described *mcr-2.1* was predominant, we also detected a new *mcr-2* variant in two isolates from Belgium, which was termed *mcr-2.8*. MCR-2 isolates were mostly classified as ETEC or ETEC-like, while one isolate from Spain represented an atypical enteropathogenic *E. coli* (aEPEC; *eae*+). The ST29-aEPEC isolate carried *mcr-2* on the chromosome. Another eight isolates carried their *mcr-2* gene on IncX4 plasmids that resembled the pKP37-BE MCR-2 plasmid originally described in Belgium in 2015. Three

ST100 *E. coli* isolates from a single farm in Belgium carried the *mcr-2.1* gene on a 47-kb self-transmissible IncP type plasmid of a new IncP-1 clade.

Discussion: This is the first report of *mcr-2* genes in *E. coli* isolates from Germany. The detection of a new *mcr-2* allele and a novel plasmid backbone suggests the presence of so far undetected *mcr-2* variants and mobilizable vehicles.

KEYWORDS

mobile colistin resistance, *mcr-2*, *Escherichia coli*, swine, pathotype, plasmid, IncP, IncX4

Introduction

Antimicrobial resistance against colistin has emerged worldwide and poses a serious challenge to the treatment of diseases caused by multidrug resistant Gram-negative bacteria. The value of colistin as a last resort antimicrobial is compromised by the occurrence of mobile colistin resistance (*mcr*) genes (Schwarz and Johnson, 2016). After the first description of plasmid-encoded *mcr-1* from *Escherichia coli* and *Klebsiella pneumoniae* isolated from patients, food, and animals in China in 2015 (Liu et al., 2016), nine additional *mcr* genes (*mcr-2* to *mcr-10*) and their products were described in different Gram-negative bacterial species (Xavier et al., 2016; AbuOun et al., 2017; Borowiak et al., 2017; Carattoli et al., 2017; Yin et al., 2017; Wang et al., 2018; Yang et al., 2018; Carroll et al., 2019; Wang et al., 2020a).

Since the first report of plasmid-borne *mcr-1* gene, MCR-1-producing *Enterobacterales*, mostly *Escherichia coli*, have been described to occur with different frequencies in livestock, companion animals, wildlife, food and humans across the globe (Ewers et al., 2016; Falgenhauer et al., 2016; Irrgang et al., 2016; Schwarz and Johnson, 2016; Zhang et al., 2016; Unger et al., 2017; Nang et al., 2019). Much less is known about the occurrence of the other *mcr* genes, particularly about *mcr-2* in Europe. MCR-2 is a member of the MCR-family of bacterial phosphoethanolamine transferases and shares 80.6% amino acid identity with MCR-1. The *mcr-2* gene was discovered in some *E. coli* isolates from diarrheic pigs and calves in Belgium (Xavier et al., 2016). It was located on an IncX4 incompatibility-type plasmid (pKP37-BE) of 35,104 bp in size, and it was harbored by mobile insertion element IS*Ec69* which belongs to the IS*1595* insertion sequence family (Xavier et al., 2016). This plasmid was identified in two porcine ST10 *E. coli* isolates and in one bovine ST167 isolate in the original publication.

A similar plasmid has, to the best of our knowledge, only been reported in two further studies. Among 105 colistin-resistant *Salmonella* isolates collected from 2012 to 2015 in the national surveillance program in Belgium, Garcia-Graells and co-authors identified a *mcr-2*-carrying plasmid in a *Salmonella* Derby strain isolated from a pork carcass in 2012 that was almost identical to pKP37-BE (Garcia-Graells et al., 2018). Timmermans et al. (2021) detected *mcr-2* on a pKP37-like IncX4 plasmid in a colistin-resistant *mcr-2*-positive *E. coli* strain from a fattening pig, also from Belgium, in 2016 (Timmermans et al., 2021). Apart from the findings in Belgium, the *mcr-2* gene was only scarcely detected in other European countries, such as Spain, Great Britain and Italy (Dobrzanska et al., 2020; Miguela-Villoldo et al., 2020). In contrast, various studies from non-European countries, predominantly from Asia, confirmed the occurrence of *mcr-2* in Gram-negative bacteria not only from animal (Dutta et al., 2018; Zhang et al., 2018b; Ahmed et al., 2019; Rhouma et al., 2019; Zhang et al., 2019; Islam et al., 2020; Javed et al., 2020; Cilia et al., 2021; Ketkhao et al., 2021) but also from human sources (Zhang et al., 2018a; Ahmed et al., 2019; Mitra et al., 2020; Ara et al., 2021; Ejaz et al., 2021; Imtiaz et al., 2021; Stosic et al., 2021).

Soon after the discovery of *mcr-2*, Poirel et al. (2017) identified a novel allele of *mcr-2* on the chromosome of a *Moraxella pluranimalium* strain isolated from a pig in Spain. Based on molecular data, they proposed that this gene, termed *mcr-2.2*, was likely the progenitor of the *mcr-2* gene identified in Belgium, thereafter designated as *mcr-2.1*. As of 2nd July 2021, seven *mcr-2* alleles (*mcr-2.1* to *mcr-2.7*) are available in the Bacterial Antimicrobial Resistance Reference Gene Database.[1]

In veterinary medicine, colistin has been widely used for the control of neonatal and post-weaning diarrhea in pigs, mainly caused by enterotoxigenic *Escherichia coli* (ETEC) (Luppi et al., 2016; Rhouma et al., 2017). In our microbiological diagnostic laboratory, we receive a large number of samples from pigs for

Abbreviations: AMR, antimicrobial resistance; CARD, comprehensive antibiotic resistance database; CGE, center for genomic epidemiology; EPEC, enteropathogenic *E. coli*; aEPEC, atypical EPEC; ETEC, enterotoxigenic *E. coli*; MALDI-TOF MS, matrix-assisted laser desorption/ionization- time of flight mass spectrometry; MLST, multilocus sequence typing; NCBI, national center for biotechnology information; ST, sequence type; STEC, shiga toxin producing *E. coli*; VAG, virulence-associated gene; WGS, whole genome sequencing.

1 https://www.ncbi.nlm.nih.gov/pathogens/isolates#/refgene/

E. coli pathotyping and have created an extensive collection of putative pathogenic *E. coli* from pigs in Germany and other European countries in the last decade. In this study, we aimed to examine these *E. coli* isolates for the presence of *mcr-1* and *mcr-2* genes and to perform a detailed characterization of *mcr-2*-positive strains with respect to clonal lineage, *mcr-2* gene variant and location, plasmid types and pathotype of the bacterial host.

Materials and methods

Bacterial isolates

Escherichia coli isolates (n = 9,091) had been obtained from feces or mucosal swabs (rectum or small intestine) collected from pigs during routine microbiological diagnostics at our institute from 2010 through 2020. Samples were collected mainly in Germany (n = 7,155) and in 17 other European countries including the Netherlands (n = 888), Poland (n = 349), Denmark (n = 145), Switzerland (n = 141), Belgium (n = 131), Austria (n = 87), Hungary (n = 56), Italy (n = 42), Spain (n = 28), Portugal (n = 28), and seven other countries (n = 41) (**Table 1**). The majority of samples had been collected from piglets suffering from post weaning diarrhea or edema disease.

Porcine *E. coli* isolates were screened for *mcr-1* and *mcr-2* following two different approaches. In a first approach, we included 7,614 *E. coli* isolates that were archived in our institute's strain collection from 2010 to 2017. Only those isolates had been stored that proved positive by a modified multiplex polymerase chain reaction (PCR) for the gene of at least one of the following virulence factors: adhesive fimbriae F4, F5, F6, F18 and F41; intimin; heat-labile/stable *E. coli* enterotoxins LT-I, ST-I, ST-II; Shiga toxins of type Stx2 (Casey and Bosworth, 2009). These factors are related with enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), and shiga toxin producing *E. coli* (STEC). Cultivation of samples was performed on non-selective media, namely sheep blood agar without antibiotics. Basically, one isolate per pig was stored. The maximum number of samples per farm was limited to 6 pigs per submission. In case isolates from the same pig revealed different virulence gene profiles, one representative isolate of each profile was stored.

In a second approach, we screened 1,477 *E. coli* isolates. The isolates were obtained from fecal and mucosal samples from swine that were sent for routine PCR-based pathotyping to our diagnostic laboratory from May 2018 to December 2020. They were predominantly from Germany (n = 997) and from eight other European countries (**Table 1**). The isolates were streaked on sheep blood agar containing 4 mg/L colistin and were incubated at 37°C overnight. All isolates that showed growth, irrespective of the presence or absence of virulence-associated genes (VAGs), were stored in a glycerin stock at −80°C until further use.

Polymerase chain reaction screening for *mcr-1* and *mcr-2* genes and clonal grouping of *mcr-2*-positive isolates by pulsed-field gel electrophoresis

Escherichia coli isolates collected from 2010 to 2017 were separately cultured overnight in lysogeny broth. Broth cultures of 10 *E. coli* isolates at a time were pooled and screened for *mcr-1* and *mcr-2* using recently published primers and protocols (Liu et al., 2016; Xavier et al., 2016). In case pooled material gave a positive PCR result, single isolates were separately tested by the same PCRs. If pooled material was tested negative, all respective isolates were regarded as negative. *E. coli* isolated from colistin selective media were individually tested for *mcr-1* and *mcr-2* as described before. Prior to whole genome sequencing *mcr-2*-positive *E. coli* isolates were submitted to macrorestriction analysis (*Xba*I) to determine their clonal identity according to a previously published protocol (Ewers et al., 2014). *Xba*I-generated PFGE profiles were compared using BioNumerics software (Version 6.6, Applied Maths, Belgium) and cluster analysis of Dice similarity indices based on UPGMA.

Whole genome sequencing

DNA for whole genome sequencing was extracted from *E. coli* isolates using the DNA Blood & Tissue Kit according to the manufacturer's instruction (Qiagen, Hilden, Germany), followed by library preparation, using Nextera XT library (Illumina, San Diego, USA). DNA was sequenced using Illumina HiSeq 1500 with multiplexing of 70 samples per flow cell using 250 bp paired end reads with a coverage >90 x. Raw reads were adapter-trimmed by Flexbar v.3.0.3 (Resource Identification Portal RRID:SCR_013001), corrected using BayesHammer and assembled *de novo* using SPAdes v3.12.1 (RRID:SCR_000131). Assembled draft genomes were annotated using Prodigal (Prodigal, RRID: SCR_011936).

Antimicrobial susceptibility testing and resistance gene screening

Minimum inhibitory concentrations (MICs) of colistin were determined by the broth microdilution method according to EUCAST guidelines.[2] The isolates were further evaluated against 17 other antimicrobial agents by using the VITEK2 compact system (AST-GN38, AST-N248; bioMérieux, Nürtingen, Germany). Results were interpreted according to EUCAST break point tables (EUCAST, 2020) for tigecycline

2 http://www.eucast.org

and polymyxin B and according to CLSI guidelines (CLSI, 2018) for the remaining antimicrobial agents.

The web-based tool ResFinder 4.1,[3] hosted at the Center for Genomic Epidemiology (CGE), was used to identify resistance genes and chromosomal mutations related to β-lactam (*ampC* promoter mutation), fluoroquinolone (mutations in *gyrA*, *gyrB*, *parA*, and *parC*), and colistin resistance (*pmrAB*) based on WGS data (Zankari et al., 2012). Resistance gene screening was carried out by BLASTn (90% identity and 90% query coverage) analysis against homologous genes present in the Comprehensive Antibiotic Resistance Database.[4]

Genomic location of *mcr-2* and transconjugation assays

The genomic location of the *mcr-2* gene was verified by S1 nuclease digestion of genomic DNA followed by electrophoretic separation and Southern hybridization. A digoxigenin-labeled DNA probe ("DIG luminescent detection Kit", Boehringer Mannheim GmbH, Mannheim) targeting a 567-bp PCR fragment specific for the *mcr-2* gene using the aforementioned primers (Xavier et al., 2016) was used. In addition, we performed an *in silico* search of whole genome sequences with mlplasmids v. 1.0.0 (Arredondo-Alonso et al., 2018). To test whether the colistin resistance determinant was transferable, conjugation was performed by the broth filter mating method at 37°C using plasmid-free sodium azide resistant *E. coli* K12-J53 (J53 AziR) as recipient. Prior to the conjugation assays, all *mcr-2*-positive isolates were tested for their susceptibility to sodium azide. Transconjugants were selected on Endo agar plates containing 100 mg/L sodium azide and 2 mg/L colistin sulfate or containing 100 mg/L sodium azide and 4 mg/L colistin sulfate (Sigma-Aldrich, Germany, Karlsruhe, Germany). To confirm successful plasmid transfer, antimicrobial susceptibility testing of transconjugants, a PCR targeting the *mcr-2* gene and plasmid profiling was performed as described above.

Plasmid analysis

To characterize the identified *mcr-2* gene harboring contigs, all contigs were aligned using Geneious (v. 8.1.9, Biomatters Ltd., Auckland, New Zealand) (Geneious, RRID:SCR_010519) to the respective gene. Contigs containing *mcr-2* were aligned to publicly available plasmid sequences from GenBank (GenBank, RRID:SCR_002760) using BLASTn analysis (RRID:SCR_004870). All contigs of a respective isolate were then aligned to the reference plasmid that revealed highest similarity. In addition, contigs were mapped to the selected reference plasmids using the Geneious Map to Reference. *In silico* constructed plasmids were further examined for mobile genetic elements, using ISfinder (RRID:SCR_003020). To display circular comparisons between plasmids we used the blast ring image generator software BRIG Version 0.95

3 https://cge.food.dtu.dk/services/ResFinder/

4 https://card.mcmaster.ca

TABLE 1 Frequency of *mcr-1* and *mcr-2* in *E. coli* isolates collected from pigs in Germany and other European countries (2010 – 2020).

Country	2010–2017					2018–2020					
	E. coli isolates	*mcr-1*		*mcr-2*		*E. coli* isolates	Col non-S*	*mcr-1*			*mcr-2*
	n	n	%	n	%	n	n	n	% Col-non-S	% all	n
Germany	6,158	707	11.5	2	0.03	997	64	36	56.3	3.6	0
The Netherlands	757	3	0.4	0	0	131	0	0	0	0	0
Denmark	140	1	0.7	0	0	5	0	0	0	0	0
Switzerland	129	0	0	0	0	12	0	0	0	0	0
Belgium	113	11	9.7	9	8.0	18	0	0	0	0	0
Poland	102	9	8.8	0	0	247	0	0	0	0	0
Austria	73	0	0	0	0	14	0	0	0	0	0
Spain	28	16	57.1	1	3.6	0	0	0	0	0	0
Portugal	28	17	60.7	0	0	0	0	0	0	0	0
Italy	42	25	59.5	0	0	0	0	0	0	0	0
Hungary	12	4	33.3	0	0	44	0	0	0	0	0
Other countries**	32	0	0	0	0	9	0	0	0	0	0
Total	7,614	793	10.4	12	0.2	1,477	64	36	56.3	2.4	0

*Col non-S: *E. coli* isolates show growth on sheep blood agar containing 4 mg/L colistin.

**United Kingdom (13), Luxembourg (5), Norway (5), Ireland (4), Greek (3), Romania (2), and Slovenia (9).

(RRID:SCR_007802). Plasmid maps were generated using GenomeVx (Conant and Wolfe, 2008).

PlasmidFinder 2.1[5] was applied to determine plasmid replicons. In addition, the assignment of MCR-2 plasmids to an incompatibility group was carried out by phylogenetic analysis of the sequences of the replication initiator protein TrfA and of plasmid "backbone" gene proteins TrfB, KlcA, KleA, KorC, TraD, TraE, TraF, TraG, TraJ, TraK, TraL, TrbA, TrbB, TrbC, TrbD, TrbF, TrgB, TrbI, TrbJ, and TrbK (Smith et al., 1993; Luppi et al., 2016).

Phylogenetic comparison of *mcr-2* genes

To compare our *mcr-2* genes with currently known *mcr-2* genes, we screened the literature (PubMed, RRID:SCR_004846) and DNA sequence data repositories (GenBank, RRID:SCR_002760; EMBL, RRID:SCR_004473). Nucleotide and deduced amino acid sequences of *mcr-2* genes were downloaded from GenBank and aligned with Mafft v7.407 (RRID:SCR_011811). The resulting alignment was used to calculate a maximum likelihood-based phylogeny with RAxML v.8.2.10 (RRID:SCR_006086) with 100 bootstraps under the assumption of the gtr-gamma DNA substitution model (gamma BLOSUM62 protein substitution model).

Detection of serotype and virulence-associated genes

The sero(geno)types of *E. coli* strains were determined using the web-based serotyping tool SerotypeFinder 2.0[6] (Joensen et al., 2015). Screening for VAGs was carried out by NCBI BLASTn (RRID:SCR_004870) analysis against homologous genes present in an in-house database of 800 VAGs, gene variants or genomic islands from a subset of the VirulenceFinder database and in-house created and manually curated VAG reference sequences. We searched for genes that were previously linked with porcine intestinal pathogenic *E. coli* pathovars but also for VAGs of extraintestinal pathovars, such as uropathogenic *E. coli* (UPEC). Genes belonged to different categories (adhesin, toxin, iron uptake system, capsule synthesis, colicins, effector proteins, and secretion systems). Coverage length and sequence identity thresholds were 80% and 90%, respectively. *E. coli* isolates were further analyzed for the presence of the *fimH* gene and the allele type by aligning to a FimH database using FimTyper 1.0.[7]

Phylogenetic grouping, multilocus sequence typing and core genome analysis

Phylogenetic groups were determined by using the ClermonTyping method and its associated web-interface ClermonTyper, that allows a given strain sequence to be assigned to *E. albertii, E. fergusonii*, *Escherichia* clades I-V, *E. coli sensu stricto* as well as to the seven main *E. coli* phylogroups A, B1, C, E, D, F, and B2 (Clermont et al., 2013; Beghain et al., 2018). MLST 2.0[8] (Larsen et al., 2012) was applied to identify the multilocus sequence type (RRID:SCR_010245) of *E. coli* isolates following the Achtman scheme,[9] which represents a 7-gene-scheme including genes *adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*. Phylogenetic and population genetic relationships were determined by applying a gene-by-gene approach on the dataset to generate a core genome alignment and subsequently a phylogenetic tree. The core genome alignment was assembled by a gene-wise alignment (Mafft v7.407: RRID:SCR_011811) of 1,366 core genes that were present in at least 99% of the strains (sequence similarity min. 70%, sequence coverage min. 90%) and were concatenated afterward. The resulting alignment was used to infer a phylogeny with 100 bootstrap replicates using RAxML v.8.2.10 (RAxML, RRID:SCR_006086) with a General Time Reversible model and gamma correction for among site rate variation.

Results

Number and origin of *Escherichia coli* carrying *mcr-1* and *mcr-2* genes

Among the first collection of 7,614 *E. coli* isolates obtained from 2010 to 2017, 793 (10.4%) isolates were positive for *mcr-1* (**Table 1**). The rates of *mcr-1*-positive strains with respect to countries differed significantly, ranging from low (0–0.7%) (e.g., Switzerland, Austria, Netherlands, and Denmark) to moderate (8.8%–11.5%; Poland, Belgium and Germany) and high (33.3%–60.7%; Hungary, Spain, Italy and Portugal).

However, due to the biased sample material and the low number of *E. coli* isolates available from several countries, the data cannot be regarded as true prevalence data for most of the countries.

In Germany, where the highest number of samples was available, the rate of *mcr-1* among porcine *E. coli* isolates increased from 2010 (8.9%) to 2015 (15.2%), dropped slightly in the year 2016 (11.4%) and revealed the lowest percentage in 2017 (6.3%) (**Figure 1**, **Supplementary Table 1**).

5 https://cge.food.dtu.dk/services/PlasmidFinder/

6 https://cge.food.dtu.dk/services/SerotypeFinder/

7 https://cge.food.dtu.dk/services/FimTyper/

8 https://cge.food.dtu.dk/services/MLST/

9 https://pubmlst.org/mlst/

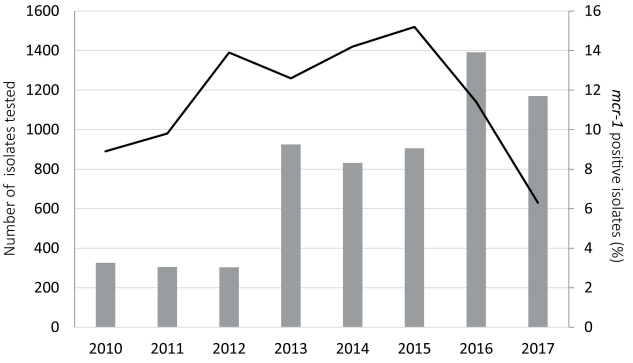

FIGURE 1
Annual rates of *mcr-1* (line) among 6,158 *E. coli* isolates (annual numbers as columns) collected from pigs in Germany from 2010 to 2017.

In contrast to *mcr-1*, the *mcr-2* gene was only rarely detected in the first collection of 7,614 isolates. Twelve isolates were positive for *mcr-2*, representing 0.2% of the total number of isolates tested (**Table 1**). The majority of *mcr-2*-positive isolates was obtained from Belgium (9 isolates/within country rate of 8.0%), followed by Germany (2/0.03%) and Spain (1/3.6%). The Belgian isolates were all isolated in 2015 and originated from three different swine farms (farms I-III) (**Table 2**). The Spanish isolate dates back to October 2013 (farm IV) and the two strains from Germany were obtained in August and December 2014 on two different farms (farms V and VI).

Among the 1,477 *E. coli* isolates that we pre-screened on sheep blood agar containing 4 mg/L colistin, 64 isolates (4.3%) showed growth and were regarded as colistin non-susceptible (Col-non-S) isolates. Based on PCR analysis, none of the 64 Col-non-S isolates possessed *mcr-2*, while 36 isolates, i.e., 56.3% of Col-non-S strains and 2.4% from all 1,477 isolates tested, harbored the *mcr-1* gene (**Table 1**). All *mcr-1*-positive isolates were from Germany, accounting to 3.6% among 997 isolates tested in the years 2018-2020.

Clonal relatedness of *mcr-2*-positive *Escherichia coli*

PFGE analyses separated the 12 *mcr-2*-positive *E. coli* isolates into eight different groups (A–H). Macrorestriction patterns revealed the clonality of three isolates from two pigs on farm I (pulsotype A) and of two isolates from two pigs from farm II (pulsotype B), respectively (data not shown). On farm II, *mcr-2*-positive isolates displayed different PFGE patterns (pulsotypes B and C), suggesting that they were not clonally related to those on farm I and III. Five different pulsotypes (A–E) were identified among the nine isolates from three farms in Belgium (n = 9). Isolates from Spain and Germany differed from Belgian isolates in their PFGE profiles (pulsotypes F–H). Among the total of eight PFGE profiles, seven different multilocus sequence types were determined (**Table 2**). We found ST100 in four isolates from Belgium collected on two different farms (farm I and III). On farm III, two ST10 isolates (IHIT323204 and IHIT32397) were additionally present. Sequence type ST5786, which is a single locus variant of ST10 (IHIT32302 and IHIT32399), and ST3057 (IHIT32303) were identified in isolates obtained from farm II. The isolate from Spain belonged to ST29 and the two German isolates were assigned to ST93 and a single locus variant of ST93 (ST11875) that differed in the *gyrB* allele (ST93: *gyrB*-6; ST11875: *gyrB*-405). With the exception of the ST29 isolate (phylogroup B1) and the ST3057 isolate (clade I), all *mcr-2*-positive isolates belonged to phylogenetic group A (**Table 2**).

Antimicrobial susceptibility and antimicrobial resistance genes

Minimum inhibitory concentration (MIC) data for the 12 *mcr-2*-positive *E. coli* isolates are provided in **Supplementary Table 2**. All but one repeatedly tested isolate (IHIT32302, MIC 0.5 mg/L) were resistant to colistin (MICs 4 mg/L–16 mg/L). Additional resistances were determined for ampicillin (100%), piperacillin (100%), tetracycline (91.7%), trimethoprim/sulfamethoxazole (91.7%), and chloramphenicol (33.3%) (**Table 3**). Only one isolate (ST100-IHIT32305, Belgium) was resistant to the aminoglycosides gentamicin and tobramycin, which correlated with the presence of aminoglycoside acetyltransferase gene *aac(3)-IV* in this strain (**Table 3**). None of the isolates showed resistance

TABLE 2 Characteristic features of *mcr-2*-positive *E. coli* isolates from pigs.

Strain	Month/Year of isolation	Country	Farm-pig-Isolate	MLST	cgMLST complex type	Phylo-group	Predicted serotype	Predicted pathotype	*mcr-2* gene	Plasmidome (>97.0% identity)**
IHIT31008*	02/2015	BE	I-1-1	ST100	12274	A	O138:H10	none	*mcr-2.1*	IncFII, IncFII(pSE11), IncI2, IncX1, IncX4
IHIT32395*	02/2015	BE	I-1-2	ST100	12274	A	O138:H10	ETEC	*mcr-2.1*	IncFIB(AP001918), IncFII, IncFII(pSE11), IncI2, IncX4
IHIT32396	02/2015	BE	I-2-1	ST100	12274	A	O138:H10	ETEC	*mcr-2.1*	IncFIB(AP001918), IncFII, IncFII(pSE11), IncI2, IncX4
IHIT32302	06/2015	BE	II-1-1	ST5786	3796	A	O182:H4	ETEC	*mcr-2.8*	IncFIB(AP001918), **IncX4**, IncFIA(HI1), IncHI1A, IncHI1B(R27), IncI1-I(Gamma), pO111
IHIT32399	06/2015	BE	II-2-1	ST5786	3796	A	O182:H4	ETEC	*mcr-2.8*	IncFIB(AP001918), **IncX4**, IncFIA(HI1), IncHI1A, IncHI1B(R27), IncI1-I(Gamma), pO111
IHIT32303*	06/2015	BE	II-3-1	ST3057	12275	clade I	O182:H4	ETEC-like	*mcr-2.1*	IncFIB(AP001918), **IncX4**, Col440II, IncFII, IncHI2, IncHI2A, IncY
IHIT32397	05/2015	BE	III-1-1	ST10	12276	A	O35:H6	ETEC	*mcr-2.1*	IncFIB(AP001918), **IncX4**, IncFII, IncI1-I(Gamma)
IHIT32305*	05/2015	BE	III-2-1	ST100	12277	A	O149:H10	ETEC	*mcr-2.1*	IncFIB(AP001918), **IncX4**, IncFIB(K), IncFIC(FII), IncFII(pSE11), IncI(Gamma), pO111
IHIT32304*	05/2015	BE	III-3-1	ST10	12276	A	O35:H6	ETEC-like	*mcr-2.1*	IncFIB(AP001918), **IncX4**, IncFII, IncI1-I(Gamma)
IHIT32403	10/2013	ES	IV-1-1	ST29	12280	B1	O45:H11	atypical EPEC	*mcr-2.1*	IncFIB(AP001918), Col(KPHS6), IncB/O/K/Z, IncFII, IncHI2, IndHI2A, IncX1
IHIT32401*	08/2014	DE	V-1-1	ST93	12278	A	O132:H25	ETEC-like	*mcr-2.1*	IncFIB(AP001918), **IncX4**, Col440II, IncFIA, IncHI2, IndHI2A, IncI1-I(Gamma)
IHIT32402*	12/2014	DE	VI-1-1	ST11875	12279	A	O132:Hnt	ETEC-like	*mcr-2.1*	IncFIB(AP001918), **IncX4**, IncFII, IncHI2, IncHI2A, IncY

BE, Belgium, DE, Germany, ES, Spain. *MCR-2 plasmids of these isolates could be successfully transferred to an *E. coli* K-12 recipient strain. **Plasmids that carry *mcr* genes are indicated with bold letters. The *mcr* gene of strain IHIT32403 is located on the chromosome; strains IHIT31008, IHIT32396, and IHIT32396 carry their *mcr* gene on an IncP-like plasmid.

to fluoroquinolone, third-generation cephalosporins or to carbapenems. The phenotypic antimicrobial resistance profile almost always corresponded with the presence of resistance genes in the respective isolates (**Table 3**).

Virulence genes and serotypes of *mcr-2*-positive *Escherichia coli* isolates

Based on the presence of VAGs obtained from WGS data we conducted a pathotype prediction of the *mcr-2*-positive *E. coli* isolates. Enterotoxigenic *E. coli* (ETEC) are characterized by the presence of genes for heat labile (*eltA, eltB*) and/or stable (*esta, estb*) *E. coli* enterotoxins and genes for adhesive fimbriae (F4, *fae*; F5, *fan*; F6, *fas*; F17, *f17*; F18, *fed*). With a percentage of 83.3%, most of our isolates were classified as ETEC or ETEC-like (**Table 2**), as they harbored *estb* and/or *eltA*, often in combination with genes for F4 or F18 fimbriae, respectively (**Supplementary Table 3**). The ST29 *E. coli* isolate IHIT32403 represented an atypical enteropathogenic *E. coli* (aEPEC) as it harbored intimin gene *eae* and lacked bundle-forming pili adhesin genes *bfpA-L*, which together with *eae* are indicative for typical EPEC. Only one isolate, namely ST100-IHIT31008, could not be assigned to a recognized *E. coli* intestinal or extraintestinal pathotype. Apart from enterotoxin and ETEC fimbriae genes, all isolates possessed a number of additional VAGs related to adhesion, tissue damage, iron acquisition, secretion and serum resistance. In particular aEPEC strain IHIT32403 from Spain revealed a number of additional genes that are known to play a significant role in the pathogenesis of EPEC-induced disease, including adhesin genes *cfa, efa1*, *lpf,* and *paa* as well as type III secretion system and effector protein genes (*esp, sep, lifA, nle, tccP, and tir*) (**Supplementary Table 3**).

TABLE 3 Antimicrobial susceptibility and resistance genes of *mcr-2*-positive *E. coli* isolates from pigs.

Strain	Phenotypic resistance	Antimicrobial resistance genes according to antibiotic classes*								
		BL	AMG	FQ	PMB	TET	FOL	PHE	ML	LIN
IHIT31008	AMP, PIP, TET, CHL, SXT, PMB, CST	bla_{TEM-1B}	*aadA1*, *aadA10*, *aph(6)-Id*, *aph(3")-Ib*	–	*mcr-2.1*	*tet*(B)	*sul1*, *dfrA1*, *dfrA14*	*catA1*	*mdf*(A)	*Inu*(G)
IHIT32395	AMP, PIP, TET, CHL, SXT, PMB, CST	bla_{TEM-1B}	*aadA10*, *aph(6)-Id*, *aph(3")-Ib*	–	*mcr-2.1*	*tet*(B)	*sul1*, *dfrA1*, *dfrA14*	*catA1*	*mdf*(A)	*Inu*(G)
IHIT32396	AMP, PIP, TET, CHL, SXT, PMB, CST	bla_{TEM-1B}	*aadA1*, *aadA10*, *aph(6)-Id*, *aph(3")-Ib*	–	*mcr-2.1*	*tet*(B)	*sul1*, *dfrA1*, *dfrA14*	*catA1*	*mdf*(A)	*Inu*(G)
IHIT32302	AMP, PIP, TET, SXT, PMB, CST	bla_{TEM-1B}	*aadA1*, *aadA5*, *aph(6)-Id*, *aph(3")-Ib*	GyrA S83L	*mcr-2.8*	*tet*(B)	*sul1*, *dfrA17*	–	*mdf*(A)	*Inu*(G)
IHIT32399	AMP, PIP, TET, SXT, PMB, CST	bla_{TEM-1B}	*aadA1*, *aadA5*, *aph(6)-Id*, *aph(3")-Ib*	GyrA S83L	*mcr-2.8*	*tet*(B)	*sul1*, *sul2*, *dfrA17*	–	*mdf*(A)	*Inu*(G)
IHIT32303	AMP, PIP, TET, PMB, CST	bla_{TEM-1B}	*aadA2*	–	*mcr-2.1*	*tet*(B)	*sul1*	–	*mdf*(A)	–
IHIT32397	AMP, PIP, TET, SXT, PMB, CST	bla_{TEM-1B}	*aadA1*, *aph(6)-Id*, *aph(3")-Ib*	–	*mcr-2.1*	*tet*(A)	*sul1 sul2*, *dfrA1*	–	*mdf*(A)	–
IHIT32305	AMP, PIP, GEN, TOB, CHL, SXT, PMB, CST	bla_{TEM-1C}	*aadA1*, *aadA2*, *aph(4)-Ia*, *aph(6)-Id*, *aph(3")-Ib*, *aac(3)-IV*	GyrA S83L, ParC S80R	*mcr-2.1*		*sul2*, *sul3*, *dfrA12*	*cmlA1*	*mdf*(A)	–
IHIT32304	AMP, PIP, TET, SXT, PMB, CST	bla_{TEM-1B}	*aadA1*, *aph(6)-Id*, *aph(3")-Ib*	–	*mcr-2.1*	*tet*(A)	*sul1*, *dfrA1*	–	*mdf*(A)	–
IHIT32403	AMP, PIP, TET, SXT, PMB, CST	bla_{TEM-1B}, bla_{OXA-4}	*aadA2*, *ant(2")Ia*, *aph*(3')-Ia, *aph*(3')-XV	–	*mcr-2.1*	*tet*(A), *tet*(M)	*sul1*, *sul2*, *sul3*, *dfrA1*, *dfrA12*	*cmlA1*, *catB3*	*mdf*(A), *mph*(E), *msr*(E)	–
IHIT32401	AMP, PIP, TET, SXT, PMB, CST	bla_{TEM-1A}	*aadA1*, *aph(6)-Id*, *aph(3")-Ib*	–	*mcr-2.1*	*tet*(B)	*sul1*, *sul2*, *dfrA1*	–	*mdf*(A), *mph*(B)	–
IHIT32402	AMP, PIP, TET, SXT, PMB, CST	bla_{TEM-1B}	*aadA1*, *aph(6)-Id*, *aph(3")-Ib*	–	*mcr-2.1*	*tet*(B)	*sul1*, *sul2*, *dfrA1*	–	*mdf*(A), *mph*(B)	–

*BL, beta-lactam, AMG, aminoglycoside, FQ, fluoroquinolone, PMB, polymyxin B, TET, tetracycline, FOL, folate pathway antagonist, PHE, phenicol, ML, macrolide, LIN, lincosamide.

Sero(geno)typing revealed the presence of serotypes O138:H10, O182:H4, O35:H6, O149:H10, and O132:H25/Hnt among ETEC and ETEC-like isolates and of serotype O45:H11 in the aEPEC isolate (**Table 2**).

Identification of a novel *mcr-2* variant

At the time of writing, seven valid *mcr-2* genes (*mcr-2.1* to *mcr-2.7*) have been recognized. Gene *mcr-2.1* was discovered in *E. coli* isolates from pigs and cattle in Belgium (Xavier et al., 2016) and *mcr-2.2* was detected in *Moraxella pluranimalium* strain 248-01T (CCUG 54913) isolated from a pig in Spain in 2001 (Poirel et al., 2017). Another five genes, available in GenBank, were confirmed as reference sequences for *mcr-2* genes after curation of records by the NCBI staff (see **Figure 2** for NCBI reference numbers). Nucleotide sequences obtained for the *mcr-2* genes (1,617 bp) of our *E. coli* isolates revealed that all but two isolates harbored *mcr-2.1* (**Table 2**). Two isolates from Belgium carried a novel variant of *mcr-2*, which had highest similarity (99.94%) to *mcr-2.1* from *E. coli* KP37-BE that harbored the originally described *mcr-2* gene (NG_051171). The novel *mcr-2* allele differed from *mcr-2.1* by encoding a single amino acid variation at position 390 (Met→Thr). Overall, *mcr-2.1* to *mcr-2.7* and our novel *mcr-2* allele had a similarity ranging from 94.19% (*mcr-2.4* vs. novel *mcr-2* from this study) to 99.94%. A comparison of the deduced amino acid sequences (538 aa) of *mcr-2* genes revealed similarity levels of 96.47% (MCR-2.4 vs. MCR-2.5) to 99.81% (MCR-2.1 vs. novel MCR-2 from this study). A maximum likelihood-based phylogeny of *mcr-2* gene alleles and MCR-2 proteins is provided in **Figure 2**.

Genomic location and transferability of *mcr-2* genes

We identified *mcr-2* on two different plasmids (n = 11 isolates) and on the chromosome of our *E. coli* strains (n = 1

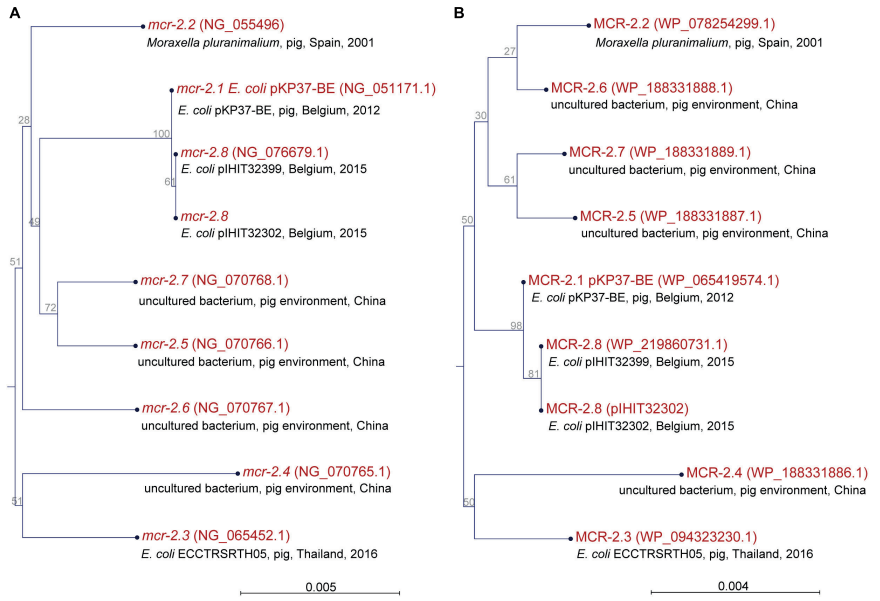

FIGURE 2
Phylogeny of seven confirmed and a novel *mcr-2* genes **(A)** and deduced MCR-2 proteins **(B)** based on a maximum-likelihood method.

isolate). This was initially confirmed by S1 nuclease PFGE and Southern blotting (Supplementary Figure 1). Based on BLASTn analysis of constructed plasmid sequences, the MCR-2 plasmids of six *E. coli* isolates from Belgium and of the two German isolates were found to be highly similar (>99.8%) to the *mcr-2*-bearing IncX4 plasmid pKP37-BE (GenBank LT598652.1). Like pKP37-BE, they were 35 kb in size, belonged to incompatibility group IncX4, and possessed *mcr-2* as the sole resistance gene. An overview of plasmid incompatibility groups and estimated plasmid sizes of all *mcr-2*-positive *E. coli* found in this study is given in Supplementary Table 4.

Three other isolates from Belgium revealed 47 kb MCR-2 plasmids (pIHIT31008-MCR-2, pIHIT32395-MCR-2, and pIHIT32396-MCR-2) that were identical in size and structure and also harbored *mcr-2* as the sole resistance gene (Table 2). The genetic organization of pIHIT31008-MCR-2 as a representative of these plasmids is shown in Supplementary Figure 2. BLASTn analysis of the pIHIT31008-MCR-2 nucleotide sequence against the GenBank database revealed that this plasmid shared conserved backbones with previously published plasmids of 41.4 to 46.5 kb in size that were identified in *E. coli*, *E. fergusonii* and *Salmonella* Enteritidis. Seven plasmids with highest query coverage (84.0%–88.0%) and query cover plasmid identity (96.95%–100%) to our plasmid are provided in Supplementary Table 5. They were identified in bacteria from food in China (*S. enterica*, pCFSA664-2), pooled sheep fecal samples in the UK (*E. coli*, pRHB15-C18_3; *E. fergusonii*, pRHB23-C01_5), pig feces in Canada (*E. coli*, plasmid unnamed_novel_0), and also from human patient samples collected in Australia (*S.* Enteritidis, pAUSMDU00010527), Japan (*E. coli*, pTHO-015-2), and the U.S.A. (*E. coli*, pYDC107_41) from 2008 to 2018. In contrast to our plasmid, the previous plasmids commonly lacked the *mcr-2*-containing cassette (IS*Ec69*-*mcr-2*-ORF-IS*Ec69*) and an insertion sequence element IS*91* that disrupted the type IV secretion system gene *virB4* genetic region on our 47 kb plasmids (Figure 3).

Figure 4A shows the integration site of the 3,489 bp *mcr-2* gene cassette into the common plasmid backbone region of the published plasmids. The *mcr-2* gene cassette of pIHIT31008-MCR-2 and of the two other 47 kb plasmids is almost identical (99.9%) to that described for pKP37-BE. Like in that IncX4 plasmid, the 1,617 bp *mcr-2* gene is flanked by directly oriented copies of insertion sequence element IS*Ec69* of the IS*1595* family (Partridge, 2017). We also identified a 297 bp open reading frame downstream of *mcr-2* on this element, which encodes a

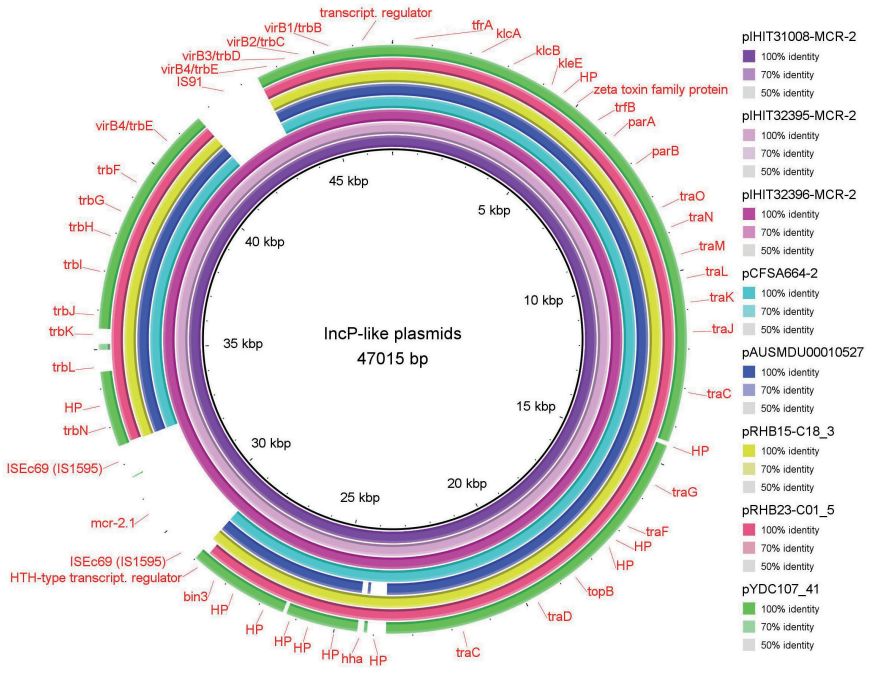

FIGURE 3
Circular representation of novel MCR-2 plasmids compared with five of the most similar reference plasmids available in GenBank (see **Supplementary Table 4**).

PAP2 membrane-associated lipid phosphatase with 41% identity to *Moraxella osloensis* phosphatidic acid phosphatase (71% query coverage).

The novel 47 kb MCR-2 plasmid was not predicted to have an Inc-type using PlasmidFinder. However, a BLASTn search of the IHIT31008 *trfA* gene, which encodes the initiation of plasmid replication, revealed four hits with a 100% nucleotide sequence identity to the IncP-1-like *trfA* replication genes of plasmids pAUSMDU00010527, pCFSA664-2, pRHB15-C18_3, and pRHB23-C01_5). In addition, the *trfA* genes of plasmids pYCD107, pTHO, and plasmid unnamednovel_0 showed 99.7% to 99.9% similarity to our genes (**Supplementary Table 5**).

A Neighbor-joining tree based on the alignment of TrfA amino acid sequences of representative members of the different IncP1 plasmid subfamilies α, β1, β2, δ, γ, ε, and ζ, our 47 kb MCR-2 plasmid and the aforementioned plasmids with identical backbones was created (**Supplementary Figure 3**). Interestingly, this showed that our MCR-2 plasmid and those with the shared backbone fall within the IncP1 family, but are phylogenetically different from the currently known IncP1 sub-families, indicating that they might represent a new subfamily. This clustering was also supported when performing a phylogeny inferred from concatenated sequences of the 22 plasmid backbone gene encoded proteins TrfA, TrfB, KfrA, KfrB, KlcA, KlcB, KorC, TraC, TraD, TraF, TraG, TraJ, TraK, TraL, TrbB, TrbC, TrbD, TrbF, TrbG, TrbI, TrbJ, and TrbK (data not shown) (Krol et al., 2012).

Seven out of eleven *mcr-2*-bearing plasmids from this study were successfully transferred to the recipient strain *E. coli* K12-J53. In case of strains IHIT32396 (47 kb IncP-1-like plasmid), IHIT32302, IHIT32399, and IHIT32397 (all IncX4 plasmids) conjugation experiments failed to transfer the pMCR-2 plasmid into the *E. coli* laboratory recipient. The seven pMCR-2 transformants showed a MIC of 4–8 mg/L for colistin, 16 to 32-fold higher than the empty J53 recipient (MIC = 0.25 mg/L).

The atypical EPEC isolate IHIT32403 carried its *mcr-2.1* gene on the chromosome. Attempts to transfer *mcr-2* by transconjugation failed, further confirming a chromosomal

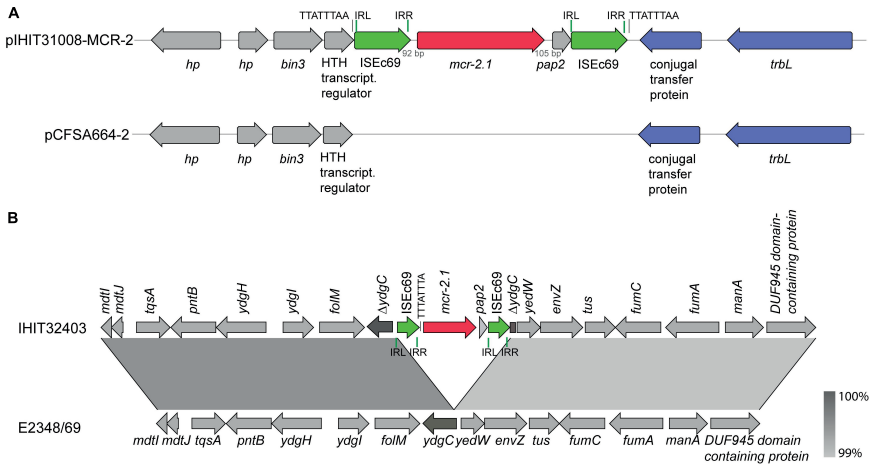

FIGURE 4
Genetic characterization of *mcr-2* genes. **(A)** Scheme of the integration site of the *mcr-2*-containing cassette into a plasmid backbone present in pCFSA664-2 (CP033354.2; *Salmonella enterica* ssp. *enterica* from food, China, 2015) and other plasmids, shown in **Figure 3**. **(B)** Linear comparison of a partial region (18,296 bp) of the genome of *mcr-2*-positive atypical EPEC strain IHIT32403 (diarrheic pig, Spain, 2013, ST29, O45:H11, phylogroup B1) and prototype EPEC strain E2348/69 (infantile diarrhea, UK, 1969, ST15, O45:H11, phylogroup B2; NZ_BDOY01.1). Boxed arrows represent the position and transcriptional direction of open reading frames. Insertion sequences are highlighted with green arrows, transfer genes with light blue arrows. Green vertical bars represent inverted regions (IR) at the left (IRL) and right site (IRR) of each IS element; black vertical bars represent 8-bp direct repeat sequences. Region of >99% identity are shaded in gray.

location of the gene. A linear comparison of a partial region (18,296 bp) of the genome of this ST29-B1 isolate of serotype O45:H11, that was obtained from a diarrheic pig in Spain in 2013, and of prototype O45:H11-ST15-B2 EPEC strain E2348/69, obtained from infantile diarrhea in Great Britain in 1969 (NZ_BDOY01.1) is shown in **Figure 4B**. The two regions were 100% identical, except for the insertion of gene cassette IS*Ec69-mcr-2-pap2*-IS*Ec69* into the inner membrane protein gene *ydgC* in isolate IHIT32403.

Discussion

The colistin resistance gene *mcr-1* gene is disseminated in many countries on all continents except Antarctica, and has been reported from various Gram-negative bacterial species from animals, human and the environment, such as *E. coli*, *Klebsiella* spp., *Salmonella enterica* serovars, *Enterobacter* spp. and *Moraxella* spp. (Nang et al., 2019; Anyanwu et al., 2021; Valiakos and Kapna, 2021). Our study revealed a prevalence of 11.5% *mcr-1*-positive isolates among pathogenic *E. coli* isolates collected from German pig farms in 2010-2017. A retrospective study on preselected bacterial cultures from asymptomatic fattening pigs, isolated in 2011 and 2012 in Germany, showed a similar prevalence of 9.9% (Roschanski et al., 2017). In a more recent study, Effelsberg et al. (2021) investigated the prevalence of mobile colistin resistance genes *mcr-1* to *mcr-5* in *Enterobacterales* from 81 pig farms in North-West Germany. In that study, the authors included fecal samples from pigs and stool samples from directly exposed humans working on the respective farms in 2018-2020. Two (1.4%) out of 138 stool samples from farmers, farm workers and their family members were tested positive for *mcr-1*, though a direct transmission could not be verified. The *mcr-1* gene was detected in 5.7% of the porcine samples (Effelsberg et al., 2021).

Considering the overall rate of *mcr-1* determined in the present study, including isolates from other European countries, prevalences were 10.4% in 2010-2017 and 2.4% in 2018-2020. From 2018 to 2020 we used selective culturing of bacteria on sheep blood agar containing colistin. Therefore, we cannot not exclude that some isolates carrying the *mcr-1* gene were missed. Thus, no conclusions on prevalence dynamics can be drawn from comparison of these two data, but noteworthy, annual rates of *mcr-1*-positive *E. coli* isolates started to decrease already in 2016. Similar results were reported from Spain. Miguela-Villoldo et al. described a steady increase of *mcr-1* in bacteria from cecal samples from pigs between 2004 and 2015 and a downward trend between 2017 and

2021 (Miguela-Villoldo et al., 2022). Another group from Spain screened 200 *E. coli* isolates collected in 1999 to 2018 from swine and reported a peak of colistin resistance (17.5% of the strains) in 2011–2014 (Aguirre et al., 2020). Our observation that *mcr-1* occurrence is much less frequent in samples collected after 2015 is in line with the studies reported.

It needs to be verified whether observed decreases are already a consequence of the restrictions recommended by the WHO for the use of important antimicrobials, including colistin (WHO, 2017). Indeed, an observational study from Great Britain revealed that the stoppage of colistin usage in pig farms could lead to a lower persistence of mobile colistin resistance genes on a long-term (Duggett et al., 2018). Based on data from longitudinal studies performed in China, authors could determine an association between significantly reduced colistin sales and a decreased frequency of *mcr-1*-producing *E. coli* in pig feces and in the intestinal tract of healthy humans as well as decreased rates of human infections with colistin resistant *E. coli* after 2017. It was suggested that this might be a direct consequence of banning colistin as an animal growth promoter by the Chinese government in April 2017 (Wang et al., 2020b; Zhao et al., 2022). On the other hand, no significant association could be found in a cross-sectional study on 48 pig farms in 2011-2012 between presence of *mcr-1* resistance genes in isolated *E. coli* and antimicrobial treatment of the sampled fattening pigs (Hille et al., 2018). In another study, healthy weaned piglets were inoculated with a colistin-resistant *E. coli* strain harboring *mcr-1*. Following this, one group of piglets was force-fed with colistin sulfate for five consecutive days. The double dose of colistin sulfate was given to a second group of piglets and a placebo group received water over the same period of time. A selection of *mcr*-positive strains was not observed, as the prevalence of the inoculated *mcr-1*-positive *E. coli* remained almost at the same levels in all groups over 25 days (Viel et al., 2018).

The *mcr-2* gene, which was detected soon after *mcr-1*, was globally found on rare occasions and predominantly in Asian countries, including Bangladesh, China, India, Pakistan, Thailand, and Turkey (Valiakos and Kapna, 2021). Nine out of 40 studies performed in Europe reported infrequent to moderate findings of *mcr-2* (0.15% – 11.4%) in samples, isolates or in the microbiota of livestock animals in Great Britain, Spain, Italy, and especially in Belgium in the years 2001 to 2019 (Xavier et al., 2016; AbuOun et al., 2017; Carattoli et al., 2017; Poirel et al., 2017; Garcia-Graells et al., 2018; Dobrzanska et al., 2020; Miguela-Villoldo et al., 2020; Bertelloni et al., 2022) (**Supplementary Table 6**). Although we tested 9,091 *E. coli* isolates from Germany and other European countries, only 12 isolates were *mcr-2*-positive. Out of these, nine isolates were obtained from three different pig farms in Belgium, which underlines the local spread of *mcr-2* in this country. Interestingly, the two *mcr-2*-positive *E. coli* isolates from two different pig farms in Germany, collected in 2014, are, to the best of our knowledge, the first reported *mcr-2* findings in this country. In two studies from 2011/2012 and 2011-2018 healthy animals from Germany, in particular pigs, were sampled and Gram-negative bacteria were tested for the presence of *mcr-1* to *mcr-2* and *mcr-1* to *mcr-9*, respectively. While 9.9% of the 436 tested mixed bacterial cultures and 45% of 407 tested *Salmonella enterica* isolates were *mcr-1*-positive, none of the cultures and isolates harbored *mcr-2* (Roschanski et al., 2017; Borowiak et al., 2020). In 2016 and 2017, raw municipal wastewater was sampled all over Germany and analyzed in a metagenomics approach. Genes *mcr-3*, *mcr-4*, *mcr-5*, and *mcr-7* were ubiquitous in all 14 samples, but only one proved positive for *mcr-1* and none for *mcr-2* (Kneis et al., 2019). Likewise, *mcr-2* was not detected in a very recent study performed on 456 samples obtained from pigs and humans in Germany, where all isolated *Enterobacterales* were tested for the genes *mcr-1* to *mcr-5* (Effelsberg et al., 2021). The systematic review by Valiakos et al. reported *mcr-2* in five studies from swine, three from bovine and three from poultry (Valiakos and Kapna, 2021). Total numbers of *mcr-2* were low, in particular in Europe and Africa. The vast majority of *mcr-2*-positive bacteria in that survey were *E. coli*. An exception was one *Moraxella pluranimalium*-like isolate harboring a *mcr-2.2* variant isolated from pooled cecal samples of healthy pigs (AbuOun et al., 2017). A more recent study from Egypt tested *Enterobacterales* isolates, collected between 2018 and 2020 from bovine milk samples associated with mastitis for the genes *mcr-1* to *mcr-9*. Among 117 tested isolates, 12.0% possessed *mcr-2* and another 28.2% carried *mcr-1*, *mcr-3*, *mcr-4* or *mcr-7* genes (Tartor et al., 2021).

In non-European countries *mcr-2* mediated colistin resistance was systematically tested in 64 studies (**Supplementary Table 6**). Twenty-five studies (38.5%) found bacteria harboring *mcr-2* resistance genes. Peak prevalences were as high as 56.3% in healthy pigs in 2016 and 14.9% in healthy chickens in 2015-2016 in China (Zhang et al., 2018b; Zhang et al., 2019).

Also wild animals, reptiles and pets have been identified as a source of *mcr-2*-positive bacteria. In 2018 and 2019, 26/168 (15.5%) *E. coli* isolates from hunted wild boar harbored *mcr-2* (Cilia et al., 2021). A study from Egypt identified *mcr-2* in 2.5% of 122 Gram-negative bacterial isolates from wild birds (Ahmed et al., 2019). In pet animals, the prevalence of *mcr-1* ranged from 0% to 12.5%, that of *mcr-2* from 0% to 0.9% (Borowiak et al., 2020; Ilbeigi et al., 2021; Wang et al., 2021; Bertelloni et al., 2022; Hamame et al., 2022).

In 2018, Partridge et al. (2018) proposed a nomenclature scheme for *mcr* genes and their variants, therefore systematizing future registration of new discoveries of genes and allele numbers (Partridge et al., 2018). Their scheme suggested pre-publication submission of new *mcr* sequences to the International Nucleotide Sequence Database Collaboration.[10]

10 https://www.insdc.org/

New *mcr* gene variants are defined by deduced amino acid sequence and assigned by the National Center for Biotechnology Information (NCBI). To date, a regularly updated overview of known *mcr* genes and variants is available in the Bacterial Antimicrobial Resistance Reference Gene Database, maintained by the NCBI.[11] So far, seven genetically different *mcr-2* variants are described in the literature (*mcr-2.1* – *mcr-2.7*). We here report an additional genetic variant, termed *mcr-2.8*, which is closely related to *mcr-2.1* and was found in *E. coli* isolates from pigs in Belgium. All other *mcr-2* genes in our samples were *mcr-2.1* (**Table 2**), which is also the most frequent variant worldwide.

Out of 12 *mcr-2*-positive bacterial isolates, ten could be typed as enterotoxigenic *E. coli* (ETEC) or ETEC-like and one as an atypical enteropathogenic *E. coli* (aEPEC). Another isolate from Belgium could not be grouped to any known *E. coli* pathotype as it lacked all taxonomical relevant combinations of virulence genes. ETEC are known for their frequent association with profuse neonatal and post-weaning diarrhea in pigs and calves (Foster and Smith, 2009; Liu et al., 2014). In an epidemiological study from 2018, ETEC was prevalent in 67% of post-weaning piglets suffering from diarrhea, followed by aEPEC (21.7%) (Garcia-Menino et al., 2018). In the same study, aEPEC was the most commonly detected pathovar (60.3%) among samples from diarrheic suckling piglets. The Global Burden of Disease study, performed between 1990 and 2016 in the USA, analyzed the impact of human diarrhea caused by ETEC on social and economic factors of health. At all age groups, ETEC was responsible for about 3.2% of deaths due to diarrhea in 2016 (Khalil et al., 2018). In humans, aEPEC are also regarded as important bacterial pathogens that are particularly involved in persistent diarrhea in children under five years of age (Afset et al., 2004; Mora et al., 2016; Snehaa et al., 2021). Unfortunately, the occurrence of *mcr* genes was not tested in the studies reporting on human ETEC and aEPEC isolates. Therefore, it remains to be determined whether *mcr*-mediated colistin resistance is relevant in *E. coli* strains causing diarrhea in humans.

Six *mcr-2* genes and the two novel *mcr-2* variants were found on IncX4 plasmids, which are known to play a vital role in the distribution of *mcr-1* genes among *Enterobacterales* (Doumith et al., 2016; Jamin et al., 2021; Abou Fayad et al., 2022; Xie et al., 2022). The IncX4 plasmids detected in our study showed high sequence similarity (>99.8%) to the originally described plasmid pKP37-BE from *E. coli* in 2011/2012 (Xavier et al., 2016). This is in line with previously reported occurrences of pKP37-BE-like plasmids, indicating a steady appearance of this plasmid in Belgium in association with *mcr-2* (Garcia-Graells et al., 2018; Timmermans et al., 2021). Our findings support the idea that IncX4 plasmids also play a role in the spread of *mcr-2* genes.

So far, the only plasmid groups identified in association with *mcr-2* were IncX4 and IncHI1B/IncFIB (Stosic et al., 2021). We found one new *mcr-2*-harboring plasmid (IncP-like plasmid), which was detected in three pigs on the same farm in Belgium. Plasmid transferability was observed in two of three *E. coli* isolates. This newly described plasmid represents to the best of our knowledge the third plasmid group beside IncX4 and IncHI1B/IncFIB harboring the *mcr-2* gene. The low frequency of *mcr-2*-IncP-like plasmids in our strain collection suggests that this plasmid type has rarely spread yet. However, only 9 of 39 studies mentioned in **Supplementary Table 6** explored the genomic location of *mcr-2* and characterized the plasmids in more detail (Xavier et al., 2016; AbuOun et al., 2017; Poirel et al., 2017; Garcia-Graells et al., 2018; Stosic et al., 2021; Tartor et al., 2021; Timmermans et al., 2021; Trongjit and Chuanchuen, 2021; Phuadraksa et al., 2022). Thus, occurrence of IncP-like plasmids carrying the *mcr-2* gene may be underestimated.

This study has some limitations. We have few information about pig housing conditions, hygiene standards, previous antimicrobial treatment and animal trafficking. Due to differing numbers of sample submissions from the different European countries throughout the years, the collected data are not representative of the target population. At the time of writing, ten different *mcr* genes (*mcr-1* to *mcr-10*) were reported. In this study, we concentrated on mobilizable colistin resistant genes *mcr-1* and *mcr-2*. Thus, future investigations on the distribution of the remaining *mcr* genes among the strain collection would be preferable. The chosen laboratory method since 2018, i.e., selective culturing of isolates on sheep blood agar containing 4 mg/L colistin, may have led to a fewer detection rate of *mcr*-positive isolates with low or diminished growth rates under these conditions (Smelikova et al., 2022).

On the other hand, our study has several strengths. We performed an extensive screening of plasmid-mediated colistin resistance genes (*mcr-1* and *mcr-2*) on a large pool of putative pathogenic *E. coli* collected from pigs over a longer period of time. Moreover, *mcr-2*-positive isolates and their plasmids underwent detailed genotyping, while also the transferability of *mcr-2* genes was explored.

Conclusion

We could confirm a continuous and substantial decrease in the percentage of porcine *E. coli* isolates harboring *mcr-1* from 2015 to 2017. As expected, *mcr-2*-mediated colistin resistance was much less frequent than that conferred by *mcr-1*. We here described the new *mcr-2.8* variant and a new *mcr-2.1*-bearing IncP1-like plasmid, which is capable of transferring colistin resistance to susceptible *E. coli* by conjugation. These novel variants and recent reports of *mcr-2*-positive bacteria in previously less frequently tested samples like bovine milk, pet animals and reptiles suggest that colistin

11 https://www.ncbi.nlm.nih.gov/pathogens/refgene/#mcr

resistance genes are highly variable and represent a constant threat to animal and human welfare. There is an urgent need for a longitudinal monitoring of *mcr* genes in Gram-negative bacteria from different sectors, including humans, animals, food products and the environment. This would ensure that the distribution of colistin resistant strains and putative novel resistance mechanisms are detected at an early stage. Gained knowledge about the distribution and epidemiology of *mcr* genes might help to prevent epidemic occurrence of multidrug resistant bacteria.

Data availability statement

Read genome sequences used in this study were submitted to the Sequence Read Archive (SRA) platform available from NCBI (https://trace.ncbi.nlm.nih.gov/Traces/sra) under biosample accession numbers: SAMN17614371 – SAMN17614382 and Sequence Read Run (SRR) numbers: SRR13570475 – SRR13570486.

Author contributions

CE and RB supervised the entire project. CE, LG, and RB drafted the manuscript. CE, KK, and RB designed the study. EP-B, CE, TS, and LG have provided raw data and/or analyzed the data and conducted part of the laboratory experiments. CE, TS, and LG performed analyses of sequencing data. All authors critically reviewed the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1076315/full#supplementary-material

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Supplementary Tables

Supplementary Table 1:
Annual rates of *mcr-1* and *mcr-2* among porcine *E. coli* isolates collected between 2010 and 2017

Supplementary Table 2:
MIC data for *mcr-2* positive *E. coli* isolates from pigs

Supplementary Table 3:
Virulence-associated genes (90% coverage, ≥ 90% identity) in *mcr-2* positive *E. coli* isolates and predicted pathotype

Supplementary Table 4:
Plasmid incompatibility groups and estimated plasmid sizes of *mcr-2* positive *E. coli* from pigs

Supplementary Table 5:
Characteristics of plasmids that share a common backbone to the novel 47-kb MCR-2 plasmid of porcine *E. coli* IHIT31008

Supplementary Table 6:
Occurrence of *mcr-2* and other *mcr* genes in samples from pigs and other animals, from animal meat, humans and the environment according to 104 publications. Only studies that included a screening for the *mcr-2* gene were considered.

Supplementary Table 1: Annual rates of *mcr-1* and *mcr-2* among porcine *E. coli* isolates collected between 2010 and 2017

Country	2010-2017				
	Isolates	*mcr-1*		*mcr-2*	
	n	n	%	n	%
All countries					
2010	392	29	7.4	0	0
2011	349	30	8.6	0	0
2012	346	42	12.1	0	0
2013	1,006	142	14.1	1	0.1
2014	1,179	134	11.4	2	0.2
2015	1,315	165	12.5	9	0.7
2016	1,612	165	10.2	0	0
2017	1,415	86	6.1	0	0
	7,614	**793**	**10.4**	**12**	**0.2**
Germany (total)					
2010	326	29	8.9	0	0
2011	305	30	9.8	0	0
2012	303	42	13.9	0	0
2013	925	117	12.6	0	0
2014	832	118	14.2	2	0.2
2015	906	138	15.2	0	0
2016	1,391	159	11.4	0	0
2017	1,170	74	6.3	0	0
	6,158	**707**	**11.5**	**2**	**0.03**
Northern Germany*					
2010	62	2	3.2	0	0
2011	39	2	5.1	0	0
2012	30	7	23.3	0	0
2013	216	17	7.9	0	0
2014	222	46	20.7	1	0.5
2015	233	29	12.4	0	0
2016	438	51	11.6	0	0
2017	260	19	7.3	0	0
	1,500	**173**	**11.5**	**1**	**0.07**
Western Germany*					
2010	136	24	17.6	0	0
2011	153	20	13.1	0	0
2012	105	6	5.7	0	0
2013	178	20	11.2	0	0
2014	194	27	13.9	1	0.5
2015	289	45	15.6	0	0
2016	387	45	11.6	0	0
2017	313	22	7.0	0	0

Country	2010-2017				
	Isolates	*mcr-1*		*mcr-2*	
	n	n	%	n	%
	1,755	**209**	**11.9**	**1**	**0.06**
Eastern Germany*					
2010	40	1	2.5	0	0
2011	27	6	22.2	0	0
2012	38	8	21.1	0	0
2013	64	6	9.4	0	0
2014	282	37	13.1	0	0
2015	149	42	28.2	0	0
2016	241	36	14.9	0	0
2017	253	23	9.1	0	0
	1,094	**159**	**14.5**	**0**	**0**
Southern Germany*					
2010	88	2	2.3	0	0
2011	86	2	2.3	0	0
2012	130	21	16.2	0	0
2013	467	74	15.8	0	0
2014	134	8	5.9	0	0
2015	235	22	9.4	0	0
2016	325	27	8.3	0	0
2017	344	10	2.9	0	0
	1,809	**166**	**9.2**	**0**	**0**

*Northern Germany: including Federal States Schleswig-Holstein, Hamburg, Lower Saxony and Bremen; Western Germany: including Federal States North Rhine-Westphalia, Hesse, Rhineland-Palatinate and Saarland; Eastern Germany: including Federal States Mecklenburg-Vorpommern, Berlin, Brandenburg, Saxony, Saxony-Anhalt and Thuringia; Southern Germany: including Federal States Bavaria and Baden-Württemberg

Supplementary Table 2: MIC data for *mcr-2* positive *E. coli* isolates from pigs

Strain-ID	AMP	AMC	PIP	LEX	CPD	CFT	CEP	IPM	AMK	GEN	TOB	ENR	MAR	TET	NIT	CHL	PMB	CST*	SXT
IHIT31008	≥ 32	4	≥ 128	8	≤ 0,25	≤ 1	≤ 1	≤ 1	≤ 2	≤ 1	≤ 1	≤ 0,12	≤ 0,5	≥ 16	64	≥ 64	8	4	≥ 3/20
IHIT32395	≥ 32	4	64	8	≤ 0,25	≤ 1	≤ 1	≤ 1	≤ 2	≤ 1	≤ 1	≤ 0,12	≤ 0,5	≥ 16	64	≥ 64	≥ 16	16	≥ 3/20
IHIT32396	≥ 32	4	≥ 128	8	≤ 0,25	≤ 1	≤ 1	≤ 1	≤ 2	≤ 1	≤ 1	≤ 0,12	≤ 0,5	≥ 16	64	≥ 64	≥ 16	16	≥ 3/20
IHIT32302	≥ 32	4	≥ 128	8	0,5	≤ 1	≤ 1	≤ 1	≤ 2	≤ 1	≤ 1	0,5	≤ 0,5	≥ 16	≤ 16	4	0,5	0,5	≥ 3/20
IHIT32399	≥ 32	4	≥ 128	8	0,5	≤ 1	≤ 1	≤ 1	≤ 2	≤ 1	≤ 1	1	1	≥ 16	≤ 16	8	8	4	≥ 3/20
IHIT32303	≥ 32	4	≥ 128	8	≤ 0,25	≤ 1	≤ 1	≤ 1	≤ 2	≤ 1	≤ 1	≤ 0,12	≤ 0,5	≥ 16	≤ 16	4	8	8	≤ 20
IHIT32397	≥ 32	4	≥ 128	8	≤ 0,25	≤ 1	≤ 1	≤ 1	≤ 2	≤ 1	≤ 1	≤ 0,12	≤ 0,5	≥ 16	32	≤ 2	8	4	≥ 3/20
IHIT32305	≥ 32	16	≥ 128	8	≤ 0,25	≤ 1	≤ 1	≤ 1	≤ 2	≥ 16	≥ 16	1	1	≤ 1	≤ 16	32	≥ 16	8	≥ 3/20
IHIT32304	≥ 32	4	64	8	≤ 0,25	≤ 1	≤ 1	≤ 1	≤ 2	≤ 1	≤ 1	≤ 0,12	≤ 0,5	≥ 16	32	≤ 2	8	4	≥ 3/20
IHIT32403	≥ 32	4	16	≤ 4	≤ 0,25	≤ 1	≤ 1	≤ 1	≤ 2	2	≤ 1	≤ 0,12	≤ 0,5	≥ 16	≤ 16	16	8	8	≥ 3/20
IHIT32401	≥ 32	4	≥ 128	≤ 4	≤ 0,25	≤ 1	≤ 1	≤ 1	≤ 2	≤ 1	≤ 1	≤ 0,12	≤ 0,5	≥ 16	≤ 16	≤ 2	4	4	≥ 3/20
IHIT32402	≥ 32	4	≥ 128	≤ 4	≤ 0,25	≤ 1	≤ 1	≤ 1	≤ 2	≤ 1	≤ 1	≤ 0,12	≤ 0,5	≥ 16	≤ 16	≤ 2	8	8	≥ 3/20

AMP = ampicillin, AMC = amoxicillin/clavulanic acid, PIP = piperacillin, LEX = cephalexin, CPD = cefpodoxim, CFT = ceftiofur, CEP = cefpirom, IPM = imipenem, AMK = amikacin, GEN = gentamicin, TOB = tobramycin, ENR = enrofloxacin, MAR = marbofloxacin, TET = tetracycline, NIT = nitrofurantoin, CHL = chloramphenicol, PMB = polymyxin B, CST = colistin, SXT = trimethoprim/sulfamethoxazole.

*MICs for colistin were determined using the Micronaut system (Merlin Diagnostika GmbH, Germany); all other antibiotics were tested by using the VITEK2 compact system (bioMérieux, Nürtingen, Germany).

Supplementary Table 3: Virulence-associated genes (90% coverage, ≥ 90% identity) in *mcr-2* positive *E. coli* isolates and predicted pathotype

Strain	Virulence associated genes and categories*				Predicted pathotype
	Adhesion	**Toxin, hemolysin**	**Iron acquisition**	**Colicins, serum resistance, secretion and effector proteins**	
IHIT31008	*ecpA-D, faeCDEFH, fdeC, hra, matB*	*astA, hlyA-D*	*entBCDEFS, fepABCD, fes, fyuA, iha, irp2, ybt*	*capU, cia, espL1, espX1, espX4, espX5, ompA, traT*	none
IHIT32395	*ecpA-D, faeCDEFH, fdeC, hra, matB, paa*	*astA, eltA, estb, hlyA-D*	*entBCDEFS, fepABCD, fes, fyuA, iha, irp1, irp2, ybt*	*capU, cia, espL1, espX1, espX4, espX5, ompA, traT*	ETEC
IHIT32396	*ecpA-D, faeCDEFH, hra, matB*	*astA, eltA, estb, hlyA-D*	*entBCEFS, fepABCD, fes, fyuA, iha, irp1, irp2, ybt*	*capU, cia, espL1, espX1, espX5, traT*	ETEC
IHIT32302	*ecpA-D, fedA, fedF, fimBCDEFHI* (*fimH24*), *matB*	*astA, eltA, estb, hlyA-D*	*chuASUWY, entBC, fepAD, fes, fyuA, iha, irp1, irp2, ybt*	*cba, cea, cib, cma, espX2, iss, ompA, ompT, traT*	ETEC
IHIT32399	*ecpA-D, fedA, fedF, fimA-I* (*fimH24*), *matB*	*astA, eltA, estb, hlyA-D*	*entBCDEFS, fepABDCG, fes,iha*	*cba, cea, cib, cma, espL1, espL4, espX1, espX4, espX5, espY1, iss, ompA, ompT, traT*	ETEC
IHIT32303	*ecpA-D, fed, fimBCDEFHI* (*fimH445*), *matB*	*astA, estb*	*chuASUWY, entBC, fepAD, fes, iha, irp1, irp2, fyuA, ybt*	*air, cea, eilA, espX2, traT*	ETEC
IHIT32397	*ecpA-D, fimB-I* (*fimH45*), *hra, matB*	*astA, estb*	*entBCDEFS, fepABCD, fes*	*cib, espL1, espL4, espX5, traJ*	ETEC-like**
IHIT32305	*ecpA-D, faeCDEFHJ, matB*	*astA, eltA, estb, hlyA-D*	*entBCEFS, fepABCD, fes, iha*	*cib, espL1, espX1, espX5, traJ*	ETEC
IHIT32304	*ecpA-D, fimB-I* (*fimH45*), *hra, matB*	*astA, estb*	*entBCDEF, fepABC, fes*	*cib, espL1, espL4, espX5, traJ*	ETEC-like**
IHIT32403	*aidA, aah, cfa, csgB, eae, ecpB-D, efa1, fed, fimB-I* (*fimH24*), *hra, lpf, paa, perABC, tsh*	*astA*	*entBCEFS, fepABCD, fes, iha*	*cma, cif, escCDEFGIJL, escN-V, espL2, espM1, espW, espX5, iss, katP, lifA, nleA-F, nleH, ompA, ompT, malX, sep, tccP, tir*	atypical EPEC
IHIT32401	*aah, fim* (*fimH25-like*), *hra*	*astA, estb*	*entBCDEFS, fepABCD, fes*	*cib, espL1, espX1, espX5, kpsD, kpsE, kpsM, kpsMII, ompA, traJ, traT*	ETEC-like**
IHIT32402	*aah, ecpA-D, fdeC, fimB-I* (*fimH27*), *matB*	*astA, estb*	*entBCDEFS, fepABCD, fes*	*espL1, espX1, espX5, iss, kpsD, kpsE, ompA, ompT*	ETEC-like**

The data result from a search of genome sequences by ResFinder 4.1 (https://cge.food.dtu.dk/services/ResFinder/) and BacWGSTdb (http://bacdb.cn/BacWGSTdb/Tools.php).

***Fimbrial/adhesin genes:** AIDA-I (adhesin involved in diffuse adherence), *aah* (AIDA-associated heptosyltransferase), *cfa* (CFA/I fimbriae), *csg* (curli fimbriae), *eae* (intimin), *ecp* (*E. coli* common pilus), *efa1* (EHEC factor for adherence), *fae* (F4 fimbriae), *fdeC* (factor adherence *E. coli*), *fed* (F18 fimbriae), *fim* (type I fimbriae), *hra* (heat-resistant hemagglutinin), *lpfA* (long polar fimbriae), *paa* (porcine attaching-effacing associated protein), *perABC* (regulatory operon bundle-forming pili), *tsh* (temperature-sensitive hemagglutinin).

Toxin/hemolysin genes: *astA* (enteroaggregative *E. coli* heat-stable enterotoxin 1), *eltA* (*E. coli* heat-labile enterotoxin A), *estb* (*E. coli* heat-labile enterotoxin B), *hly* (hemolysin)

Iron acquisition: *chuA* (hemin receptor), *ent* (enterobactin), *fep* (ferrienterobactin), *fes* (ferric enterobactin esterase), *fyuA* (yersiniabactin receptor), *iha* (enterobactin receptor), *irp* (iron repressible proteins), *ybt* (yersiniabactin).

Colicins, serum resistance, secretion and effector proteins: *capU* (hexosyltransferase homologue), *cba* (colicin-B), *cea* (colicin-E1), *cia* (colicin-Ia), *cib* (colicin-Ib), *cif* (cycling inhibiting factor, cyclomodulin), *cma* (colicin-M), *esp* (*E. coli* secreted proteins), *iss* (increased serum survival protein), *katP* (EHEC catalase peroxidase), kps (capsule biosynthesis), *lifA* (lymphostatin), *nle* (non-LEE encoded effector proteins), *ompA/T* (outer membrane proteins), *malX* (PAI marker, phosphotransferase enzyme), *sep* (secreted *E. coli* proteins), *tccP* (Tir-cytoskeleton coupling protein), *tir* (translocated intimin receptor), *traJ* (regulator transfer proteins), *traT* (complement resistance protein).

**The term "ETEC-like" was used for isolates that encoded for heat-labile and/or heat-stable toxins but lacked genes of ETEC-typical adhesive fimbriae.

Supplementary Table 4: Plasmid incompatibility groups and estimated plasmid sizes of *mcr-2* positive *E. coli* from pigs

Strain	Month / year of isolation	Country	Farm-Pig-Isolate	MLST	Plasmid incompatibility groups*	Estimated plasmid sizes (kb, S1-nuclease restriction)
IHIT31008	02/2015	BE	I-1-1	ST100	IncFII, IncI2, IncX1-like, IncX4	< 20, 47, 65, 150
IHIT32395	02/2015	BE	I-1-2	ST100	IncFIB-like, IncFII, IncFII(pSE11)-like, IncI2, IncX4	< 20, 47, 65, 170
IHIT32396	02/2015	BE	I-2-1	ST100	IncFIB-like, IncFII, IncFII(pSE11)-like, IncI2, IncX4	< 20, 47, 65, 170
IHIT32302	06/2015	BE	II-1-1	ST5786	IncFIA, IncFIB-like, IncHI1A-like, IncHI1B, IncI1-I(alpha), IncX4, p0111-like	< 20, 35, 110, 125, 220
IHIT32399	06/2015	BE	II-2-1	ST5786	IncFIA, IncFIB-like, IncHI1A-like, IncHI1B, IncI1-I(alpha), IncX4, p0111-like	< 20, 35, 110, 125, 220
IHIT32303	06/2015	BE	II-3-1	ST3057	IncFII, IncHI2, IncHI2A, IncX4, IncY-like	35, 95, 125, 270
IHIT32397	05/2015	BE	III-1-1	ST10	IncFII, IncI1-I(alpha), IncQ1-like, IncX4	< 20, 35, 110, 125
IHIT32305	05/2015	BE	III-2-1	ST100	IncB/O/K/Z-like, IncFIB-like, IncFIC-like, IncFII-like, IncI-like, IncX4, p0111-like	< 20, 35, 60, 85, 95, 170
IHIT32304	05/2015	BE	III-3-1	ST10	IncFII, IncI1-I(alpha), IncQ1-like, IncX4	< 20, 35, 110, 125
IHIT32403	10/2013	ES	IV-1-1	ST29	IncFIA-like, IncHI2, IncHI2A-like, IncI1-I, IncQ1-like, IncX4	35, 110, 140, 250
IHIT32401	08/2014	DE	V-1-1	ST93	IncFII, InHI2, IncHI2A-like, IncQ1-like, IncX4, IncY-like	< 20, 35, 95, 150, 250
IHIT32402	12/2014	DE	VI-1-1	ST11875	IncFII, InHI2, IncHI2A-like, IncQ1-like, IncX4, IncFIA, IncI1-I	35, 85, 95, 270

BE = Belgium, DE = Germany, ES = Spain; *threshold for minimum identity: 98%, minimum coverage range: 60%

Supplementary Table 5: Characteristics of plasmids that share a common backbone to the novel 47-kb MCR-2 plasmid of porcine *E. coli* IHIT31008

Name[a]	GenBank	Size	Bacterial host, serovar and sequence type (ST), phylogenetic group	Host/Source	Country	Year	Plasmid Inc-type[b]	Query cover plasmid/ identity	Identity entire plasmid
pCFSA664-2	CP033354.2	41,696	*S.* Enteritidis, ST17	Food	China	2015	ND	88.0%/100%	88.7%
pAUSMDU00010527	CP045957.1	41,464	*S.* Enteritidis, ST3304	Human	Australia	2017	ND	87.0%/99.91%	87.6%
pRHB15-C18_3	CP057780.1	41,696	*E. coli*, ST8185, B1	Pooled sheep faecal sample (floor)	UK	2017	ND	88.0%/99.99%	88.7%
pRHB23-C01_5	CP057569.1	41,486	*E. fergusonii*	Pooled sheep faecal sample (floor)	UK	2017	ND	88.0%/99.90%	88.1%
pYDC107_41	CP025711.1	41,544	*E. coli* O102:H6, ST964, D	Human drainage	USA	2008	ND	84.0%/97.63%	84.6%
Res13-Lact-PEA12-26 plasmid_unnamed_novel_0	CP062878.1	46,158	*E. coli* O157:H16, ST5502, A	Pig feces	Canada	2017	ND	85.0%/99.67%	77.6%
pTHO-015-2	AP022551.1	46,459	*E. coli* O25:H4, ST131, B2	Human urine	Japan	2018	ND	85.0%/96.95%	79.9%

[a] By using ResFinder 4.1 (https://cge.food.dtu.dk/services/ResFinder/), antimicrobial resistance genes were only detected in plasmid pTHO-015-2 ($bla_{CTX\text{-}M\text{-}14}$).

[b] ND indicates that the plasmid incompatibility typing, using PlasmidFinder (https://cge.food.dtu.dk/services/PlasmidFinder/), gave no result.

Supplementary Table 6: Occurrence of *mcr-2* and other *mcr* genes in samples from pigs and other animals, from animal meat, humans and the environment according to 104 publications. Only studies that included a screening for the *mcr-2* gene were considered.

Country[a]	Year of isolation	Source (no.) of samples[b]	Bacterial species[c], no. of isolates (no. of ColR isolates) [d]	*mcr-1* % (no. of positive isolates per no. of tested isolates)	*mcr-2* % (no. of positive isolates per no. of tested isolates)	*mcr-3 – mcr-10* no. of pos. isolates[e]	REF
EUROPE							
BEL	2011-2012	Passive surveillance diarrhoea • **P (53)** • CA (52)	Ec: 105 ColR	12.4% (13/105) P: 13.2% (7/53) CA: 11.5% (6/52)	11.4% (12/105) P: 20.8% (11/53) CA: 1.9% (1/52)	n.t.	[1]
BEL	2012-2015	• Food chain – POUL, **pork**, others (National surveillance program)	Sal: 1,415 (105 ColR)	1.9% 2/105 [pork carcass, 2012]	0.95% (1/105) [pork carcass, S. Derby, ST40, pKP37-BE-like IncX4, 2012]	n.t.	[2]
BEL	2012-2016	• **P – healthy, fattening farm (4)** • CA – healthy veal calves (32) • POUL - healthy (1) • CA meat (3)	Ec: 40 ColR	77.5% (31/40) [1 *mcr-1* & *mcr-3* & *mcr-5*]	2,5% (1/40) [P, 2016, pKP37-like IncX4]	*mcr-3* (1) *mcr-4* (8) *mcr-5* (1) [1 *mcr-1* & *mcr-3* & *mcr-5*] *mcr-6 – mcr-10* n.t.	[3]
CHE	2008-2018	• H - clinical	Ent: 97 (including 4 reference samples)	3.2% (3/93)	0% (0/93)	n.t.	[4]
CHE	2016	Healthy animals at slaughter, caecal • **P (325)** • CA (241) • POUL (100)	Ent (Hal, Ec, Ecl, Kp) P: 4% ColR (13/325) CA: 3.3% (8/241) POUL: 0% (0/100)	0% (0/21)	0% (0/21)	n.t.	[5]
CHE	2016	• H – UTI, urine	Ent: 2,049 (6 ColR)	0.05% (1/2,049)	0% (0/6)	n.t.	[6]
CHE	2016	• H – healthy, stool (1,091) • H – primary care patients, stool (53)	62 Ent selective plates (4 mg/L Col) (18 ColR - Hal: 9, Ec: 3, Ecl: 4, Kp: 1, Rorn: 1)	0% (0/62)	0% (0/62)	n.t.	[7]
CZE	2018-2019	• H – hospitalised, rectal swabs and stool samples	enriched cultures of 1,922 samples (qPCR)	0.21% (4/1,922)	0% (0/1,922)	*mcr-3* to *mcr-8* (0) *mcr-9, -10* n.t.	[8]
DEU	2011-2012	• **P – healthy, 58 fattening farms**; boot swabs, pooled fecal samples	various GN species: 436	9.9% samples; 25.9% farms	0% (0/436)	n.t.	[9]
DEU	2011-2018	• Healthy animals (**P**, POUL, CA, Pet), food (POUL meat, **pork**)	Sal: 407 ColR	45% (183/407) [8 *mcr-1* & *mcr-9*]	0% (0/407)	*mcr-3* (0) *mcr-4* (53) *mcr-5* (18) *mcr-6, -7, -8* (0) *mcr-9* (8) [8 *mcr-1* & *mcr-9*]	[10]

Country[a]	Year of isolation	Source (no.) of samples[b]	Bacterial species[c], no. of isolates (no. of ColR isolates) [d]	*mcr-1* % (no. of positive isolates per no. of tested isolates)	*mcr-2* % (no. of positive isolates per no. of tested isolates)	*mcr-3 – mcr-10* no. of pos. isolates[e]	REF
						mcr-10 n.t.	
DEU	2016-2017	• Municipal water - metagenome analysis	14 samples	71.4% of samples (1/14)	0% of samples (0/14)	*mcr-3, -4, -5, -7* ubiquitous in all samples *mcr-2, -6, -8, -9* (0) *mcr-10* n.t.	[11]
DNK	2009-2017	• H - clinical	Sal: ca. 2,500 genomes	0.04% (1/2500) [1 *mcr-1* & *mcr-3*]	0% (0/2,500)	*mcr-3* (10) [1 *mcr-1* & *mcr-3*] *mcr-4 – mcr-10* n.t.	[12]
DNK	2014-2017	• H – blood stream infection	Ec ESBL/AmpC: 872 genomes CPOs: 317 genomes	0.08% (1/1,183)	0% (0/1,183)	*mcr-3* (1) *mcr-4 – mcr-10* n.t.	[13]
ESP	2005-2014	• **P – PWD diagnostic samples** • White stork – fecal droppings	P – Ec: 70 (10/year) White stork – Ec: 20	P: 20.0% (14/70) White stork: 25.0% (5/20)	0% (0/90)	*mcr-4* (1) *mcr-3, -5* (0) *mcr-6 – mcr-10* n.t.	[14]
ESP	2006-2016	• **P – enteric colibacillosis**	Ec: 35 *mcr*-positive (35/499 investigated isolates)	3.8% (19/499)	0% (0/499)	*mcr-3* (0) *mcr-4* (18) *mcr-5* (2) *mcr-6 – mcr-10* n.t.	[15]
ESP	2006-2017	• **P – PWD**	Ec: 186 ETEC & STEC (126 ColR)	29.4% (37/126) [1 *mcr-1* & *mcr-4*, 1 *mcr-1* & *mcr-5*]	0% (0/126)	*mcr-3* (0) *mcr-4* (102/126) [1 *mcr-1* & *mcr-4*] *mcr-5* (5/126) [1 *mcr-1* & *mcr-5*] *mcr-6 –mcr-10* n.t.	[16]
ESP	2001	• **P –** healthy, nasal turbinate	*Moraxella pluranimalium* sp. nov.: 1 isolate	-	positive	*mcr-3 – mcr-10* n.t.	[17, 18]
ESP	2015	• CA – healthy, < 1 year, slaughterhouse, caecal content (636 animals, 318 farms)	GN: 152 potential ESBL/AmpC (6 ColR)	100% (6/6) [1 *mcr-1* & *mcr-3*]	0% (0/6)	*mcr-3* (1) [1 *mcr-1* & *mcr-3*] *mcr-4 – mcr-10* n.t.	[19]
ESP	2018	• **P – slaughterhouses, caecal content (272)**	GN: 486 (249 Ec)	54,1% (263/486)	0.2% (1/486) [Ec, O83:H42, ST648, 2018]	n.t.	[20]
EUR	2002-2014	Healthy animals at slaughter • CA (3,101) • **P (4,563)** • CH (4,316)	Ec: 10,206, Sal: 1,774 CA: 36 ColR P: 85 ColR CH: 119 ColR	Ec: 0.7%, Sal: 0.1% CA: 0% (0/36) P: 29.4% (25/85) CH: 37.8% (45/119)	0% (0/240 ColR)	n.t.	[21]
FRA	2016	• H – patients (653), rectal swabs	GN: 9 with acquired ColR	0% (0/9)	0% (0/9)	n.t.	[22]
FRA	2017	• CA – veal calves at slaughter (170)	Ec: 268 (27 ColR)	12.7% (34/268) [3 *mcr-1* & *mcr-3*]	0% (0/268)	*mcr-3* (10) [3 *mcr-1* & *mcr-3*] *mcr-4 – mcr-10* n.t.	[23]

Country[a]	Year of isolation	Source (no.) of samples[b]	Bacterial species[c], no. of isolates (no. of ColR isolates) [d]	*mcr-1* % (no. of positive isolates per no. of tested isolates)	*mcr-2* % (no. of positive isolates per no. of tested isolates)	*mcr-3 – mcr-10* no. of pos. isolates[e]	REF
FRA	2019-2020	Healthy pets in shelters, feces • dogs (52) • cats (105)	GP: 154 GN: 64	6.4% (14/218)	0% (0/218)	*mcr-3, -4, -5, -8* (0) *mcr-6, -7, -9, -10* n.t.	[24]
FRA	2020	**P – healthy, 2-8 months, fecal samples (80)**	Extracted DNA samples (80)	1.3% (1/80) [*E. coli*] [1 *mcr-1* & *mcr-3*]	0 (0/80)	*mcr-3 (1)* [1 *mcr-1* & *mcr-3*] *mcr-4, -5, -8* (0) *mcr-6, -7, -9, -10* n.t.	[25]
GBR	2014-2015	• **P – healthy, at abattoirs, caecal content (57 farms)**	Ec, *Klebsiella*, Sal, *Moraxella*: 657	0.15% (1/657) [*M. porci*]	0.15% (1/657) [*M. pluranimalium*-like]	*eptA* genes: 0.9% (6/657) [*M. osloensis*] *mcr-3 – mcr-10* n.t.	[26]
GBR	2014-2017	Public health surveillance • H, food, animal environment	Sal: 33,205 genomes	0.1% (32/33,205)	0% (0/33,205)	*mcr-3* (19) *mcr-4* (0) *mcr-5* (1) *mcr-6 – mcr-8* (0) *mcr-9, -10* n.t.	[27]
GBR	2019	• CA – calves, experimental study	Microbiota after florfenicol treatment	no	yes	n.t.	[28]
ITA	2000-2020	Feces, organs • Arthropoda: housefly, crayfish (13); birds (51); mammals (63); reptiles (98) • Environment/feed (11)	Sal: 236 (42 ColR)	0.84% (2/236) [1 *mcr-1* & *mcr-4*]	2.96% (7/236) [1 *mcr-2* & *mcr-4*] [1 isolate each of donkey, sheep, housefly; 4 of reptiles]	*mcr-4* (4) [1 *mcr-1* & *mcr-4*, 1 *mcr-2* & *mcr-4*] *mcr-6* (1) *mcr-8* (2) *mcr-3, -5, -7, -9* (0) *mcr-10* n.t.	[29]
ITA	2013-2017	• H – patients	Kp: 369 CPR (127 Col-non-S)	0% (0/127)	0% (0/127)	n.t.	[30]
ITA	2014/2015	• Fattening turkey • Broiler chickens • **P – fattening farm** • Bovines < 12 months	Turkey – iEc: 170 (39 ColR), ESBL/AmpC Ec: 224 (58 ColR), Sal: 146 (12 ColR) Broilers – iEc: 170 (9 ColR), ESBL/AmpC Ec: 244 (13 ColR) Sal: 90 (0 ColR) Pig – iEc: 168 (1 ColR), ESBL/AmpC Ec: 214 (14 ColR) Bovines – iEc: 170 (8 ColR), ESBL/AmpC Ec: 179 (7 ColR)	Turkey: iEc: 97.4% (38/39), ESBL/AmpC Ec: 100% (58/58), Sal: 25.0% (3/12) Broilers: iEc: 88.9% (8/9), ESBL/AmpC Ec: 84.6% (11/13) Sal: n.t. Pig: iEc: 100% (1/1), ESBL/AmpC Ec: 92.9% (13/14) Bovines: iEc: 62.5% (5/8), ESBL/AmpC Ec: 57.1% (4/7)	0%	*mcr-3* (4, bovine ESBL/AmpC Ec) *mcr-4* (2, ESBL/ AmpC Ec pig & iEC bovine) *mcr-5* (0) *mcr-6 – mcr-10* n.t.	[31]
ITA	2015-2016	• **P – PWD**	Ec: 51	72.5% (37/51)	0% (0/14 *mcr-1* neg.)	n.t.	[32]
ITA, ESP, BEL	2015-2016	• **P – PWD**	Ec: 125 (50 ColR) ITA: 34, Spain: 43, BEL: 48	25.6% (32/125)	2.4% (3/125) (BEL: P 2, CA 1; see Ref. [1])	*mcr-4* (11) *mcr-3* (0)	[33]

Country[a]	Year of isolation	Source (no.) of samples[b]	Bacterial species[c], no. of isolates (no. of ColR isolates) [d]	*mcr-1* % (no. of positive isolates per no. of tested isolates)	*mcr-2* % (no. of positive isolates per no. of tested isolates)	*mcr-3 – mcr-10* no. of pos. isolates[e]	REF
						mcr-5 – mcr-10 n.t.	
ITA	2016-2017	• H – clinical	Ent: 19,053 (12,441 Ec) (90 ColR)	28.9% (26/90), all Ec	0% (0/90)	*mcr-3, -4, -5* (0) *mcr-6 – mcr-10* n.t.	[34]
ITA	2016-2017	POUL • Broiler – cloacal swabs (13) • Slaughterhouse – product samples (72): environmental, skin, liver, meat	Sal: 85 (6 ESBL, 3 ColR)	3.5% (3/85)	0% (0/85)	*mcr-3 – mcr-5* (0) *mcr-6 – mcr-10* n.t.	[35]
ITA	2018-2019	• Wild boar – hunted	Ec: 168 (47 ColR)	29.2% (49/168) [23 *mcr-1* & *mcr-2*]	29.2% (49/168) [23 *mcr-1* & *mcr-2*]	n.t.	[36]
NLD	2014-2015	• H – patients attending hospital (576)	621 fecal samples	0.35% (2/576 patients)	0% (0/576)	n.t.	[37]
NLD, DEU, DNK, BEL	2015	• CH – retail meat (214)	Extracted DNA samples (214) NLD: 67 DEU: 44 DNK: 9 NLD/DEU: 80 NLD/DEU/BEL: 12 Unknown: 2	24.8% (53/214) NLD: 30.3% (21/67) DEU: 43.2% (19/44) DNK: 11.1% (1/9) NLD/DEU: 13.8% (11/80) NLD/DEU/BEL: 0% (0/12) Unknown: 50.0% (1/2)	0% (0/214)	n.t.	[38]
POL	2011-2016	Fecal samples • Turkeys, broilers, layers (74), **P (1)**, and CA (1)	Ec: 5,878 (128 ColR)	62.5% (80/128 ColR)	0% (0/128)	*mcr-3, -4, -5* (0) *mcr-6 – mcr-10* n.t.	[39]
ROU	2014-2017 2011-2012 2011-2012	• H – clinical • H – poultry abattoir workers • Broilers – fecal	H – clinical: Ent: 543 ColR and/or CPR H – abattoir workers: 15 Ec, 3GCR Broilers: 92 Ec, 3GCR	0% (0/543) 0% (0/15) 11.9% (11/92)	0% (0/543) 0% (0/15) 0% (0/92)	n.t.	[40]
Non-European countries							
BGD	not specified	• H (100), CA (50), goat (100), POUL (250) • POUL – farm environment (150) • Street food (160)	Ec: 358	0.73% (3/410) [3 POUL isolates]	0.49% (2/410) [2 street food isolates]	n.t.	[41, 42]
BGD	2017-2018	• H – UTI, urine (142)	Ent: 123	9.8% (12/123)	1.6% (2/123) [Ec & Kp]	*mcr-3, -4, -5* (0) *mcr-6 – mcr-10* n.t.	[43]
BGD	2017-2018	• POUL – healthy, feces (104)	Ec: 104 ESBL (98 ColR)	13.5% (14/104)	0% (0/104)	*mcr-3, -4, -5* (0) *mcr-6 – mcr-10* n.t.	[44]
BGD	2017-2018	• POUL – dropping samples (100; 20 farms)	Ent: 149 (92 ColR)	28.9% (43/149) [2 *mcr-1* & *mcr-2*]	3.4% (5/149) [2 *mcr-1* & *mcr-2*]	*mcr-3, -4, -5* (0) *mcr-6 – mcr-10* n.t.	[45]

Country[a]	Year of isolation	Source (no.) of samples[b]	Bacterial species[c], no. of isolates (no. of ColR isolates) [d]	*mcr-1*	*mcr-2*	*mcr-3 – mcr-10*	REF
				% (no. of positive isolates per no. of tested isolates)		no. of pos. isolates[e]	
BGD	2018	• POUL (20 broiler farms, 3 sampling times, 60 pooled samples)	Ec: 1,200	25.4% (305/1,200)	0% (0/1,200)	*mcr-3, -4, -5* (0) *mcr-6 – mcr-10* n.t.	[46]
BOL	2016	• H – children, healthy, feces	GN: 337 (cultivated on MacConkey with 2 mg/L Colistin)	38.3% (129/337)	0% (0/337)	n.t.	[47]
BRA	2013-2016	• POUL – meat cuts (33 cuts, 24 markets)	Sal: 60 (7 ColR)	3.3% (2/60)	0% (0/60)	*mcr-3, -4* (0) *mcr-5 – mcr-10* n.t.	[48]
BRA	2015-2016	• POUL – healthy, trachea/cloaca (107) & APEC (2)	Ec: 109	57.9% (62/109)	0% (0/109)	*mcr-3, -4* (0) *mcr-5* (3) *mcr-6* - mcr-*9* (0) *mcr-10* n.t.	[49]
KHM	2017	• **P – healthy, feces (91 farms)**	Ec: 261 (52 ColR)	80.8% (42/52) [11 *mcr-1* & *mcr-3*]	0% (0/52)	*mcr-3* (20) [11 *mcr-1* & *mcr-3*] *mcr-4, -5* (0) *mcr-6 – mcr-10* n.t.	[50]
CAN	2011-2012	• CH – healthy, feces (12)	Ec potential ESBL/AmpC: 108 (0 ColR)	0% (0/108)	0% (0/108)	n.t.	[51]
CAN	2017	• **P – healthy; fecal microbiota** (5 sampling dates over growing period)	Extracted DNA samples (fecal samples 33-62 pigs per sampling date)	positive at 5 sampling dates	positive at 3 sampling dates	n.t.	[52]
CHN	2004-2012	• **P,** CA, CH, ducks – clinical	Total - Ec: 624 P - Ec: 113 CH - Ec: 404 Ducks - Ec: 44 CA Ec: 63	Total: 2.7% (17/624) P: 0.9% (1/113) CH: 3.2% (13/404) Ducks: 6.8% (3/44) CA: 0% (0/63)	0% (0/624)	*mcr-3* (0) *mcr-4 – mcr-10* n.t.	[53]
CHN	2008-2014	• CH – cloacal swabs	Ec: 821	44 among ColR isolates (total no. of ColR unclear)	0%	*mcr-3, -4, -5* (0) *mcr-6 – mcr-10* n.t.	[54]
CHN	H: 2011-2014 C: 2013	• H – clinical (2,353 Ent) • CH – slaughterhouse	H: 964 Kp (6 ColR), 1,389 Ec (23 ColR) CH: 47 Kp (11 ColR), 121 Ec (10 ColR)	H: 0.4% (4/964) Kp, 1.7% (23/1,389) Ec CH: 0% Kp, 8.3% (10/121) Ec	0% (0/13 *mcr-1* neg. & ColR)	n.t.	[55]
CHN	2013	• Flies on a university campus (297)	Extracted DNA samples: 297 GN: 189	DNA: 36.7% (109/297) GN: 4.8% (9/189)	DNA: 1.4% (4/297) GN: 0% (0/189)	DNA: *mcr-3* (33) GN: *mcr-3* (0) *mcr-4 – mcr-10* n.t.	[56]
CHN	2014, 2016	• **P – healthy, feces (1,552)** • POUL – healthy, nasal, oropharyngeal, anal/cloacal swabs (1,836)	Extracted DNA samples: P: 1,454 CH: 1,498 Geese: 109 Ducks: 130 Pigeons: 99	P: 79.2% (1,152/1,454) CH: 31.8% (476/1,498) Geese: 71.7% (78/109) Ducks: 5.5% (7/130) Pigeons: 13.1% (13/99)	P: 56.3% (819/1,454) CH: 5.5% (82/1,498) Geese: 5.5% (6/109) Ducks: 2.3% (3/130) Pigeons: 0% (0/99)	*mcr-3:* P: 18.7% CH: 5.2% Geese: 11.9% Ducks: 13.8% Pigeons: 5.1%	[57]

Country[a]	Year of isolation	Source (no.) of samples[b]	Bacterial species[c], no. of isolates (no. of ColR isolates) [d]	*mcr-1* % (no. of positive isolates per no. of tested isolates)	*mcr-2* % (no. of positive isolates per no. of tested isolates)	*mcr-3 – mcr-10* no. of pos. isolates[e]	REF
						mcr-4 – mcr-10 n.t.	
CHN	2015-2016	• **P**, CH, CA – healthy, intensive feeding farms, fecal swabs (2,199)	P – Ec: 811 (440 Col-R) CH – Ec: 1,232 (443 Col-R) CA – Ec: 156 (42 Col-R)	P: 68.9% (303/440) [88 *mcr-1* & *mcr-2*] CH: 87.6% (388/443) [32 *mcr-1* & *mcr-2*] CA: 71.4% (30/42) [4 *mcr-1* & *mcr-2*]	PI: 46.8% (206/440) [88 *mcr-1* & *mcr-2*] CH: 14.9% (66/443) [32 *mcr-1* & *mcr-2*] CA: 19.1% (8/42) [4 *mcr-1* & *mcr-2*]	n.t.	[58]
CHN	2016	• H – women attending hospital, vaginal swabs (134)	Extracted DNA samples	0.7% (1/134) [*mcr-1* & *mcr-5*]	1.5% (2/134)	*mcr-3* (2) *mcr-4* (17) *mcr-5* (1) [*mcr-1* & *mcr-5*] *mcr-6 – mcr-10* n.t.	[59]
CHN	2016	• Farming soil samples (96)	Ec ESBL: 42 (10 ColR) Kp ESBL: 11 (2 ColR)	50 % (6/12 ColR)	0% (0/12 ColR)	*mcr-3, -4* (0) *mcr-5 – mcr-10* n.t.	[60]
CHN	2019	• cats and dogs – healthy and clinical, fecal samples (1550)	Kp: 1190 (ColR)	12.5% (149/1190) [4 *mcr-1* & *mcr-3*, 3 *mcr-1* & *mcr-5*]	0.9% (11/1190)	*mcr-3* (15) [4 *mcr-1* & *mcr-3*] *mcr-4* (6) *mcr-5* (16) [3 *mcr-1* & *mcr-5*] *mcr-7, -8* (0) *mcr-9* (5) *mcr-10* (4) *mcr-6* n.t.	[61]
EGY	2017-2018	• Resident wild birds (80) • Migratory waterfowls (60) • Surface water (20) • H – farmer, stool (50)	RB – Ec: 33, Kp: 22, Kox: 4, Pa: 8 MB – Ec: 29, Kp: 9, Kox: 6, Pa: 11 SW – Ec: 7, Kp: 7, Kox: 1, Pa: 3 H – Ec: 10, Kp: 15, Kox: 2, Pa: 4	RB: 10.4% (7/67) MB: 20.6% (11/55) [2 *mcr-1* & *mcr-2*] SW: 16.6% H: 9.6% [1 *mcr-1* & *mcr-2*]	RB: 1.4% (1/67, Pa) MB: 3.6% (2/55, Ec, Kp) [2 *mcr-1* & *mcr-2*] SW: 11.1% (2/18, Ec, Kp) H: 9.6% (3/31, Ec, Kp) [1 *mcr-1* & *mcr-2*]	n.t.	[62]
EGY	2018-2020	• CA – milk samples (total number not given) • clinical mastitis (70), subclinical mastitis (11), raw milk (36)	Ec: 42 (24 ColR) Cb: 20 (0 ColR) Kp: 18 (14 ColR) Am: 17 (15 ColR) Pa: 10 (8 ColR) Ent: 10 (0 ColR)	Ec: 4.8% (2/42) Kp: 22.2% (4/18) Am: 37.5% (6/16) Pa: 30% (3/10)	Ec: 19% (8/42) Kp: 16.7% (3/18) Am: 12.5% (2/16) Pa: 10% (1/10)	*mcr-3* (16) *mcr-4* (1) *mcr-7* (1) *mcr-5, -6, -8, -9* (0) *mcr-10* n.t.	[63]
EGY	2018-2020	• CA – milk samples (570)	Ec: 90 (24 ColR) Pm: 33 (4 ColR)	0% (0/10)	0% (0/10)	*mcr-3 – mcr-9* (0)	[64]

Country[a]	Year of isolation	Source (no.) of samples[b]	Bacterial species[c], no. of isolates (no. of ColR isolates) [d]	*mcr-1* % (no. of positive isolates per no. of tested isolates)	*mcr-2* % (no. of positive isolates per no. of tested isolates)	*mcr-3 – mcr-10* no. of pos. isolates[e]	REF
		• clinical mastitis (350), subclinical mastitis (95), raw milk (125)	Kp: 25 (14 ColR) Am: 17 (14 ColR) Ecl: 15 (0 ColR) Cb: 4 (0 ColR)			*mcr-10* (1 Kp from raw milk)	
EGY	2019	• H – patients (324 samples)	Ec & Kp: 200 (24 ColR)	8.3% (2/24)	0% (0/24)	n.t.	[65]
EGY	not specified	• H - clinical in ICU	Ec & Kp: 100 (70 ColR)	0% (0/100)	0% (0/100)	n.t.	[66]
GHA	2018-2019	• H - clinical	Ec: 135 (2 CPOs)	0.7% (1/135)	1.5% (2/135)	*mcr-3 – mcr-8* (0) *mcr-9, -10* n.t.	[67]
HKG	2016	• H – routine stool samples (672) from 616 individuals	Ent: 79 Col-non-S	17.7% (14/79)	0% (2/79)	n.t	[68]
IND	2013-2015	• H – clinical	Kp: 8 ColR	0% (0/8)	0% (0/8)	n.t.	[69]
IND	2017-2018	• H – consecutive samples ocular infections	GN: 60 (24 Col-R, including 15 intrinsically resistant isolates)	6.3% (1/16 ColR tested)	25.0% (4/16 ColR tested, Bcep, Pa)	n.t.	[70]
IND	2018-2019	• H – clinical, urine (109), pus (24), respiratory (42), blood (5), miscellaneous (20)	Ec, CPOs: 113 (3 ColR) Kp, CPOs: 79 (22 ColR) Ent, CPOs: 8 (2ColR)	0% (0/27 ColR tested)	0% (0/27 ColR tested)	n.t.	[71]
IRN	2008-2016	• Various animal species	Broiler: 183 APEC; CA: 94 STEC, 36 MPEC; ostrich: 35 septicemic Ec, 70 fecal Ec; sheep: 51 STEC, 31 commensal Ec; pigeon: 33 STEC; dog: 74 commensal Ec	0% (0/607)	0% (0/607)	n.t.	[72]
IRN	2017	• H – clinical	Ec: 351 (38 Col-non-S) Kp: 119 (26 Col-non-S)	Ec: 1.7% (6/351) Kp: 1.7% (2/119)	Ec: 0% (0/351) Kp: 0% (0/119)	n.t.	[73]
IRN	2019	• CA (38) • CH (47) • Urban sewage (30)	CA – Ec: 18 (1 ColR) CH – Ec: 30 (1 ColR) Urban sewage – Ec: 17 (1 ColR)	33.3% (1/3 ColR)	0% (0/3 ColR)	*mcr-3, -4, -5, -6* (0) *mcr-7 – mcr-10* n.t.	[74]
IRN	not specified	• POUL – fecal samples (156 samples, 23 farms)	Sal: 30	0% (0/30)	0% (0/30)	n.t.	[75]
IRQ	2016-2018	• clinical and environmental samples	Ab: 121 (92 ColR)	73.5% (89/121)	64.5% (78/121)	*mcr-3* (82) *mcr-4 – mcr-10* n.t.	[76]
KOR	2007-2016	• **P – diarrhoeic weaned piglets (100 pig herds)**	Ec: 364	1.1% (4/364) [3 *mcr-1* & *mcr-3*]	0% (0/364)	*mcr-3* (8) [3 *mcr-1* & *mcr-3*] *mcr-4 – mcr-10* n.t.	[77]
JPN	2000-2014	national veterinary antimicrobial resistance monitoring • CA – healthy (3,134)	Ec: 9,306 (732 ColR)	All animals: 0.42% (39/,9306); 5.3% (39/732 ColR) CA: 0.16% (5/3134)	0% (0/732)	n.t.	[78]

Country[a]	Year of isolation	Source (no.) of samples[b]	Bacterial species[c], no. of isolates (no. of ColR isolates) [d]	*mcr-1*	*mcr-2*	*mcr-3 – mcr-10*	REF
				% (no. of positive isolates per no. of tested isolates)		no. of pos. isolates[e]	
		• **P – healthy (2,052)** • Broilers – healthy (2,017) • Layers – healthy (2,103)		P: 0.97% (20/2,052) Broilers: 0.69% (14/2,017) Layers: 0% (0/2,103)			
JPN	2008-2015	• **P – healthy/PWD (120)** • H – clinical (514)	Ec: 676	P – healthy: 2.4% (1/42) P – PWD: 30% (36/120) [5 *mcr-1* & *mcr-5*] H: 0% (0/514)	0% (0/676)	*mcr-3* (10) *mcr-4* (0) *mcr-5* (35) [5 *mcr-1* & *mcr-5*] *mcr-6 – mcr-10* n.t.	[79]
JPN	2015	• Retail meat (111) - CH (55), **pork (32)**, beef (24)	CH – Ec: 154 (9 ColR) Pork – Ec: 55 (1 ColR) Beef – Ec: 47 (0 ColR)	CH: 5.2% (8/154) Pork: 1.8% (1/55) Beef: 0% (0/47)	0% (0/9 ColR)	n.t.	[80]
MYS	not specified	• Raw food samples – CH meat (50), bean sprouts (50)	CH meat – Ec: 23 Bean sprouts – Ec: 6	CH meat: 52.2% (12/23) Bean sprouts: 0 (0/6)	0% (0/29)	n.t.	[81]
PAK	not specified (study over 18 months)	• H – clinical (38,500) • POUL meat, CH fecal; respiratory secretion (630)	H: 5,893 GN (17 ColR) Animal: 630 GN (126 ColR)	H: 23.5% (4/17) Animal: 82.5% (104/126)	H: 0% (0/17) Animal: 1.6% (2/126, Pm)	*mcr-3, -4, -5* (0), *mcr-6* to *mcr-10* n.t.	[82]
PAK	not specified	• H – clinical (6,879)	GN: 718 (57 ColR)	31.6% (18/57) [Ec, Kp, Ab, Pa]	1.8% (1/57) [Kp, NDM-1/CTX-M-1]	*mcr-3, -4, -5* (0) *mcr-6 – mcr-10* n.t.	[83]
PAK	not specified	• H – clinical	Kp: 200	12.0% (24/200) [14 *mcr-1* & *mcr-2*]	8.5% (17/200)	n.t.	[84]
SEN	2011	• CH – healthy, feces (50)	Ec potential ESBL/AmpC: 93 (2 ColR)	0% (0/2)	0% (0/2)	n.t.	[51]
SGP	2017	• H – diarrheal stool samples (201)	Ent: 23 SuperPolymyxin screening agar (19 ColR/Col-non-S)	63.2% (12/19)	0% (0/19)	n.t.	[85]
THA	2007-2013	• **P – healthy (1), clinical (2)**	Ec ESBL: 3 (3 ColR)	66.7% (2/3)	33.3% (1/3)	*mcr-3* (2) *mcr-4* (0) *mcr-5 – mcr-10* n.t.	[86]
THA	2007-2018	• **P – healthy, fecal (354); clinical, fecal (100)**	Ec: 454 (217 ColR)	10.4% (47/454) [32 *mcr-1* & *mcr-3*]	1.1% (5/454) [5 *mcr-2* & *mcr-3*]	*mcr-3* (204) [32 *mcr-1* & *mcr-3*, 5 *mcr-2* & *mcr-3]* *mcr-4* (0) *mcr-5 – mcr-10* n.t.	[87]
THA	2016-2019	• H – clinical	Ent: 6,996 MDR (4,516 ColR, thereof 4,235 CPE)	0.3% (13/4,235) 1.03% E 0.12% Kp [1 Ec *mcr-1* & *mcr-3*]	0% (0/4,235)	*mcr-3* (1) [Ec, *mcr-1* & *mcr-3*] *mcr-4 - mcr-9* (0) *mcr-10* n.t.	[88]
THA	2016-2017	• **P – healthy, longitudinal study (4 farms)**	Ec: 100 ColR	64% (64/100) [24 Ec *mcr-1* & *mcr-2*]	38% (38/100) [24 Ec *mcr-1* & *mcr-2*]	n.t.	[89]

Country[a]	Year of isolation	Source (no.) of samples[b]	Bacterial species[c], no. of isolates (no. of ColR isolates) [d]	*mcr-1*	*mcr-2*	*mcr-3 – mcr-10*	REF
				% (no. of positive isolates per no. of tested isolates)		no. of pos. isolates[e]	
THA	2017-2020	• **P – healthy, longitudinal study (1 farm), fecal samples (70)** • Wastewater (50) • H – farm workers, rectal swabs (50)	Ec: 33 (ColR)	P: 24.3% (17/70) [2 *mcr-1* & *mcr-3*] Wastewater: 18% (9/50) H: 8% (4/50)	0% (0/170)	*mcr-3* (5) [2 *mcr-1* & *mcr-3*] *mcr-4, -5, -6, -7, -8* (0) *mcr-9 – mcr-10* n.t.	[90]
THA	2018	• H – healthy farmer, stool	Kp: 1 ColR (case report)	0% (0/1)	100% (1/1) [*mcr-1* & *mcr-3*]	*mcr-3* (1) [*mcr-1* & *mcr-3*] *mcr-4 – mcr-10* n.t.	[91]
THA	not specified	• H – healthy, feces (3)	Ec: 3 ColR	66.7% (2/3) [1 *mcr-1* & *mcr-3*]	33.3% (1/3) [*mcr-2* & *mcr-3*]	*mcr-3* (2) (1 *mcr-1* & *mcr-3*, 1 *mcr-2* & *mcr-3*] *mcr-4 – mcr-10* n.t.	[92]
TUN	2011-2013	• Camel calves – healthy (23), diarrhea (29); 25 extensive camel farms	Ec: 51 ColS Ecl: 1 ColR	0% (0/52)	0% (0/52)	n.t.	[93]
TUR	not specified	• CA, sheep	Ec: 49 O157	0% (0/49)	6.1% (3/49) [3 *mcr-2* & *mcr-3*]	*mcr-3* (5) [3 *mcr-2* & *mcr-3*] *mcr-4, -5* (0) *mcr-6 – mcr-10* n.t.	[94]
TUR	not specified	• H - clinical	Kp: 38	0% (0/38)	0% (0/38)	*mcr-3* (0) *mcr-4 – mcr-10* n.t.	[95]
TUR	2015-2016	• H – clinical	Ent: 329 (Kp: 217, Sal 75, Ec 31, Ecl 3, Kox 2, Eaer 1)	0% (0/329)	0% (0/329)	n.t.	[96]
TUR	2018-2021	• H – clinical	Kp: 150 CPE (78 ColR)	0% (0/150)	0% (0/150)	*mcr-3 – mcr-5* (0) *mcr-6 – mcr-10* n.t.	[97]
TUR	2021	• H – inpatients in ICU, oral swabs (96)	Ab: 21 (0 ColR)	0% (0/21)	4.8% (1/21)	*mcr-3 – mcr-5* (0) *mcr-6 – mcr-10* n.t.	[98]
USA	2006-2014	• **Livestock (180),** wildlife (320), watersheds (240), leafy vegetables (220), sediment, soil, fruit, other vegetables (10 each)	Ec: 1,000 STEC	0% (0/1,000)	0% (0/1,000)	n.t.	[99]
USA	not specified	• H (109) • nonhuman isolates (2)	Ent: 111 (Kp: 61, Ec: 33, Ecl: 7, Kox: 2, Ka: 2, Cb: 2, Ea:1, Sal: 3) (28 ColR)	13.5% (15/111) [Ec: 11, Kp: 1, Sal: 3]	0.9% (1/111) [Ec: 1]	*mcr-3 – mcr-10* n.t.	[100]
VNM	2011	• CH – healthy, feces (51)	Ec potential ESBL/AmpC: 126 (11 ColR)	VNM: 100% (11/11 ColR)	0% (0/11)	n.t.	[51]
VNM	2015-2017	• Food samples – CH (116), **pork (112)**, fish (112), shrimp (112)	CH – Ec ESBL: 77 Pork – Ec ESBL: 62 Fish – Ec ESBL: 47	CH: 53.2% (41/77) Pork: 11.3% (7/62) Fish: 6.4% (3/47)	0% (0/208)	*mcr-3* (9) [8 *mcr-1* & *mcr-3*] *mcr-4 – mcr-8* (0)	[101]

Country[a]	Year of isolation	Source (no.) of samples[b]	Bacterial species[c], no. of isolates (no. of ColR isolates) [d]	*mcr-1* % (no. of positive isolates per no. of tested isolates)	*mcr-2* % (no. of positive isolates per no. of tested isolates)	*mcr-3 – mcr-10* no. of pos. isolates[e]	REF
			Shrimp – Ec ESBL: 22	Shrimp: 22.7% (5/22) [8 *mcr-1* & *mcr-3*]		*mcr-9, -10* n.t.	
ZAF	2016	• POUL - clinical	Ec: 50 (8 isolates with maximum colistin MIC (2 µg/mL) in this study)	12.5% (1/8)	0% (0/8)	n.t.	[102]
ZAF	2017-2018	• POUL – healthy broilers, fecal samples (2400)	Cj: 26	0% (0/26)	0% (0/26)	*mcr-3, -5* (0) *mcr-4* (8) *mcr-6 – mcr-10* n.t.	[103]
WW	2014-2016	• H – clinical (INFORM global surveillance program (44,407 isolates from 39 countries)	Ent: 908 ColR	2.6% (24/908)	0% (0/908)	*mcr-3* (2) *mcr-4* (0) *mcr-5* (1) *mcr-6 – mcr-10* n.t.	[104]

[a] 3-letter country abbreviation: BEL, Belgium; BGD, Bangladesh; BOL, Bolivia; BRA, Brazil; Can, Canada; CHE, Switzerland; CHN, China; CZE, Czechia; DEU, Germany; DNK, Denmark; EGY, Egypt; ESP, Spain; EUR, Europe; FRA, France; GBR, United Kingdom; GHA, Ghana; HKG, Hong Kong; IND, India; IRN, Iran; IRQ, Iraq; ITA, Italy; JPN, Japan; KHM, Cambodia; KOR, The Republic of Korea; MYS, Malaysia; NLD, Netherlands; PAK, Pakistan; POL, Poland; ROU, Romania; SEN, Senegal; SGP, Singapore; THA, Thailand; TUN, Tunisia; TUR, Turkey; USA, United States of America; VNM, Viet Nam; ZAF, South Africa. WW, worldwide.

[b] Abbreviations of hosts: CA, cattle; CH, chicken; H, human; MB, migratory birds; P, pig; POUL, poultry; PWD, post weaning diarrhea; RB, resident wild bird; SW, surface water; UTI, urinary tract infection. Samples from pig or pig meat are marked in bold.

[c] Abbreviations of bacterial species and pathotypes: Ab, *Acinetobacter baumannii*; Am, *Aeromonas hydrophila*; Bcep, *Burkholderia cepacia*; Cb, *Citrobacter* species; Cj, *Campylobacter jejuni*; Ea, *Escherichia albertii*; Ec, *Escherichia coli*; iEc, indicator *E. coli*, Ecl, *Enterobacter cloacae*; Ent, Enterobacterales; GN, Gram negative bacteria; Hal, *Hafnia alvei*; Ka, *Klebsiella aerogenes*; Kox, *Klebsiella oxytoca*; Kp, *Klebsiella pneumoniae*; Pa, *Pseudomonas aeruginosa*; Pm, *Proteus mirabilis*; Rorn, *Raoultella ornithinolytica*, Sal, *Salmonella enterica*; APEC, Avian Pathogenic *E. coli*; MPEC, Mammary Pathogenic *E. coli*; STEC, Shiga toxin producing *E. coli*;

[d] If available, the number of ColR isolates is provided. Unless otherwise stated, ColR means acquired Col-resistance, excluding *Serratia* spp., *Providencia* spp., *Proteus* spp., and *Morganella* spp., which are intrinsically colistin non-susceptible). Abbreviations: 3GCR, 3rd-generation cephalosporin resistant bacteria; AmpC, AmpC β-Lactamase; APEC, Avian Pathogenic *E. coli*; Col-non-S, Colistin non-susceptible; ColR, colistin resistant; CPE, carbapenem-resistant Enterobacteriaceae; CPOs, carbapenemase-producing organism; CPR, carbapenem-resistant; ESBL, Extended-spectrum β-Lactamase; ETEC, enterotoxigenic *E. coli*; STEC, Shiga toxin producing *E. coli*;

[e] Unless otherwise stated, *mcr-6* to *mcr-10* genes were not investigated in the studies cited, e.g., due the fact, that these *mcr* gene variants were unknown at the time the studies were performed.

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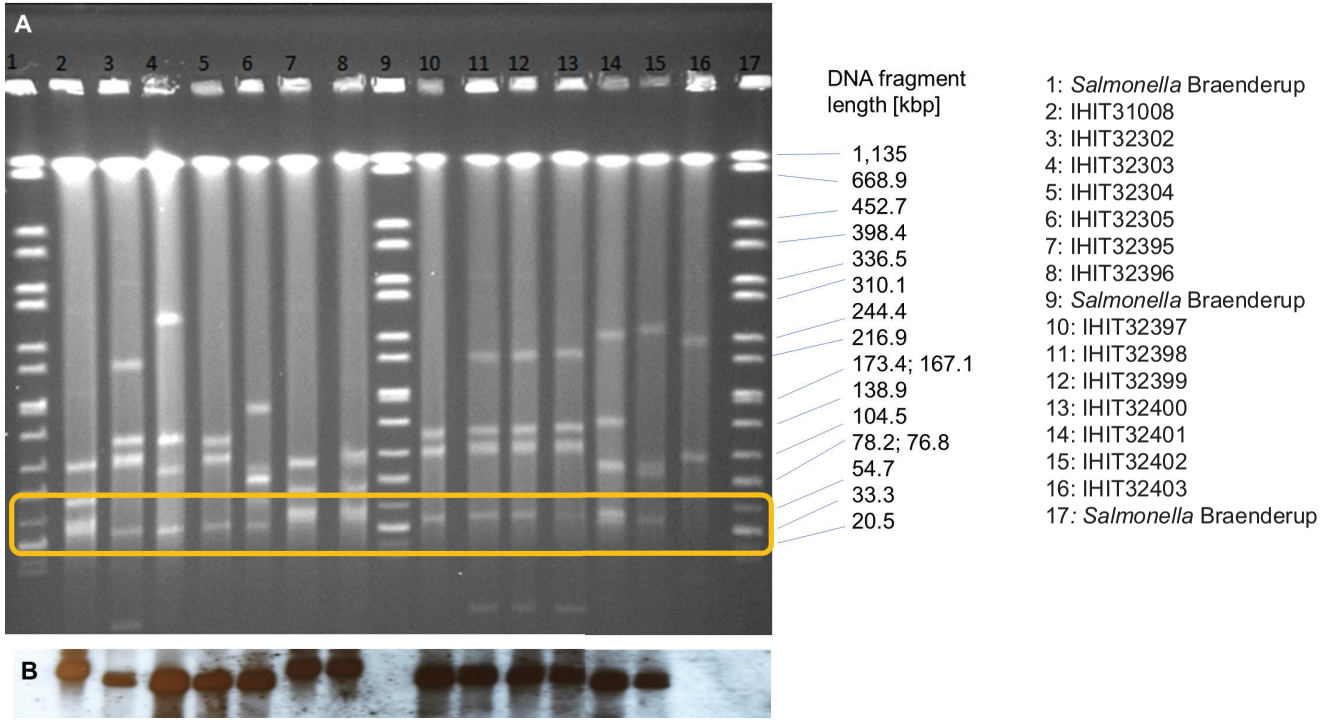
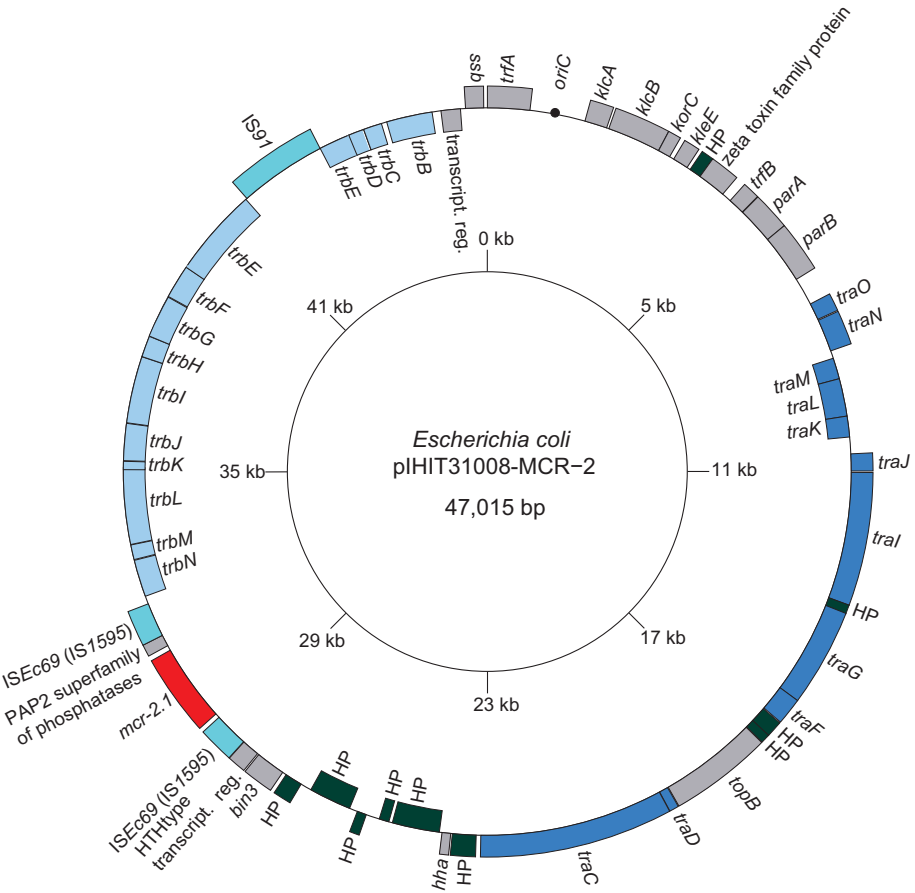
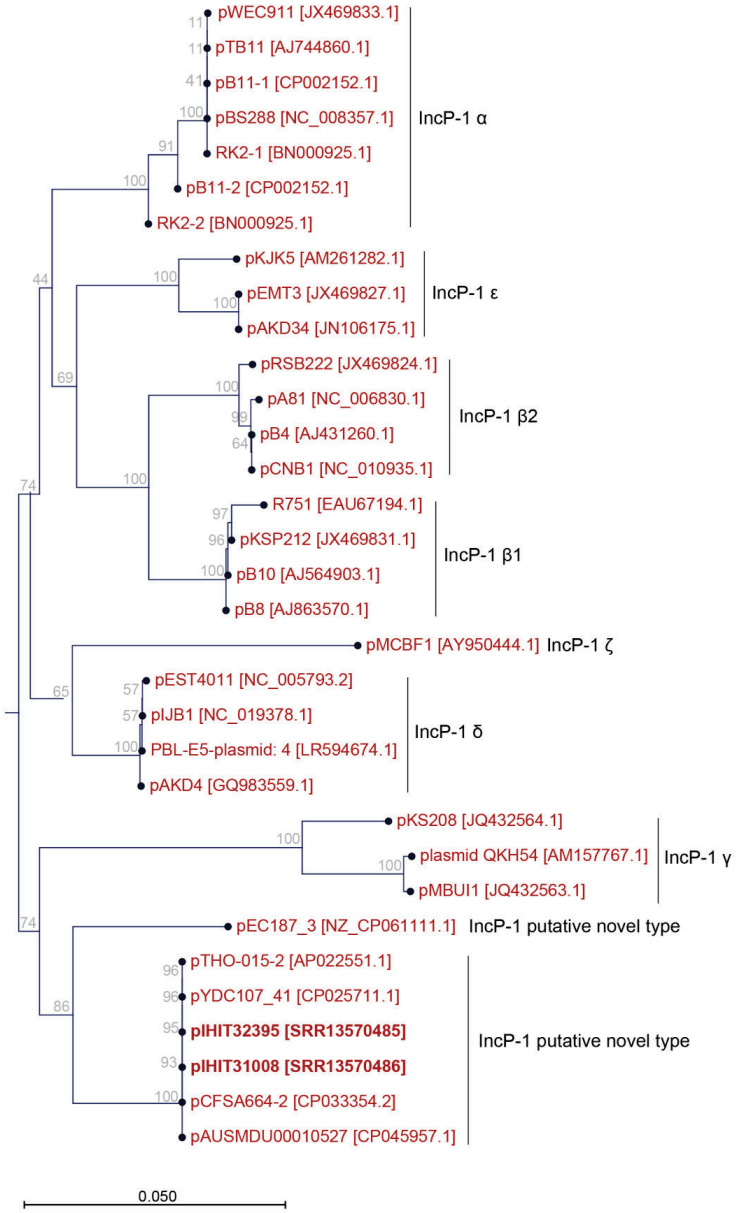


Supplementary Figure 1: S1-nuclease digested genomic DNA obtained from 14 *mcr-2*-positive *E. coli* isolates (IHIT32398 and IHIT32400 were duplicates to IHIT32399; thus, they were not included in the main text). **A** PFGE electropherogramm; **B** Southern blot after hybridization with *mcr-2* probe (the blot section corresponds to the area highlighted in Fig. A).



Supplementary Figure 2: Genetic organisation and structure of *mcr-2.1* harbouring plasmid pIHIT31008-MCR-2 from colistin-resistant porcine *Escherichia coli* isolate IHIT31008.



Supplementary Figure 3: Neighbor-joining tree based on the alignment of TrfA amino acid sequences of representative members of the different IncP1 plasmid subfamilies α, β1, β2, δ, γ, ε, and ζ, our 47 kb MCR-2 plasmids (bold letters) and publicly available plasmids with backbones almost similar to our plasmids (see Supplemental Table 5).

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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-Ip*) 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 ≤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.

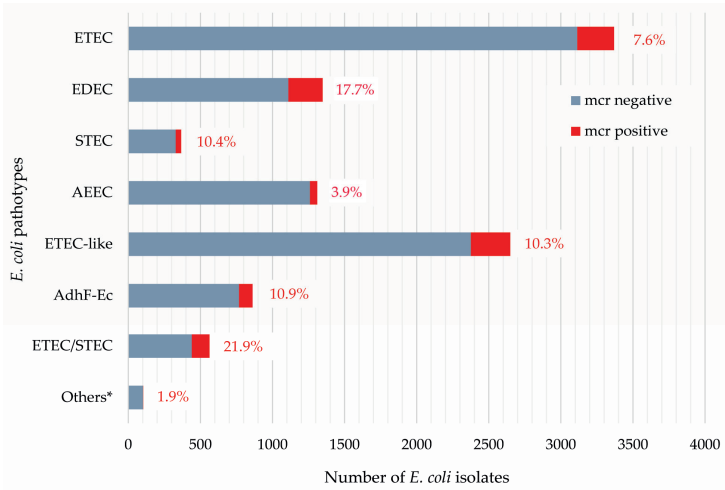

Figure 1. Presence of *mcr* genes among 10,573 porcine *E. coli* isolates based on their association to a distinct pathotype. * Others include 104 *E. coli* isolates defined as ETEC-like/STEC (n = 75), AEEC/STEC (n = 23), ETEC-like/AEEC (n = 3), AdhF-*Ec*/EDEC (n = 2), and AdhF-*Ec*/AEEC (n = 1).

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).

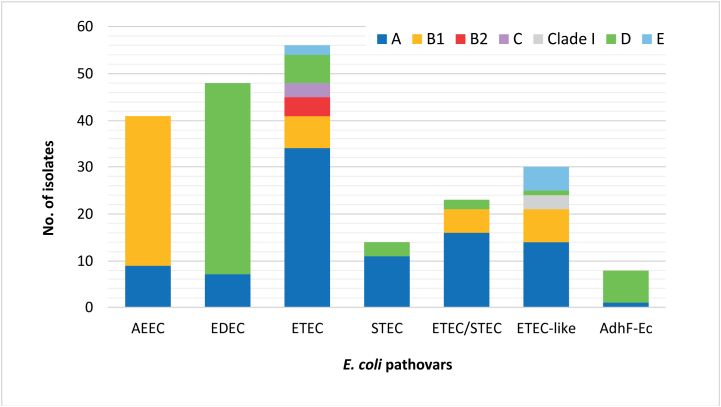

Figure 2. Distribution of phylogenetic groups among 220 *mcr*-positive porcine *E. coli* isolates according to pathotypes.

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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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.

Primer name	Sequence (5´-3´)	Target gene	Amplicon size (bp)	Positive control strain / DNA	Primer reference
Multiplex-PCR-VAGs					
STb-1	TGCCTATGCATCTACACAAT	*estb*	113	*E. coli* E57	[13,14]
STb-2	CTCCAGCAGTACCATCTCTA				
STaP-1	CAACTGAATCACTTGACTCTT	*estap*	158	*E. coli* 987P, *E. coli* B41, *E. coli* E57	[13,14]
STaP-2	TTAATAACATCCAGCACAGG				
K99-1	AATACTTGTTCAGGGAGAAA	*fanA*	230	*E. coli* B41	[13,14]
K99-2	AACTTTGTGGTTAACTTCCT				
LT-1	GGCGTTACTATCCTCTCTAT	*eltB-Ip*	272	*E. coli* G7	[13,14]
LT-2	TGGTCTCGGTCAGATATGT				
F18-1	TGGTAACGTATCAGCAACTA	*fedA*	313	*E. coli* E57	[13,14]
F18-2	ACTTACAGTGCTATTCGACG				
P987-1	AAGTTACTGCCAGTCTATGC	*fasA*	409	*E. coli* 987P	[13,14]
P987-2	GTAACTCCACCGTTTGTATC				
F-EaeA-1	ATATCCGTTTTAATGGCTATCT	*eae*	425	*E. coli* TTP-1	[15]
F-EaeA-2	AATCTTCTGCGTACTGTGTTCA				
K88-1	GAATCTGTCCGAGAATATCA	*faeG*	499	*E. coli* G7	[13,14]
K88-2	GTTGGTACAGGTCTTAATGG				
F41-1	AGTATCTGGTTCAGTGATGG	*fimF41a*	612	*E. coli* B41	[13,14]
F41-2	CCACTATAAGAGGTTGAAGC				
Stx2e-1	AATAGTATACGGACAGCGAT	*stx2*	733	*E. coli* E57, *E. coli* TTP-1	[13,14]
Stx2e-2	TCTGACATTCTGGTTGACTC				
Multiplex-PCR-I					
CLR F	CGGTCAGTCCGTTTGTTC	*mcr-1*	309	*E. coli* IHIT22995 (this study)	[16]
CLR R	CTTGGTCGGTCTGTAGGG				
MCR2-IF	TGTTGCTTGTGCCGATTGGA	*mcr-2*	567	*E. coli* IHIT31008 [7]	[17]
MCR2-IR	AGATGGTATTGTTGGTTGCTG				
MCR3-F	TTGGCACTGTATTTTGCATTT	*mcr-3*	542	*E. coli* IHIT37100 (this study)	[18]
MCR3-R	TTAACGAAATTGGCTGGAACA				
Mcr-4 FW	ATTGGGATAGTCGCCTTTT	*mcr-4*	488	Salmonella R3445 [8]	[12]

Mcr-4 RV	TTACAGCCAGAATCATTATCA				
MCR5_rev	TCATTGTGGTTGTCCTTTTCTG	*mcr-5*	771	Salmonella 13-SAO1718 [9]	[1]
MCR-5-mp-fw	TGCATGTTTTCCCTCAATGG				This study
Multiplex-PCR-II					
mcr-6-mp-fw	AGCTATGTCAATCCCGTGAT	*mcr-6*	252	Genomic DNA - Top10F′+ pCR2.1-*mcr-6* (provided by M. Borowiak)	[19]
mcr-6-mp-rev	ATTGGCTAGGTTGTCAATC				
mcr-7-mp-fw	GCCCTTCTTTTCGTTGTT	*mcr-7*	551	Genomic DNA - Top10F′+ pCR2.1-*mcr-7* (provided by M. Borowiak)	[19]
mcr-7-mp-rev	GGTTGGTCTCTTTCTCGT				
mcr-8-mp-fw	TCAACAATTCTACAAAGCGTG	*mcr-8*	856	Genomic DNA - Top10F′+ pCR2.1-*mcr-8* (provided by M. Borowiak)	[19]
mcr-8-mp-rev	AATGCTGCGCGAATGAAG				
mcr-9-mp-fw	TTCCCTTTGTTCTGGTTG	*mcr-9*	1011	*E. coli* IHIT41513 (this study)	This study
mcr-9-mp-rev	GCAGGTAATAAGTCGGTC				
mcr-10-mp-F	TATCCTGAGCCGTCTTGAAC	*mcr-10*	386	*Enterobacter kobei* IHIT44343 (this study)	This study
mcr-10-mp-R	GGATCAGCGAAGCGAGCAT				

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Table S2: Overview of all porcine *E. coli* isolates included in this study and their classification into pathotypes according to the VAG pattern.

VAG pattern	ETEC	ETEC-like	EDEC	AEEC	AdhF-*Ec*	ETEC/STEC	STEC	AdhF-Ec/AAEC	AdhF-Ec/EDEC	AEEC/STEC	ETEC-like/AAEC	ETEC-like/STEC	Total number
eae				1310									**1310**
eae, fedA								1					**1**
eae, estap											3		**3**
eae, stx2										23			**23**
eltB-Ip		1											**1**
eltB-Ip, faeG	6												**6**
eltB-Ip, fedA	12												**12**
estap		29											**29**
estap, faeG	5												**5**
estap, fanA	62												**62**
estap, fasA	60												**60**
estap, fedA	40												**40**
estap, fanA, fimF41a	22												**22**
estap, stx2												25	**25**
estap, fedA, stx2						16							**16**
eltB-Ip, estap, faeG	2												**2**
eltB-Ip, estap, fedA	1												**1**
eltB-Ip, estb		142											**142**
eltB-Ip, estb, faeG	1451												**1451**
eltB-Ip, estb, fedA	205												**205**
eltB-Ip, estb, faeG, fedA	3												**3**
eltB-Ip, estb, fedA, stx2						60							**60**
eltB-Ip, estb, estap		4											**4**
eltB-Ip, estb, estap, faeG	508												**508**
eltB-Ip, estb, estap, fedA	54												**54**
eltB-Ip, estb, estap, faeG, fasA	2												**2**
eltB-Ip, estb, estap, faeG, fedA	4												**4**
eltB-Ip, estb, estap, faeG, fedA, stx2						1							**1**
eltB-Ip, estb, estap, fedA, stx2						19							**19**
eltB-Ip, estb,estap, faeG, fanA	1												**1**
estb		1708											**1708**
estb, faeG	23												**23**
estb, fedA	16												**16**
estb, estap		766											**766**
estb, estap, faeG	291												**291**

estb, estap, fanA	67												**67**
estb, estap, fasA	17												**17**
estb, estap, fedA	513												**513**
estb, estap, faeG, fanA	1												**1**
estb, estap, faeG, fedA	3												**3**
estb, estap, faeG, fedA, stx2						3							**3**
estb, estap, fedA, stx2						454							**454**
estb, estap, stx2												44	**44**
estb, stx2												6	**6**
estb, stx2, fedA						10							**10**
stx2							367						**367**
stx2, fedA			1348										**1348**
stx2, faeG, fedA									2				**2**
faeG					44								**44**
fanA					1								**1**
fasA					2								**2**
fedA					790								**790**
fimF41a					23								**23**
faeG, fasA					1								**1**
faeG, fedA					1								**1**
Total number	3369	2650	1348	1310	862	563	367	1	2	23	3	75	**10,573**

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 that involved the screening of other animals/humans were included only for data as defined under (i) and (ii).

Country [a]	Year of isolation	Source of samples [b]	No. of *E. coli* isolates tested for *mcr* genes [c]	*mcr-1*	*mcr-2*	*mcr-3*	*mcr-4*	*mcr-5*	*mcr-6 – mcr-10*	Reference
				% (no. of positive isolates per no. of tested isolates)					no. of pos. isolates	
AUT	n.t.	**Clinical samples of suckling and weaning pigs**	102 Ec (22.5% ETEC, 4.9% EDEC)	2.9% (3/102)	0% (0/102)	0% (0/102)	0% (0/102)	0% (0/102)	n.t.	[44]
BEL	2011-2012	**Diarrheic pigs**	53 ColR Ec	13.2% (7/53)	20.8% (11/53)	n.t.	n.t.	n.t.	n.t.	[17, 45]
CHN	2015-2016	**PWD**	5 Ec: 3 hybrid ETEC/STEC, 2 aEPEC	100% (5/5) (3 *mcr-1* & *mcr-3*)	0% (0/5)	60% (3/5) (3 *mcr-1* & *mcr-3*)	0% (0/5)	0% (0/5)	0/5	[49]
CHN	2017-2018	**5 swine farms**	86 Ec which were positive for at least one VAG associated with porcine ExPEC	100% (86/86)	0% (0/86)	0% (0/86)	0% (0/86)	0% (0/86)	n.t.	[46]
CHN	2020-2021	**Diarrheic pigs**	19 ETEC	0% (0/19)	0% (0/19)	0% (0/19)	0% (0/19)	0% (0/19)	0/19	[50]
ESP	1999-2018	**Clinical cases of diarrhoea in neonatal and post-weaned piglets**	200	7% (14/200)	0% (0/200)	0% (0/200)	13% (27/200)	3% (6/200)	n.t.	[32]
ESP	2005-2014	**PWD**	70 (10/year)	20.0% (14/70)	0% (0/90)	0% (0/90)	1.4% (1/70)	0% (0/70)	n.t.	[8]
ESP	2006-2016	**Enteric colibacillosis**	481 Ec tested for *mcr-1*	25.6% (123/481) (3 *mcr-1* & *mcr-4*,	0% (0/65)	0% (0/65)	4.6% (3/65)	6.2% (4/65)	n.t.	[26]

Country [a]	Year of isolation	Source of samples [b]	No. of *E. coli* isolates tested for *mcr* genes [c]	*mcr-1*	*mcr-2*	*mcr-3*	*mcr-4*	*mcr-5*	*mcr-6 – mcr-10*	Reference
				% (no. of positive isolates per no. of tested isolates)					no. of pos. isolates	
			65/123 *mcr-1*-pos. Ec tested for *mcr-2, -3, -4, -5*	4 *mcr-1* & *mcr-5*)			(3 *mcr-1* & *mcr-4*)	(4 *mcr-1* & *mcr-5*)		
ESP	2006-2016	**Enteric colibacillosis**	35 *mcr*-positive (35/499 investigated isolates)	54.3% (19/35) (2 *mcr-1 & mcr-4*)	0% (0/35)	0% (0/35)	45.7% (16/35) (2 *mcr-1 & mcr-4*)	5.7% (2/35)	n.t.	[10]
ESP	2006-2016	**Diarrheic piglets and pork meat**	11 APEC-, UPEC-, and ExPEC-like	45.5% (5/11)	0% (0/11)	0% (0/11)	0% (0/11)	0% (0/11)	0/11	[51]
ESP	2006-2017	**PWD**	186 Ec: 86.5 % ETEC (161/186) 5.4% STEC (10/186) 8.1% hybrid ETEC/STEC (15/186)	19.9% (37/186) (2 *mcr-1* & *mcr-4*, 2 *mcr-1* & *mcr-5*)	0% (0/186)	0% (0/186)	54.8% (102/186) (2 *mcr-1* & *mcr-4*, 1 *mcr-4* & *mcr-5*)	2.7% (5/186) (2 *mcr-1* & *mcr-5*, 1 *mcr-4* & *mcr-5*]	n.t.	[3,9]
ITA	2015-2016	**PWD**	51	72.5% (37/51)	0% (0/14 *mcr-1*-negative)	n.t.	n.t.	n.t.	n.t.	[11]
ITA	After 2019	**Supected enteritis**	1	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	0% (0/1)	*mcr-9* (1/1) *mcr-6, -7, -8, -10* (0)	[52]
ITA, ESP, BEL	2015-2016	**PWD**	125 (50 ColR) ITA: 34, Spain: 43, BEL: 48	25.6% (32/125)	2.4% (3/125) (BEL: P 2, CA 1; see Ref. [1])	0% (0/125)	8.8% (11/125)	n.t.	n.t.	[12]
JPN	2011-2015	**4 pigs with edema**	4 Ec (4/4 STEC)	100% (4/4)	n.t.	n.t.	n.t.	n.t.	n.t.	[47]

Country [a]	Year of isolation	Source of samples [b]	No. of *E. coli* isolates tested for *mcr* genes [c]	*mcr-1*	*mcr-2*	*mcr-3*	*mcr-4*	*mcr-5*	*mcr-6 – mcr-10*	Reference
				% (no. of positive isolates per no. of tested isolates)					no. of pos. isolates	
		disease from one farm								
JPN	2004-2014	**Diseased swine**	684 (309/684 ColR)	13% (90/684)	n.t.	n.t.	n.t.	n.t.	n.t.	[48]
JPN	2008-2015	**PWD**	120	30% (36/120) (5 *mcr-1* & *mcr-5*)	0% (0/120)	8.3% (10/120)	0% (0/120)	28.3% (34/120) (5 *mcr-1* & *mcr-5*)	*mcr-9* (1/120) *mcr-6, -7, -8, -10* (0/120)	[30,31]
KOR	2007-2016	**PWD (100 herds)**	364	1.1% (4/364) (3 *mcr-1* & *mcr-3*)	0% (0/364)	2.2% (8/364) (3 *mcr-1* & *mcr-3*)	n.t.	n.t.	n.t.	[35]
KOR	2011-2018	**Diseased pigs**	31	n.t.	n.t.	45.2% (14/31)	n.t.	n.t.	n.t.	[36]
THA	2007-2013	**Clinically sick pigs**	2 ColR	100% (2/2) (2 *mcr-1 & mcr-3*)	0% (0/2)	100% (2/2) (2 *mcr-1 & mcr-3*)	0% (0/2)	n.t.	n.t.	[37]
THA	2011-2018	**Diarrheic pigs**	100	20% (20/100) (13 *mcr-1* & *mcr-3*)	0% (0/100)	70% (70/100) (13 *mcr-1* & *mcr-3*)	0% (0/100)	n.t.	n.t.	[38]
THA	2017-2020	**Healthy farrowing sows and suckling piglets**	70 ColR Ec: 20 *mcr*-pos. Ec tested for VAGs present in ETEC and EHEC - non-pathogenic (8/20), ETEC-like (6/20), ETEC (5/20), hybrid	24.3% (17/70)	0% (0/70)	4.3% (3/70)	0% (0/70)	0% (0/70)	*mcr-6, -7, -8* (0/70) *mcr-9, -10* n.t.	[33]

Country [a]	Year of isolation	Source of samples [b]	No. of *E. coli* isolates tested for *mcr* genes [c]	*mcr-1*	*mcr-2*	*mcr-3*	*mcr-4*	*mcr-5*	*mcr-6 – mcr-10*	Reference
				% (no. of positive isolates per no. of tested isolates)					no. of pos. isolates	
			EHEC-ETEC (1/20)							
THA	2018-2019	**Diarrheic pigs during edema disease outbreak**	37 (12 non-pathogenic Ec, 9 ETEC-like, 7 AEEC, 5 ETEC, 2 Adhesive Fimbriae Ec, 1 STEC, 1 EDEC)	48.6% (18/37) (10 *mcr-1* & *mcr-3*)	0% (0/37)	54.1% (20/37) (*10 mcr-1* & *mcr-3*)	0% (0/37)	0% (0/37)	0/37	[39]
TWN	2012-2016	**Diseased swine**	In total 492 Ec (2012: 96 Ec 2013: 252 Ec 2016: 144 Ec)	29.7% (146/492)	n.t.	n.t.	n.t.	n.t.	n.t.	[29]

[a] 3-letter country abbreviation: AUT, Austria; BEL, Belgium; CHN, China; ESP, Spain; ITA, Italy; JPN, Japan; KOR, The Republic of Korea; THA, Thailand; TWN, Taiwan.

[b] Abbreviation: PWD, Post-weaning diarrhea

[c] Abbreviations: AEEC, attaching and effacing *E. coli*; aEPEC, atypical enteropathogenic *E. coli*; APEC, avian pathogenic *E. coli*; ColR, colistin resistant; Ec, *E. coli*; EDEC, edema disease *E. coli*; EHEC, enterohaemorrhagic *E. coli*; ETEC, enterotoxigenic *E. coli*; ETEC-like, enterotoxigenic-like *E. coli*; ExPEC, extraintestinal pathogenic *E. coli*; STEC, Shiga toxin producing *E. coli*; UPEC, uropathogenic *E. coli*; VAGs, virulence-associated genes.
n.t. = not tested

microorganisms

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Repeated Occurrence of Mobile Colistin Resistance Gene-Carrying Plasmids in Pathogenic *Escherichia coli* from German Pig Farms

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Abstract: The global spread of plasmid-mediated mobile colistin resistance (*mcr*) genes threatens the vital role of colistin as a drug of last resort. We investigated whether the recurrent occurrence of specific *E. coli* pathotypes and plasmids in individual pig farms resulted from the continued presence or repeated reintroduction of distinct *E. coli* strains. *E. coli* isolates (n = 154) obtained from three pig farms with at least four consecutive years of *mcr* detection positive for virulence-associated genes (VAGs) predicting an intestinal pathogenic pathotype via polymerase chain reaction were analyzed. Detailed investigation of VAGs, antimicrobial resistance genes and plasmid Inc types was conducted using whole genome sequencing for 87 selected isolates. Sixty-one *E. coli* isolates harbored *mcr-1*, and one isolate carried *mcr-4*. On Farm 1, *mcr*-positive isolates were either edema disease *E. coli* (EDEC; 77.3%) or enterotoxigenic *E. coli* (ETEC; 22.7%). On Farm 2, all *mcr*-positive strains were ETEC, while *mcr*-positive isolates from Farm 3 showed a wider range of pathotypes. The *mcr-1.1* gene was located on IncHI2 (Farm 1), IncX4 (Farm 2) or IncX4 and IncI2 plasmids (Farm 3). These findings suggest that various pathogenic *E. coli* strains play an important role in maintaining plasmid-encoded colistin resistance genes in the pig environment over time.

Keywords: *Escherichia coli*; ETEC; EDEC; mobile colistin resistance; *mcr-1*; plasmid; IncX4; IncHI2; IncI2; swine

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1. Introduction

The emergence and spread of multidrug-resistant bacteria is a rising problem that threatens the effective treatment of infectious diseases in humans and animals [1]. *Escherichia* (*E.*) *coli* is a ubiquitous Gram-negative bacterium that is regularly found in the intestinal tract of humans and animals. Intestinal pathogenic *E. coli* (InPEC) strains can cause a number of different diseases, including diarrhea.

In pigs, neonatal and post-weaning diarrhea is a widespread and often severe disease, resulting in significant economic losses in the swine industry worldwide [2]. Certain *E. coli* pathotypes are associated with causing enteric diseases in piglets, e.g., enterotoxigenic *E. coli* (ETEC) and atypical enteropathogenic *E. coli* (aEPEC) [3]. *E. coli* isolates producing the Shiga toxin subtype Stx2e, encoded by the *stx2e* gene, are the causative agent of edema disease in weaned piglets [4]. Colistin has been widely used to treat intestinal infections in swine caused by InPEC [5]. In 2015, the use of colistin came under scrutiny due to the emergence of colistin-resistant bacteria due to a transferable colistin resistance gene [6]. A recent study from 2022 reported that 51.9% of 662 veterinarians surveyed stopped the use of colistin and 33.4% reduced their usage. The main indication for the use of colistin was gastrointestinal disease in pigs [6]. The increasing occurrence of antimicrobial resistance

(AMR) against this last resort antibiotic incites new debates for additional regulations of its usage, especially in veterinary medicine [7].

Until a few years ago, acquired colistin resistance in bacteria was mainly attributed to chromosomal mutations such as modifications of two-component systems like *pmrA/pmrB* and *phoP/phoQ* [8,9]. In 2015, Liu et al. described for the first time a mobile colistin resistance gene that was found on a transmissible plasmid in one *E. coli* isolate from a Chinese pig, thereafter named *mcr-1* [10]. Since then, ten different *mcr* genes (*mcr-1*–*mcr-10*) have been reported with numerous variants, mostly found in Gram-negative bacterial species [11–19]. Over the years, *mcr*-mediated colistin resistance has been reported in Enterobacterales isolated from several sources, including humans, livestock, companion animals, wildlife and the environment [20–23].

The conjugative transfer of *mcr*-harboring plasmids between bacteria plays a vital role in the dissemination of AMR against colistin [24]. The first discovered *mcr-1* gene was located on a plasmid (pHNSHP45) of 64,015 bp in size, possessing a typical IncI2-type backbone [10]. Since then, *mcr-1* has been found on numerous plasmid types, such as IncHI1, IncHI2, IncF, IncN, IncP, IncX4 and IncN1-IncHI2 [25–27]. Resistance genes *mcr-2*, *mcr-3*, *mcr-6*, *mcr-7*, *mcr-8*, *mcr-9* and *mcr-10* were detected frequently on plasmids of types IncX4, IncHI2, IncHI2, IncI2, IncF type, IncHI2/HI2A and IncF, respectively [24].

The temporal and spatial distribution of *mcr*-positive isolates among pathogenic *E. coli* at individual farm levels has rarely been studied. Miguela-Villoldo et al. investigated *mcr-1* prevalence in healthy Spanish food-producing pigs from 1998 to 2021, selecting 50 caecal pig samples across 14 years [28]. While the frequency of *mcr-1*-positive samples increased from 2004 (16%) to 2015 (66%), a downward trend was observed from 2017 (54%) to 2021 (17%). In Germany, the *mcr-1* gene was detected in 15 different pig-fattening farms where pooled feces and boot swabs had been collected from 2011 to 2012 [29]. Further analyses of one representative *mcr-1*-containing *E. coli* isolate per farm showed that *mcr-1* was mainly located on IncX4 plasmids (n = 9) but also on IncHI2 (n = 3), IncX4/N (n = 1), IncHI2/FIB/FII/X3 (n = 1) and integrated into the chromosome (n = 1).

This study investigated the occurrence and genomic location of *mcr* genes in pathogenic *E. coli* isolates obtained from three German pig farms over at least four years, which were chosen from a comprehensive in-house database. The genomes of a representative set of isolates were sequenced to determine the presence of virulence-associated genes (VAGs) characteristic for intestinal pathogenic *E. coli* (InPEC) pathotypes and AMR genes as well as their plasmid location. Based on core genome and plasmid comparisons, the repeated occurrence of distinct *E. coli* clones, as well as distinct resistance and virulence plasmids on different farms, was examined.

2. Materials and Methods

2.1. Study Inclusion Criteria for Farms

For this retrospective survey on the repeated occurrence of *mcr*-positive porcine pathogenic *E. coli*, suitable pig farms were selected based on specific criteria. From our database of more than 3000 registered pig farms in Germany that had sent samples for molecular typing of *E. coli* isolates in the past, we selected only farms from which at least 25 pathogenic *E. coli* isolates had been obtained and preserved over the years (n = 23 farms). This was based on our recently published collection of 10,573 *E. coli* isolates, each harboring at least one of ten VAGs, which were tested using PCR for the presence of *mcr-1* to *mcr-10* genes [30]. Briefly, *E. coli* isolates were obtained from feces or mucosal swabs (rectum or small intestine). Upon arrival at the laboratory, the samples 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). After approximately 18 h of incubation at 37 °C, up to six morphologically different, putative *E. coli* colonies were picked per sample and tested for the presence of VAGs. More details of sample collection and processing were published recently [30].

Subsequently, pig farms with less than 25 pigs sampled (n = 11) or no detection of *mcr* genes (n = 5) were excluded from the study, as well as farms with no isolates for six consecutive years (n = 3). Finally, only farms were selected where *mcr*-positive *E. coli* had been isolated from pig fecal samples for at least four consecutive years.

2.2. Whole Genome Sequencing

The genomic DNA of *E. coli* bacteria was extracted 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 achieve an average coverage of 90-fold. Quality control, including contamination removal and adapter trimming, was carried out using an in-house pipeline. De novo assemblies were generated using the SPAdes Genome Assembler (v3.15.5) with the "-isolate" flag [31]. The Bakta pipeline (v1.8.2) was employed using species-specific databases for genomic annotation of the bacterial genomes [32].

2.3. Phenotypic Resistance Testing, Antimicrobial Resistance Genes, Virulence-Associated Genes

All 87 whole genome-sequenced *E. coli* isolates were tested for antimicrobial susceptibilities using the broth microdilution method. An individual panel layout was used from the MICRONAUT system (Merlin Diagnostics, Bornheim-Hersel, Germany). Fourteen antimicrobial substances and/or combinations (in µg/mL: amikacin (0.25–32), amoxicillin/clavulanic acid (1/0.5–16/8), ampicillin (1–16), cefotaxime (0.016–2), ceftazidime (0.031–8), colistin (0.125–8), enrofloxacin (0.004–2), florfenicol (1–16), gentamicin (0.125–8), piperacillin/tazobactam (0.5/4–64/4), spectinomycin (4–64), sulfamethoxazole (4–256), tetracycline (0.25–8) and trimethoprim (0.25–8)) were included in this layout. *E. coli* strains ATCC 25922 and NCTC 13846 were used for quality control. Minimum inhibitory concentration (MIC) values were interpreted by means of defined clinical breakpoints set by the Clinical and Laboratory Standards Institute (CLSI) for veterinary and human Enterobacterales isolates [33]. For antibiotics without a defined breakpoint for *E. coli* (in the case of cefotaxime, sulfamethoxazole and trimethoprim), human breakpoints of Enterobacterales according to CLSI [34] were used, except for colistin, which was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines [35]. No veterinary or human breakpoints are available for enrofloxacin and spectinomycin from either CLSI or EUCAST. For both substances, the epidemiological cut-off (ECOFF) was used for *E. coli* provided by EUCAST (https://mic.eucast.org/search/, accessed on 5 March 2024), separating the *E. coli* population into a population without acquired or mutational resistance (wild-type) and a population with phenotypically detectable acquired resistance mechanisms (non-wild-type) against enrofloxacin and spectinomycin.

Antimicrobial resistance (AMR) genes and chromosomal point mutations related to antimicrobial resistance were identified in all whole genome-sequenced isolates by using the online tool ResFinder 4.1, available on the website of the Center for Genomic Epidemiology (CGE) (https://cge.food.dtu.dk/services/ResFinder/, accessed on 17 December 2023). All isolates were investigated for VAGs using VirulenceFinder 2.0 (https://cge.food.dtu.dk/services/VirulenceFinder/, accessed on 17 December 2023). The results were additionally verified with multiple genome analysis accessible online from BacWGSTdb (http://bacdb.cn/BacWGSTdb/Tools.php, accessed on 17 December 2023).

2.4. Determination of Genoserotypes, Clonotypes, Multilocus Sequence Types, Core Genome MLS Types and Phylogroups

The genoserotypes of the whole genome-sequenced *E. coli* isolates were determined by applying the web-based SerotypeFinder 2.0, provided by the Center for Genomic Epidemiology (https://cge.food.dtu.dk/services/SerotypeFinder/, accessed on 22 November 2023). The internal 469- and 489-nucleotide sequences of the *fumC* and *fimH* genes, respectively, were used for clonotyping [36]. Clonotypes (CH types), which are the com-

binations of allele assignments for *fumC* and *fimH*, were determined using CHTyper 1.0 (https://cge.food.dtu.dk/services/CHTyper/, accessed on 1 January 2024).

Multilocus sequence types (STs) were determined by applying MLST 2.0 (https://cge.food.dtu.dk/services/MLST/, accessed on 22 November 2023), which is based on the Achtman seven-gene MLST scheme that includes seven housekeeping genes (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA* and *recA*). *E. coli* isolates were defined as clonal when they displayed the same sequence type in addition to identical virulence and resistance gene profiles.

The core genome was calculated using Roary [37]. Phylogenetic analysis was performed by using the web-based tool ClermontTyper (IAME, http://clermontyping.iame-research.center/index.php, accessed 18 December 2023), allowing us to assign tested strains to *E. albertii*, *E. fergusonii*, *Escherichia* cryptic clades I-V, *E. coli sensu strico* as well as to the main phylogroups A, B1, B2, C, D, E, F and G [38,39]. The remaining non-sequenced *E. coli* isolates were tested in a quadruplex PCR, allowing for the assignment to the eight main phylogroups A, B1, B2, C, D, E, F, G and *Escherichia* cryptic clades I-V [39,40].

2.5. Genomic Location of Virulence-Associated Genes and mcr Genes, Plasmid Analysis

To determine the location of *mcr* genes, two methods were used. Whole genome sequence data were analyzed for the location of AMR and virulence genes using the tool "Chromosome & Plasmid Overview" implemented in the software package Ridom SeqSphere$_+$ (http://www3.ridom.de/seqsphere, accessed 22 January 2024). To identify plasmid replicon types, PlasmidFinder 2.1 (https://cge.food.dtu.dk/services/PlasmidFinder/, accessed 22 January 2024) and Ridom SeqSphere+ were employed. The BacWGST database (http://bacdb.cn/BacWGSTdb/Tools.php, accessed on 17 December 2023) and RidomSeqSphere+ were applied to detect closely related plasmids via sequence comparison. To illustrate circular comparisons between the plasmids, we used the blast ring image generator software BRIG Version 0.95 [41].

To confirm the results of sequence analysis regarding the genomic location of *mcr-1* genes, an additional method was employed. The location of *mcr-1* genes in 38 *E. coli* isolates was determined via the S1 nuclease digestion of genomic DNA and pulsed-field gel electrophoresis (PFGE), followed by Southern blot hybridization (SBH). For this method, digoxigenin-labeled DNA probes ("DIG luminescent detection Kit" Boehringer Mannheim GmbH, Mannheim, Germany) were generated, targeting the PCR fragments specific for the *mcr-1* gene [10].

3. Results

3.1. Farm Selection

Farms in Hesse (n = 2) and North Rhine Westphalia (n = 1), Germany, met the chosen study inclusion criteria. Farm 1 sent samples (41 × feces, 1 × intestine + feces, and 1 × *E. coli* isolate) from May 2002 to October 2019. Samples were obtained from 43 pigs, most of them suffering from diarrheal disease. From this sample material, 50 *E. coli* isolates were cultivated that were positive for at least one VAG associated with certain pathotypes of InPEC as determined by PCR [30]. Twenty-two (44%) of these *E. coli* isolates, obtained from July 2009 to September 2012, tested positive for *mcr-1* genes (Table 1).

Farm 2 submitted samples from 69 pigs between June 2004 and February 2021. Samples were mainly feces (n = 43), followed by directly submitted *E. coli* isolates (n = 22) and by isolates obtained from the intestine and feces (n = 4). From these samples, 78 VAG-positive *E. coli* isolates were stored. Twenty-four (30.8%) isolates, obtained from July 2013 to February 2018, proved *mcr-1*-positive (Table 1).

Table 1. Sample collection from three farms and predicted pathotypes from all *E. coli* isolates and *mcr*-positive *E. coli* isolates per farm.

			***E. coli* Isolates Possessing InPEC-Related VAGs ***			***mcr*-Positive *E. coli* Isolates Possessing InPEC-Related VAGs**		
Farm No.	**No. of Sampled Pigs**	**Sample Type (No.)**	**No.**	**Predicted Pathotypes ****	**Sample Collection Period**	**No.**	**Predicted Pathotypes**	**Isolate Collection Period**
Farm 1	43	feces (41), intestine + feces (1), isolate (1)	50	EDEC (23), ETEC (21), STEC (3), ETEC-like (2), AdhF-*Ec* (1)	05/2002–10/2019	22	EDEC (17), ETEC (5)	07/2009–09/2012
Farm 2	69	feces (43), isolate (22), intestine + feces (4)	78	ETEC (53), ETEC-like (7), EDEC (6), AEEC (6), AdhF-*Ec* (3), ETEC/STEC (2), STEC (1)	06/2004–02/2021	24	ETEC (24)	07/2013–02/2018
Farm 3	25	isolate (23), feces (2)	26	ETEC/STEC (14), ETEC (4), EDEC (3), ETEC-like (3), STEC (2)	10/2014–04/2019	16	ETEC/STEC (11), EDEC (2), ETEC (1), ETEC-like (1), STEC (1)	10/2014–04/2019

* VAGs = virulence-associated genes; InPEC = positive for at least one of the following virulence factors (and encoding genes): F4 (*faeG*), F5 (*fanA*), F6 (*fasA*), F18 (*fedA*) and F41 (*fimF41a*) (adhesive fimbriae); LT-Ia, LT-Ib (*eltB-Ip*), ST-Ia (*estap*) and ST-II (*estb*) (heat-labile/stable enterotoxins); stx2, including stx2e (*stx2*) (Shiga toxin) or intimin (*eae*) in a modified multiplex PCR [30]. ** Pathotypes: AdhF-*Ec*, positive for at least one adhesive fimbriae gene (*faeG, fanA, fasA, fedA* and *fimF41a*); AEEC, positive for *eae*; EDEC, positive for *fedA* and *stx2*; ETEC, positive for at least one adhesive fimbriae gene (*faeG, fanA, fasA, fedA* and *fimF41a*) and at least one enterotoxin gene (*eltB-Ip, estap* and *estb*); ETEC-like, positive for at least one enterotoxin gene (*eltB-Ip, estap* and *estb*); ETEC/STEC, positive for at least one adhesive fimbriae gene (*faeG, fanA, fasA, fedA* and *fimF41a*) and at least one enterotoxin gene (*eltB-Ip, estap* and *estb*) and *stx2*; STEC, positive for *stx2*.

From October 2014 to April 2019, 26 VAG-positive *E. coli* were isolated from 25 sampled pigs of Farm 3. Most isolates (n = 23) were received through submissions from other veterinary diagnostic laboratories for further molecular typing in our institute, supplemented by two fecal samples. In total, 16 (61.5%) *mcr*-positive pathogenic *E. coli* were retrieved over more than four years (Table 1).

In total, 154 intestinal pathogenic *E. coli* isolates were obtained from 137 pigs housed on the selected three farms from May 2002 to February 2021. The total number of *mcr*-positive isolates was 62 (40.3%).

3.2. E. coli Pathotypes

A prediction of pathotypes was conducted for all 154 *E. coli* isolates included in this study based on the presence of certain VAGs obtained from PCR analysis, as described previously [30]: adhesive fimbriae *E. coli* (in the following termed AdhF-*Ec*), positive only for at least one adhesive fimbriae gene (*faeG, fanA, fasA, fedA* and *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* and *fimF41a*) and at least one enterotoxin gene (*eltB-Ip, estap* and *estb*); ETEC-like, positive only for at least one enterotoxin gene (*eltB-Ip, estap* and *estb*); ETEC/STEC hybrid (in the following simply termed ETEC/STEC), positive for at least one adhesive fimbriae gene (*faeG, fanA, fasA, fedA* and *fimF41a*) and at least one enterotoxin gene (*eltB-Ip, estap* and *estb*) and *stx2*; STEC, positive only for *stx2*.

The most common pathotypes detected in all farms were ETEC (n = 78; 50.7%) and EDEC (n = 32; 20.8%). ETEC/STEC (n = 16; 10.4%) and ETEC-like (n = 12; 7.8%) were found in farms 2 and 3 and all three farms, respectively. AEEC (n = 6; 3.9%), STEC (n = 6; 3.9%) and AdhF-*Ec* (n = 4; 2.6%) were rarely identified. Details about the distribution of *E. coli* pathotypes according to farms are given in Table 1.

Additionally, with regard to *mcr-1*-positive pathogenic *E. coli*, ETEC (*n* = 29; 48.3%) and EDEC (*n* = 18; 30%) were the most common pathotypes, followed by ETEC/STEC (*n* = 11; 18.3%) and STEC (*n* = 2; 3.3%). On Farm 1, 81% of the *mcr-1*-positive isolates were EDEC (*n* = 17) and 19% ETEC (*n* = 4) (Table 1). On Farm 2, all *mcr-1*-positive isolates were ETEC. The distribution of *mcr-1*-positive isolates on Farm 3 was as follows: ETEC/STEC (*n* = 11; 73.3%), followed by EDEC (*n* = 2; 13.3%), STEC (*n* = 1; 6.7%), and ETEC (*n* = 1; 6.7%).

3.3. Virulence Associated Genes and Virulence Plasmids

The genomes of 87 *E. coli* isolates were sequenced to conduct further molecular analysis, specifically regarding VAGs, as well as *mcr* detection and genomic localization. The isolates included 38 *mcr-1.1*-positive, 1 *mcr-4.8*-positive and 48 *mcr*-negative *E. coli* isolates (Table 2). At least one isolate per year and farm was selected for genome sequencing. If there were several *mcr*-positive isolates of different pathotypes per year and farm, at least one representative isolate per pathotype was selected. The *mcr*-negative isolates (one per pathotype) were additionally sequenced from the years with detected *mcr* occurrence.

The majority of the VAGs identified via PCR (Section 3.1) were also identified in the genomic data (Table 2). The adhesive fimbriae F18 (encoded by the *fedA* gene) were further classified into F18 fimbrial subtypes F18ab (*fedAab*) and F18ac (*fedAac*). In total, 19 isolates encoded for the F18ab subtype, while 21 isolates encoded for F18ac. One isolate carrying the *fedA* gene was not further typeable (Farm 1, IHIT52950). EDEC, defined as isolates positive for *fedA* and *stx2e*, was predicted in 94.7% of all F18ab-positive bacteria. Isolates encoding for F18ac mostly harbored enterotoxin genes (ETEC; 61.9%) or enterotoxin and Shiga toxin genes (ETEC/STEC; 33.3%). All of the isolates that tested positive for F4 fimbriae (encoded by *faeG*) in the PCR were identified as the F4ac subtype (*faeGac*) via genome analysis. This subtype is known to be the most prevalent in piglets with post-weaning diarrhea [2]. Genomic data from 30 isolates positive for the *stx2* gene via PCR revealed that they encoded for Shiga toxin subtype Stx2e (*stx2e*), which plays a pivotal role in the pathogenesis of edema disease in swine [4].

Over the years, *E. coli* isolates from all three farms repeatedly harbored similar virulence plasmids that either carried fimbrial genes, enterotoxin genes or both. Two reference plasmids were identified in the NCBI database, which showed high similarities to these virulence plasmids. To illustrate similarities between the study and reference plasmids, we selected one representative isolate per year and farm for BRIG analysis (Figure 1). Table 2 provides details on the *E. coli* strains used as representative isolates, including isolation year, pathotype, and ST.

Reference plasmid p15ODTXV (NCBI Reference Sequence: MG904998.1) was identified in an ETEC/STEC strain isolated from a diarrheic pig in Switzerland in 2014/2015. It carried *estap*, *estb*, *fedA* and *hlyDBAC* virulence genes. Representative plasmids identified in four isolates from Farm 3 were highly similar to the IncFII/IncX1 multivirulence reference plasmid used in Figure 1A. Virulence plasmids detected in Farm 1 (*n* = 19) and Farm 2 (*n* = 10) resembling p15ODTXV showed varying similarities over the years and mostly carried either *fedAab* (*n* = 15) or *fedAac* (*n* = 11; Table S1) genes.

Plasmids with high similarity to the IncFII reference plasmid pUMNK88_K88 (NCBI Reference Sequence: CP002730.1) were found in all three farms. In 2007, one ETEC strain from a pig with neonatal diarrhea in the USA was found to contain the pUMNK88_K88 plasmid, which carried *faeG* as a virulence gene. BLAST analysis revealed two very similar IncFII plasmids harbored by two strains positive for *faeGac* from Farm 1 and Farm 3, respectively (Table S1). In Farm 2, IncFII plasmids encoding for *faeGac* were found in ten ETEC strains (all ST100) isolated in the years 2005 to 2018 (Figure 1B). A comparison of the plasmids from this study with reference plasmids demonstrated structural resemblance of virulence plasmids over several years within and, in part, also across farms (e.g., plasmid pUMNK88_K88).

Table 2. Characteristics of 87 (27/44/16) *Escherichia coli* isolates, sorted by Farms 1–3, MLST and date of isolation.

Strain ID	Source *	Date of Isolation	VAGs **	Pathotype	MLST	Clonotype	Genoserotype ***	Phylogroup	*mcr* Gene/*pmrB* Mutation	Colistin MIC (µg/mL)
Farm 1 (*n* = 27)										
IHIT46527	Feces	06/2005	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	-	1
IHIT48337	Feces	12/2005	*fedAab*, *stx2e*	EDEC	1	2-54	Ont:H1	D	-	8
IHIT46528	Feces	01/2006	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	-	4
IHIT46530	Feces	07/2009	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	*mcr-1.1*	8
IHIT45339	Feces	08/2009	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	*mcr-1.1*	8
IHIT46531	Feces	12/2009	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	*mcr-1.1*	4
IHIT46532	Feces	01/2010	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	*mcr-1.1*	8
IHIT46533	Feces	03/2010	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	*mcr-1.1*	8
IHIT48339	Feces	04/2010	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	*mcr-1.1*	8
IHIT32406	Feces	06/2010	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	*mcr-1.1*	8
IHIT48341	Feces	06/2010	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	*mcr-1.1*	8
IHIT45342	Feces	07/2011	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	*mcr-1.1*	8
IHIT46538	Feces	09/2012	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	*mcr-1.1*	8
IHIT46553	Isolate	10/2019	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	-	0.5
IHIT52949	Feces	06/2011	*estb*, *estap*, *fedAac*	ETEC	23	4-54	O8:H17	C	-	0.25
IHIT48327	Feces	04/2004	*estb*, *eltB-Ip*, *fedAac*	ETEC	42	28-65	O147:H14	D	*pmrB* V161G	2
IHIT48328	Feces	07/2004	*estb*, *estap* #, *fedAac*	ETEC	42	28-65	Ont:H14	D	*pmrB* V161G	0.5
IHIT48326	Feces	05/2002	*estb*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	-	1
IHIT52948	Feces	02/2008	*estb*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	-	0.25
IHIT45341	Feces	07/2011	*estb*, *eltB-Ip*, *faeGac*	ETEC	100	27-65	O149:H10	A	*mcr-1.1*	8
IHIT48351	Feces	12/2015	*estb* #, *eltB-Ip*, *faeGac*	ETEC	100	27-0	Ont:H10	A	-	0.5
IHIT46534	Feces	06/2010	*estb*, *estap*, *fedAac*	ETEC	131	40-683	O25:H4	B2	*mcr-1.1*	4
IHIT48340	Feces	06/2010	*estb*, *estap*, *fedAac*	ETEC	131	40-683	O25:H4	B2	*mcr-1.1*	4
IHIT48343	Feces	06/2011	*estb*, *estap* #, *fedAac*	ETEC	131	40-683	O25:H4	B2	*mcr-1.1*	8
IHIT52950	Feces	07/2012	*fedA*	AdhF-*Ec*	641	6-289	O121:H10	B1	-	0.5
IHIT48325	Feces	05/2002	*stx2e*	STEC	710	153-1582	Ont:H30	A	-	0.5
IHIT52947	Feces	12/2007	*stx2e*	STEC	12009	11-23	O142:H27	A	-	0.5
Farm 2 (*n* = 44)										
IHIT48329	Feces	07/2004	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	-	0.5
IHIT48336	Feces	08/2005	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	-	8
IHIT48354	Int + Fec	11/2020	*fedAab*	AdhF-*Ec*	1	2-54	O139:H1	D	-	0.5
IHIT48331	Feces	10/2004	*estb* #	ETEC-like	10	11-54	O163:H10	A	-	0.5

Table 2. *Cont.*

Strain ID	Source *	Date of Isolation	VAGs **	Pathotype	MLST	Clonotype	Genoserotype ***	Phylogroup	*mcr* Gene/*pmrB* Mutation	Colistin MIC (μg/mL)
IHIT46535	Feces	02/2011	*estb*, *estap*, *fedAac*	ETEC	10	11-24	O141:H4	A	-	0.5
IHIT46540	Isolate	07/2013	*estb*, *estap*, *fedAac*	ETEC	10	11-24	O141:H4	A	-	0.25
IHIT23335	Feces	07/2013	*estb*, *estap*, *fedAac*, *stx2e*	ETEC/STEC	10	11-24	O141:H4	A	-	0.25
IHIT48348	Feces	11/2014	*estb*	ETEC-like	10	11-45	Ont:H6	A	-	8
IHIT46541	Feces	11/2014	*estb*, *estap* #, *fedAac*	ETEC	10	11-24	O141:H4	A	*mcr-1.1*	4
IHIT46542	Isolate	01/2015	*estb*, *estap*, *fedAac*	ETEC	10	11-24	O141:H4	A	*mcr-1.1*	4
IHIT46550	Isolate	02/2018	*estb*, *estap*, *fedAac*	ETEC	10	11-24	O141:H4	A	*mcr-1.1*	4
IHIT48346	Feces	12/2012	*eae*	AEEC	20	4-25	Ont:H49	B1	-	0.5
IHIT48330	Feces	07/2004	*eae*	AEEC	29	4-24	O123:H11	B1	-	0.5
IHIT48333	Feces	12/2004	*estb*, *eltB-Ip*, *fedAac*	ETEC	42	28-65	O147:H14	D	*pmrB* V161G	4
IHIT48334	Feces	12/2004	*fedAac*	AdhF-*Ec*	42	28-65	O147:H14	D	*pmrB* V161G	4
IHIT48342	Feces	06/2010	*eae* #	AEEC	93	11-0	O5:H4	A	-	0.25
IHIT46526	Feces	06/2004	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	-	1
IHIT48332	Feces	10/2004	*estb*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	-	0.5
IHIT48335	Feces	03/2005	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	-	0.25
IHIT46529	Feces	02/2006	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	-	8
IHIT48338	Feces	08/2008	*estb*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	Ont:H10	A	-	4
IHIT46536	Feces	11/2011	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	-	0.5
IHIT46537	Feces	01/2012	*estb*, *estap* #, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	-	0.5
IHIT45353	Isolate	07/2013	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8
IHIT46539	Isolate	07/2013	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	-	0.25
IHIT25408	Isolate	03/2014	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	Ont:H10	A	*mcr-1.1*	0.5
IHIT48347	Isolate	05/2014	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8
IHIT27622	Isolate	10/2014	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8
IHIT45399	Isolate	01/2015	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8
IHIT48349	Isolate	07/2015	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	Ont:H10	A	*mcr-1.1*	8
IHIT45401	Isolate	09/2015	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8
IHIT46543	Isolate	09/2015	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	4
IHIT46546	Isolate	09/2016	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8
IHIT45407	Isolate	08/2017	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8
IHIT36144	Isolate	01/2018	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8
IHIT36146	Isolate	01/2018	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8
IHIT36426	Isolate	02/2018	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8
IHIT36427	Isolate	02/2018	*estb*, *estap*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8

Table 2. *Cont.*

Strain ID	Source *	Date of Isolation	VAGs **	Pathotype	MLST	Clonotype	Genoserotype ***	Phylogroup	*mcr* Gene/*pmrB* Mutation	Colistin MIC (µg/mL)
IHIT48355	Int + Fec	11/2020	*estb* #	ETEC-like	641	6-832	O45:H10	B1	-	2
IHIT48358	Feces	02/2021	*estb*	ETEC-like	641	6-289	O115:H10	B1	-	0.25
IHIT32748	Isolate	09/2016	*eae*	AEEC	793	168-555	O49:H10	A	-	0.5
IHIT48344	Feces	12/2011	*eae*	AEEC	799	84-305	O108:H9	E	-	0.25
IHIT48356	Int + Fec	11/2020	*stx2e*	STEC	955	2-65	O139:H1	D	-	0.5
IHIT48350	Feces	09/2015	*estb*	ETEC-like	2944	224-1082	O17/O77:H28	D	-	1
Farm 3 (*n* = 16)										
IHIT34769	Isolate	06/2017	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	*mcr-1.1*	4
IHIT48353	Isolate	06/2017	*faeGac*, *stx2e*	EDEC	1	2-54	Ont:H1	D	*mcr-1.1*	8
IHIT47062	Isolate	10/2017	*fedAab*, *stx2e*	EDEC	1	2-54	O139:H1	D	-	1
IHIT48352	Feces	12/2016	*stx2e*	STEC	10	11-23	Ont.:H32	A	*mcr-1.1*	8
IHIT39537	Isolate	04/2019	*estb*	ETEC-like	10	11-2594	O35:H6	A	*mcr-4.8*	4
IHIT47044	Isolate	10/2014	*estb*, *estap*, *fedAac*, *stx2e*	ETEC/STEC	86	6-32	Ont:H10	B1	*mcr-1.1*	0.5
IHIT47045	Isolate	10/2014	*estb*, *estap*, *fedAac*, *stx2* #	ETEC/STEC	86	6-32	O86:H10	B1	-	1
IHIT47046	Isolate	06/2015	*estb*, *estap*, *fedAac*, *stx2e*	ETEC/STEC	86	6-32	O86:H10	B1	*mcr-1.1*	4
IHIT47048	Isolate	06/2015	*estb*, *estap*, *fedAac*, *stx2e*	ETEC/STEC	86	6-32	O86:H10	B1	-	0.25
IHIT47056	Isolate	06/2016	*estb*, *estap*, *fedAac*, *stx2e*	ETEC/STEC	86	6-32	Ont:H10	B1	*mcr-1.1*	4
IHIT47057	Isolate	11/2016	*estb*, *estap*, *fedAac*	ETEC	86	6-32	O86:H10	B1	-	0.5
IHIT34315	Isolate	04/2017	*estb*, *estap*, *fedAac*, *stx2e*	ETEC/STEC	86	6-32	Ont:H10	B1	*mcr-1.1*	4
IHIT47060	Isolate	02/2017	*estb*, *eltB-Ip*, *faeGac*	ETEC	90	4-54	O8:H19	C	-	0.5
IHIT47047	Isolate	06/2015	*estb*, *eltB-Ip*, *faeGac*	ETEC	100	27-0	O149:H10	A	*mcr-1.1*	8
IHIT47065	Isolate	07/2018	*estb*	ETEC-like	118	4-331	O15:H45	E	-	0.5
IHIT47072	Isolate	03/2019	*estb*	ETEC-like	162	65-32	O8:H19	B1	-	0.25

* Int + Fec = intestine and feces. ** VAGs = virulence-associated genes for InPEC: *faeGac*, *fedAab* and *fedAac* (adhesive fimbriae); *eltB-Ip*, *estap* and *estb* (heat-labile/stable enterotoxins); *stx2* (Shiga toxin) or *eae* (intimin). VAGs marked with # were positive in the PCR but negative for the respective virulence gene according to whole genome data. *** Ont = O not typeable.

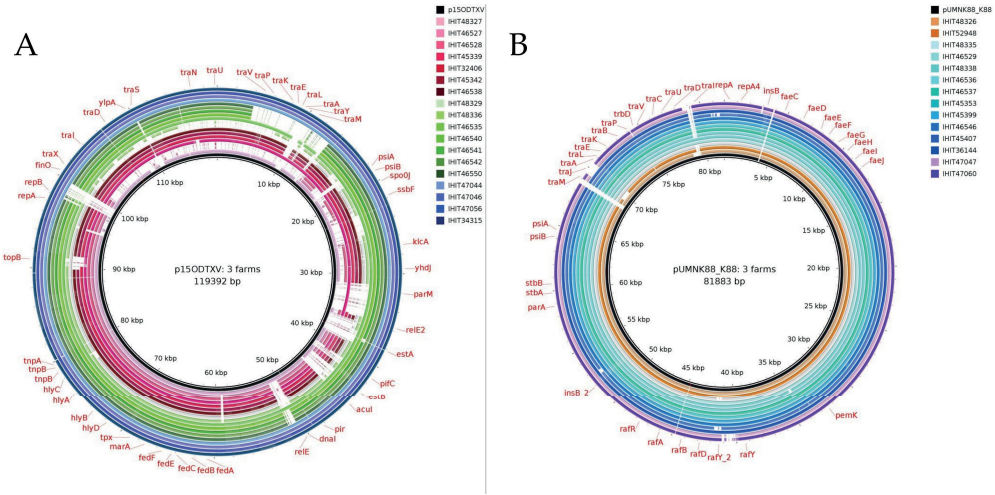

Figure 1. Schematic circular representation of *E. coli* virulence plasmids detected in all three farms over the years (details given in Table 2). For comparison, one representative isolate per year and farm was selected. The reference plasmids used were p15ODTXV (**A**); NCBI Reference Sequence: MG904998.1) and pUMNK88_K88 (**B**); NCBI Reference Sequence: CP002730.1), shown in the inner rings. The plasmids detected in isolates from the three farms are arranged according to farm and year of isolation. Isolates from the same farm are color-coded (**A**): Farm 1 = red, Farm 2 = green, Farm 3 = blue; (**B**): Farm 1 = orange, Farm 2 = turquoise/blue, Farm 3 = violet). The color of the earliest isolation is displayed as the lightest, while the color of the latest isolation is displayed as the darkest.

Table S2 provides a detailed distribution of all additionally detected VAGs among whole genome-sequenced isolates, sorted by farms and the categories: adhesion, tissue damage (hemolysin/toxin genes), invasion and protection, iron acquisition and secretion system.

3.4. Resistance Phenotypes and Genotypes

Most of the 87 isolates were resistant to ampicillin (86.2%; >16 µg/mL), sulfamethoxazole (88.5%; >256 µg/mL) and tetracycline (86.2%; >8 µg/mL). Lower resistance rates were observed with trimethoprim (42.5%; ≥16 µg/mL), gentamicin (9.2%; >8 µg/mL), florfenicol (4.6%; >16 µg/mL) and cefotaxime (1.1%; >2 µg/mL). Low MIC values were determined for enrofloxacin (83.9%; <0.125 µg/mL), assigning most of the isolates to a wild-type population, as defined by ECOFFs provided by EUCAST. More than half of the tested strains showed MIC values over 64 µg/mL for spectinomycin (62.1%), categorizing these isolates into a non-wild-type population. No isolate was resistant to amoxicillin/clavulanic acid (≥32/16 µg/mL), amikacin (≥64 µg/mL), ceftazidime (≥16 µg/mL) and piperacillin/tazobactam (≥128/4 µg/mL).

Colistin MICs ranged from 0.25 to 8 µg/mL. Fourty-one isolates proved susceptible to colistin, while fourty-six strains were resistant (52.9%; ≥4 µg/mL). Almost all *mcr*-positive isolates showed resistance to colistin, with a MIC ≥ 4 µg/mL, except for IHIT25408 (Farm 2) and IHIT47044 (Farm 3), which showed MICs of 0.5 µg/mL. The chromosomal point mutation in the *pmrB* gene (V161G), which is presumably associated with colistin resistance, was detected in four *mcr*-negative isolates (Farm 1: 2 ST42-ETEC; Farm 2: 1 ST42-AdhF-*Ec*; 1 ST42-ETEC). Two of these isolates showed phenotypic resistance to colistin (Table 2).

The same AMR genes were prevalent in all three farms, with *sul2* (85.2%), *aph(3")-Ib* (77.8%), *aph(6)-Id* (77.8%), bla_{TEM-1B} (77.8%) and *tet(A)* (74.1%) being the most frequently detected AMR genes in Farm 1. In Farm 2, the most prevalent AMR genes detected were *tet(A)* (79.6%), bla_{TEM-1B} (72.7%), *sul2* (68.2%), *aadA1* (45.5%), *aph(3")-Ib* (43.2%) and *aph(6)-Id* (43.2%). In Farm 3, the AMR genes most frequently found were *dfrA1* (75%), followed by *aph(3")-Ib* (68.8%), *aph(6)-Id* (68.8%), bla_{TEM-1B} (68.8%), *sul1* (68.8%), *sul2* (68.8%), *aadA1* (62.5) and *tet(A)* (62.5%). Chromosomal mutations in the *gyrA* and *parE* genes were identified in all three farms. The highest prevalence was observed in Farm 3, with 50% of the isolates showing the *gyrA* S83L mutation and 43.8% showing the *parE* I355T mutation. Details on the distribution of AMR genes and chromosomal point mutations are provided in Table S3.

AMR gene patterns (excluding *mcr* genes) differed among the *E. coli* isolates over time. In Farm 1, ST1-EDEC isolates from December 2005 to October 2019 revealed five different combinations of AMR genes (Table S3). In Farm 2, ST100-ETEC strains exhibited mainly two different AMR gene patterns over the years. Between November 2011 and May 2014, ST100-ETEC strains were found to carry multiple AMR genes, including *aadA5, aph(3")-Ib, aph(6)-Id,* bla_{TEM-1B}*, catB3, dfrA1, dfrA14, sul1, sul2* and *tet(A)*. However, ST100-ETEC strains isolated from October 2014 to February 2018 only tested positive for the genes bla_{TEM-1B}, *sul2* and *tet(A)*. On Farm 3, all isolates obtained over the time revealed different AMR gene patterns.

3.5. Multilocus Sequence Types, Clonotypes, Phylogenetic Groups

In the phylogenetic tree of all whole genome-sequenced *E. coli* isolates in this study, the majority of isolates from each farm clustered together, irrespective of the year of isolation (Figure 2). Clustering was also observed for *mcr*-positive isolates on each farm.

Overall, 20 known multilocus sequence types (STs) were determined (Table 2). ST1 was the predominant ST (60.9% of the isolates) on Farm 1, while the most prevalent STs on farms 2 and 3 were ST100 (n = 21; 48.8%) and ST86 (n = 7; 43.8%), respectively (Figure 2). Three *mcr-1*-positive ETEC isolates from Farm 2 (isolated between June 2010 and June 2011) belonged to ST131. This sequence type has previously been detected in ETEC strains isolated from pigs with diarrhea in Spain [3]. It is also known as the predominant *E. coli* lineage among extraintestinal pathogenic *E. coli* (ExPEC) worldwide, frequently associated with an ESBL phenotype and multidrug resistance [42].

Nine, fifteen and eight different clonotypes (CH types) were found in *E. coli* isolates from farms 1, 2 and 3, respectively (Table 2). The most frequent CH types on Farm 1 and Farm 2 were 2-54 (51.9%; all ST1-EDEC) and 27-0 (50%; all ST100-ETEC), respectively. CH type 6–32 was most common in Farm 3 (43.8%, 7 ST86-ETEC/STEC and one ST86-ETEC).

Phylogroups A (n = 58; 37.7%) and D (n = 53; 34.4%) were found to be the most common phylogroups among all 154 *E. coli* isolates (Figure 3). While the majority of *E. coli* isolates of Farm 1 were assigned to phylogroup D (n = 34; 68%), phylogroups A (n = 45; 57.7%) and B1 (n = 16; 61.5%), which were most frequent in isolates from Farms 2 and 3, respectively. Overall, only four, three and nine isolates were assigned to phylogenetic groups B2, C and E. No isolate belonged to phylogroup F or G.

Considering only isolates that were positive for *mcr* genes, a similar distribution of phylogroups per farm and in total was observed. The predominant phylogroups for farms 1 to 3 were D (n = 17; 81%), A (n = 20; 83.3%) and B1 (n = 11; 68.8%), respectively. Only phylogroups A and E (n = 4; 16.7%) were determined for *mcr*-positive isolates of Farm 2. No *mcr*-positive *E. coli* belonged to phylogroup C.

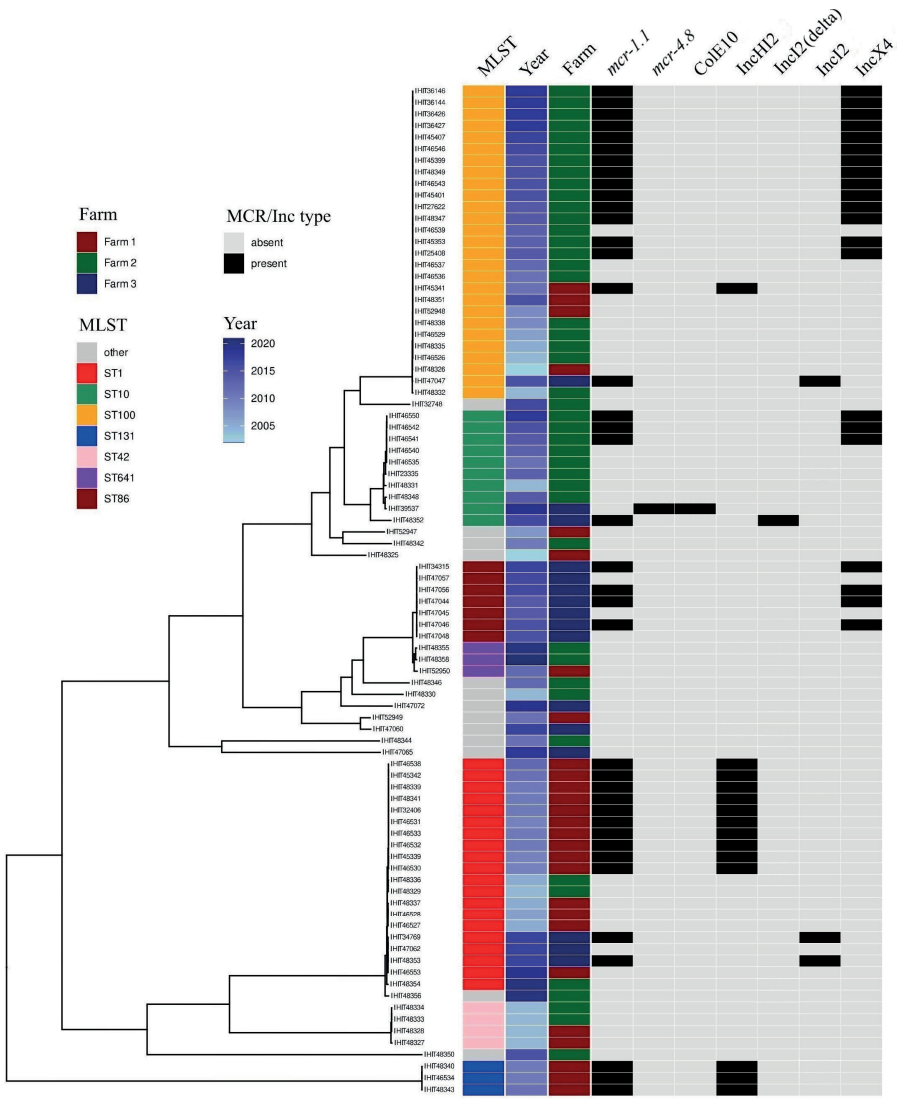

Figure 2. Neighbor-joining tree based on the comparison of 2670 core genome genes of 87 *E. coli* isolates from Farm 1, Farm 2 and Farm 3, with the respective multilocus sequence types (MLST) and isolation years color-coded. The occurrence of *mcr* genes and their location on different plasmids are shown. Farm specific clustering of isolates from different years can be observed for Farm 1, Farm 2 and Farm 3.

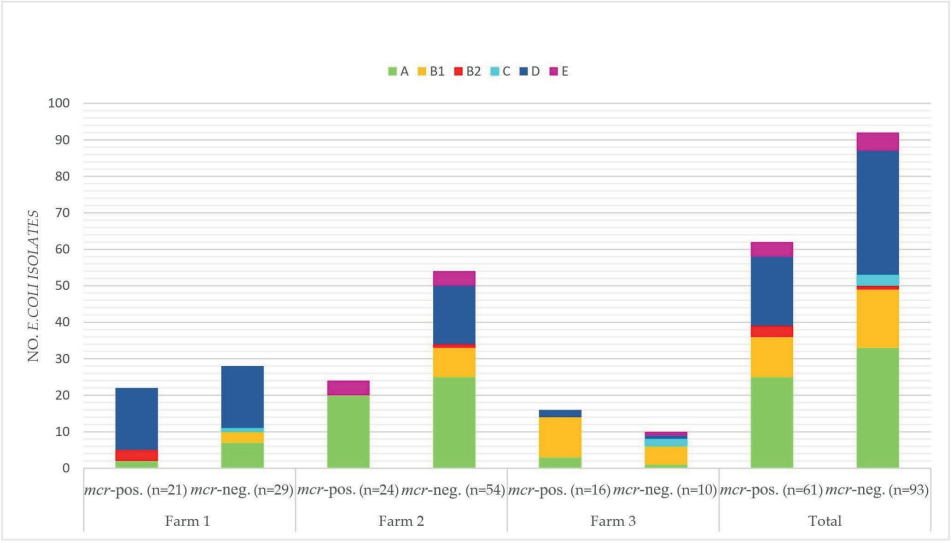

Figure 3. Overview of the phylogenetic grouping of *mcr*-positive and *mcr*-negative *E. coli* isolates sorted by Farms 1 to 3 and in total.

3.6. Genoserotypes

A total of 27 different genoserotypes were identified among 87 whole genome-sequenced *E. coli* isolates (Table 2). Fourteen isolates were not typeable (Ont). The most common genoserotype in isolates obtained from Farm 1 was O139:H1 (n = 13; 56.5%). This genoserotype has been associated with edema disease-causing *E. coli* in pigs in Europe [43]. Genoserotype O149:H10 was found repeatedly in isolates from Farm 2 (n = 18; 41.9%). Commonly reported O antigens of ETEC and EDEC isolates, such as O45, O139, O141, O147 and O149, were predominant (n = 52; 71.2%) among all typeable isolates of this study.

3.7. Genomic Location of mcr Genes and Plasmid Analysis

The genomic location of *mcr-1.1* in 38 whole genome-sequenced *E. coli* isolates was determined via Southern blot hybridization (SBH) of S1 nuclease-digested whole-cell DNA and analysis of genomic sequence data. We identified *mcr-1.1* on four different plasmids in this study. S1 nuclease PFGE and SBH initially revealed *mcr* genes on plasmids with approximate sizes of 250 kbp (n = 13), 35 kbp (n = 19) and 65 kbp (n = 3). Three isolates (IHIT34315, IHIT34769, and IHIT36427) did not reveal the genomic location of *mcr* genes through SBH despite repeated attempts.

Additionally, whole genome sequence analysis confirmed the occurrence of identical *mcr-1.1*-harboring plasmids per farm. All of the tested *mcr*-positive *E. coli* of Farm 1 carried *mcr-1.1* on IncHI2 plasmids (n = 14), while all 17 *mcr*-positive isolates of Farm 2 harbored *mcr-1.1* on IncX4 plasmids. *E. coli* isolates obtained from Farm 3 carried the *mcr-1.1* gene on IncX4 (n = 4), IncI2 (n = 3) and IncI2 (delta) (n = 1) plasmids. The *mcr-4.8* gene was located on a ColE10 plasmid, as previously reported [30].

The BacWGST database was used to identify closely related MCR-1 plasmids, which were then used as reference plasmids to illustrate circular comparisons with MCR-1 plasmids of representative isolates of this study (Figure 4).

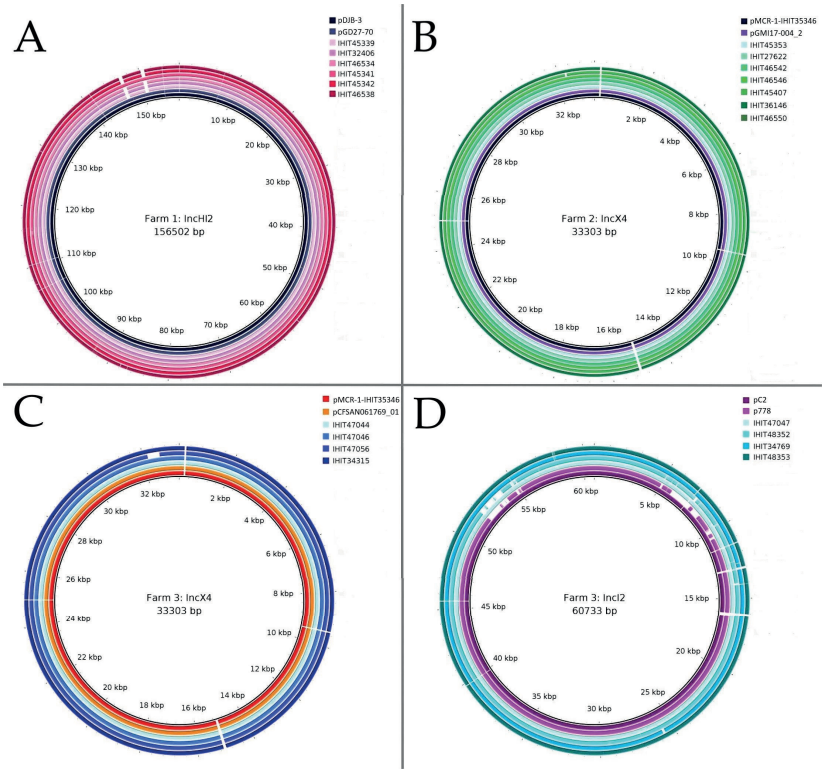

Figure 4. Schematic circular representation of detected MCR-1 plasmids from three farms in our study compared to most similar reference plasmids predicted using the BacWGST database. One representative *mcr*-positive *E. coli* isolate per year and farm was selected for comparison (details given in Table 2). If two different *mcr*-positive pathotypes were detected per year and farm, we selected both pathotypes. The plasmids from our study are arranged in order of isolation year, with the earliest (light color) on the third innermost ring and the latest (dark color) on the outermost ring in (**A**–**D**). The reference plasmids are shown in the two innermost rings. Reference plasmids (NCBI Reference Sequence in brackets) included are as follows: pDJB-3 (MK574666.1), pGD27-70 (MN232195.1) for Farm 1 (**A**); pMCR-1-IHIT35346 (KX894453.1), pGMI17-004_2 (NZ_CP028167.1) for Farm 2 (**B**); pMCR-1-IHIT35346 (KX894453.1), pCFSAN061769_01 (CP042970.1) for Farm 3 (**C**); pC2 (LC473131.1) and p778 (MN746292.1) for Farm 3 (**D**).

For comparison, we selected one *mcr*-positive representative *E. coli* isolate per year and farm. When two different pathotypes tested positive for *mcr* in the same year, both positive pathotypes were included. Six representative plasmids were detected in Farm 1 and identified as similar to two IncHI2 reference plasmids. The plasmids were found in IHIT45339 (EDEC; 2009), IHIT32406 (EDEC; 2010), IHIT46534 (ETEC; 2010), IHIT45341 (ETEC; 2011), IHIT45342 (EDEC; 2011) and IHIT46538 (EDEC; 2012), with nucleotide sequence identities ranging from 98.8% to 99.99% and coverage ranging from 90% to 97% compared to the references. The reference plasmids harboring *mcr-1.1* had been identified in *E. coli* strains isolated from swine in China (pDJB-3; NCBI Reference Sequence: MK574666.1;

used as a reference plasmid in Figure 4A) and broiler chicken in China (pGD27-70; NCBI Reference Sequence: MN232195.1).

The reference plasmid used for *mcr*-harboring plasmids from ETEC isolates in Farm 2 was the *mcr-1.1*-carrying IncX4 plasmid identified in an *E. coli* isolate obtained from pig feces in Italy in 2016 (pMCR-1-IHIT35346; identity ≥ 99.4%, coverage ≥ 95%; NCBI Reference Sequence: KX894453.1). Another very similar *mcr-1.1*-carrying IncX4 plasmid (pGMI17-004_2; NCBI Reference Sequence: NZ_CP028167.1) obtained from an *E. coli* isolate from poultry in Denmark in 2010 is also represented in Figure 4B.

The IncX4 plasmid pMCR-1-IHIT35346 was also used as a reference for all IncX4 plasmids detected in Farm 3 (identity ranging from 97.4% to 100%, coverage ≥ 98%) (Figure 4C). Additionally, one *E. coli* strain, isolated from raw milk cheese in Egypt in 2016, harbored one *mcr-1.1*-carrying IncX4, which showed high identity (≥ 99.96%, coverage ≥ 98%) to all four IncX4 plasmids from Farm 3 (pCFSAN061769_01; NCBI Reference Sequence: CP042970.1).

An *E. coli* isolate obtained from influent municipal wastewater in Japan harbored *mcr-1.1*-carrying IncI2 plasmid pC2 (NCBI Reference Sequence: LC473131.1), which was used as a reference in Figure 4D. This plasmid was highly similar to plasmids from Farm 3 (isolates IHIT47047, IHIT34769, IHIT48353), with identities ranging from 92.66% to 99.99% and coverage greater than 99%. However, IHIT48352 harbored one IncI2 (delta), which was similar to one *mcr-1.1*-carrying plasmid from a *Salmonella enterica* isolate obtained from a child in Ecuador (p778; 98.4% identity, coverage 100%; NCBI Reference Sequence: MN746292.1). None of the selected reference plasmids contained additional resistance genes besides *mcr-1.1*.

4. Discussion

The global dissemination of colistin resistance genes in bacteria from humans, animals and the environment has been reported over the last years [24,44]. The occurrence of *mcr* genes has been investigated in various studies, especially those conducted concerning pig and poultry production [45–47]. However, data on recurrent *mcr*-mediated resistance in livestock farms are scarce [48,49]. We were not able to identify clones of pathogenic *E. coli* over the years on the specific farms. However, our study described three German pig farms with the repeated occurrence of highly similar *mcr*-carrying plasmids in pathogenic *E. coli* isolates over two to more than four years, respectively.

In our study, ETEC was the prevalent pathotype for all collected isolates (78/154; 50.7%) as well as for *mcr-1*-positive isolates (30/61; 49.2%). The most common pathotypes for each farm also coincided with the prevalent pathotypes of *mcr-1*-harboring *E.coli*. ETEC has been reported to be the most common cause of post-weaning diarrhea (PWD) in swine, but is also known as a pathogen in humans [5,50]. An epidemiological study from Spain reported a significant association of ETEC (67%) with the occurrence of PWD in swine [3]. That study investigated 481 *E. coli* isolates from diarrheic pigs, of which 123 (25.6%) strains carried the *mcr-1* gene, with 57.7% belonging to the ETEC pathotype. This study also investigated the prevalence of the Shiga toxin gene *stx2*, which was found to be lower than in our study (10% vs. 35%) [3].

We identified IncFII/IncX1 and IncFII plasmids as the most frequent virulence plasmid types among the isolates from all three farms over the years. VAGs like *eltB-Ip*, *estap*, *estb*, and *fedABCEF* have previously been reported to be encoded on IncF or IncFII/IncX1 plasmids of *E. coli* strains isolated from diarrheic pigs [51,52]. Virulence plasmids have occasionally been described in ETEC and ETEC/STEC strains [52,53], but to the best of our knowledge, not in individual pig farms over the years. The striking similarity of certain virulence plasmids in single farms, such as in Farm 3 for four consecutive years (Figure 1A), suggests a local clonal distribution of virulence plasmids. However, it should be taken into account that specific virulence plasmids can be distributed across farms (Figure 1B) and countries. In summary, our data do not support clones (according to the strict definition used in our study design) of *E. coli* strains carrying specific virulence plasmids over the years.

Effelsberg et al. investigated the occurrence of *mcr-1* to *mcr-5* genes in 318 porcine fecal samples from 81 pig farms in northwest Germany collected from March 2018 to September 2020 [54]. Overall, ten farms (12.3%) provided fecal samples that contained *E. coli* harboring *mcr-1*. Two farms showed repeated presence of *mcr-1*-positive strains from two different dates of sampling. A retrospective study examined 436 boot swab and pooled fecal samples from 58 German pig-fattening farms for the presence of *mcr-1* and *mcr-2* [29]. The *mcr-1* gene was detected in 43 (9.9%) *E. coli* isolates obtained from 15 (25.9%) farms, which were almost evenly distributed in northern/western (25%), southern (25%), middle (21%) and eastern (36%) Germany in 2011 to 2012. Considering the presence of *mcr*-positive pathogenic *E. coli* strains on the farms examined in our study, the prevalence was high, with 42%, 30.8% and 61.5% for farms 1, 2 and 3, respectively. A longitudinal study from Thailand investigated the occurrence of *mcr* genes in one representative pig farm from 2017 to 2020 after the cessation of prophylactic colistin usage [48]. Samples were taken from pigs (n = 70), farm workers (n = 50) and wastewater (n = 50). While *mcr-1*-positive isolates (n = 4; 8%) were detected in humans only in 2017, the prevalence of colistin-resistant strains was 28.6% in pigs, with a declining trend over the years, and 18% in wastewater. Another study obtained mixed bacterial cultures (n = 35) from the environment of three German pig farms from 2011 to 2012 [55]. Seven *E. coli* isolates tested positive for *mcr-1*-harboring IncX4 plasmids. The positive samples were obtained from boot swabs, barn dog feces, stable flies and manure. In our study, we did not expect the recurrent identification of highly similar *mcr*-positive pathogenic *E. coli* isolates in three German pig farms over more than four years. This is a concerning observation because it suggests that the occurrence of resistant strains over the years may be largely undetected due to missing systematic observational data.

The predominant *mcr-1.1*-harboring plasmid types in our study were IncHI2 for farm 1, IncX4 for Farm 2 and both IncX4 and IncI2 plasmids for Farm 3. A systematic review by Matamoros et al. reported a total of 13 plasmid incompatibility types for *mcr-1*-carrying plasmids for 217 *Enterobacteriaceae* isolated from human, animal and environmental samples [56]. The majority of plasmid types were IncX4 (35.2%), IncI2 (34.7%) and IncHI2 (20.5%), with a significant geographical clustering of IncHI2 plasmids in Europe and a regional spread of IncI2 plasmids in Asia. A recent study from France investigated the occurrence of *mcr-1*–*mcr-5* and *mcr-9* genes in colistin-resistant *E. coli* isolates obtained from over 1500 goats of 80 breeding and five fattening goat farms [57]. In total, 149 *mcr-1*-positive *E. coli* were identified, with 146 *mcr-1* genes located on either IncX4 (38.9%) or IncHI2 (26.8%) plasmids and on the chromosome (32.2%). The *mcr-1*-carrying plasmids of types IncX4 and IncHI2 were never detected on the same farm in that study.

The most frequently detected sequence types in our study were ST100 (n = 27; all ETEC) and ST1 (n = 20; 19 EDEC, 1 AdhF-*Ec*). Kusumoto et al. (2016) investigated 967 swine-pathogenic *E. coli* strains that were isolated from diseased pigs in Japan between 1991 and 2014 [58]. Isolates that were classified as EDEC in that study predominantly belonged to ST1, while ETEC strains were mostly typed as ST100. Sequence type ST10, which was the third most common sequence type in our study (n = 10, 50% *mcr*-positive isolates), was the most prevalent ST in *mcr*-positive swine-pathogenic *E. coli* in a study from Spain [3]. Three *mcr-1.1*-positive ST131 ETEC strains (harboring ETEC-typical genes *fedAac*, *estap* and *estb*) were isolated from Farm 2 in June 2010 (n =2) and June 2011 (n = 1). All strains harbored additional virulence genes *fimH*, *fyuA*, *hlyA*, *ibeA*, *iss*, *kpsMII*, *ompA*, *papB*, *papC*, *sitA* and *traT*, which are typical for ExPEC [59].

Bok et al. analyzed 274 *E. coli* strains isolated from healthy post-weaning piglets and sows in Poland with the phylogenetic assignment of isolates mainly to phylogroups A (37.6%) and B1 (33.2%) [60]. This is in line with our findings of phylogroups A and B1 being the most prevalent phylogroups for Farm 2 and Farm 3, respectively. Another study from Thailand reported phylogroups A (44.3%), B1 (34.4%) and D (14.8%) being the predominant phylogroups for *mcr*-harboring *E. coli* from slaughtered pigs in 2014–2015 [61]. Here, we observed similar prevalences for collected *mcr*-positive *E. coli* isolates.

Our study has some limitations. This retrospective survey was based on the receipt of samples for diagnostic purposes and allows for bias due to the random nature of sample submissions. Only porcine *E. coli* isolates that proved positive for certain VAGs were documented and stored, leaving out non-pathogenic *E. coli*. In addition, viable but non-culturable (VBNC) *E. coli* were not studied. Bacteria in the VBNC state are viable but unable to grow on nutrient culture media. Pathogenic *E. coli* entering the VBNC state as a survival strategy has been reported [62], and would have been missed in our study design. Furthermore, comprehensive data were not available for all three farms, including information on farm characteristics such as potential external contamination, management practices related to animal health, and previous veterinary treatments, including the use of colistin or other antimicrobials.

The collection of a large pool of pathogenic *E. coli* isolates over a period of more than twenty years is a major advantage for the selection and evaluation of sampled pig farms in Germany. To the best of our knowledge, this study is the first to present phenotypic and molecular data on *mcr*-mediated resistance and intestinal pathogenic *E. coli* isolates from individual pig farms over an extended period of time.

5. Conclusions

We describe three German pig farms in which pathotypes of *E. coli*, including ETEC and EDEC, and various MCR plasmids have been found repeatedly over the years. Isolates obtained on each farm over 17.4, 26.6, and 4.5 years, respectively, differed from each other in their multilocus sequence types and/or VAG and AMR gene profiles and were therefore not considered clonally related. However, a comparison of the plasmid sequence data with reference plasmids demonstrated the structural resemblance of virulence plasmids over several years within and, in part, also across farms.

The data suggest that the repeated occurrence of *mcr*-carrying plasmids in pathogenic *E. coli* isolates may be due to the local long-term occurrence of *mcr*-carrying plasmids rather than reinfection with different plasmids. In affected farms, specific eradication programs, such as rigorous hygiene management and prevention of pig trafficking, may be at least as equally important and effective in reducing the burden of colistin resistance as general recommendations to reduce colistin usage.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/microorganisms12040729/s1, Table S1: 87 *E. coli* were analyzed to determine the location of virulence genes associated with intestinal pathogenic *E. coli*. Isolates are sorted by farms, sequence type (ST), and date of isolation; Table S2: Distribution of virulence-associated genes among 87 *E. coli* isolates from swine, sorted by farms, sequence types (ST), and date of isolation. Only genes that were at least present in one of the isolates with an identity of >90% (VirulenceFinder 2.0 and BacWGSTdb), are listed; Table S3: Pathotypes, sequence types (STs), resistance genes and chromosomal point mutations related to antimicrobial resistance of 87 representative whole genome-sequenced *E. coli* isolates, sorted by farms, STs, and date of isolation.

Author Contributions: Conceptualization, C.E.; methodology, C.E., R.B., L.G., T.S. and S.A.W.; validation, C.E. and L.G.; investigation, C.E., R.B., E.P.-B. and L.G.; resources, C.E., R.B. and T.S.; data curation, C.E., L.G., E.P.-B., T.S. and S.A.W.; writing—original draft preparation, L.G.; writing—review and editing, C.E., R.B. and L.G.; visualization, L.G.; supervision, C.E.; project administration, C.E. and R.B. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Ethical review and approval was waived for this study due to the fact that 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.

Data Availability Statement: All relevant data are provided in the paper and its Supplements. Raw sequence reads of 87 *E. coli* genomes are provided under NCBI Bioproject ID PRJNA916215. Further raw data can be made available on reasonable request.

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Conflicts of Interest: The authors declare no conflicts of interest.

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Table S1: 87 *E. coli* were analyzed to determine the location of virulence genes associated with intestinal pathogenic *E. coli*. Isolates are sorted by farms, sequence type (ST), and date of isolation.

Strain ID	Date of isolation	ST	Pathotype*	VAG** (adhesin)	Reference plasmid, adhesin (NCBI Reference Sequence)	VAGs** (toxin)	Reference plasmid, for one or several toxin (NCBI Reference Sequence)
Farm 1 (*n* = 27)							
IHIT46527	06/2005	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT48337	12/2005	1	EDEC	*fedAab*	p1713-1 (CP031766.1)	*stx2e*	-
IHIT46528	01/2006	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT46530	07/2009	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT45339	08/2009	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT46531	12/2009	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT46532	01/2010	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT46533	03/2010	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT48339	04/2010	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT32406	06/2010	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT48341	06/2010	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT45342	07/2011	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT46538	09/2012	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT46553	10/2019	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT52949	06/2011	23	ETEC	*fedAac*	p15ODTXV (MG904998.1)	*estb, estap*	p15ODTXV (MG904998.1)
IHIT48327	04/2004	42	ETEC	*fedAac*	p15ODTXV (MG904998.1)	*estb, eltB-Ip*	p14ODTX (MG904993.1)
IHIT48328	07/2004	42	ETEC	*fedAac*	pCV839-06-p2 (CP025752.1)	*estb, estap*^{g}	pCV839-06-p2 (CP025752.1)
IHIT48326	05/2002	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, eltB-Ip*	p14ODTX (MG904993.1)
IHIT52948	02/2008	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, eltB-Ip*	pUMNK88_Ent (NC_017640.1)
IHIT45341	07/2011	100	ETEC	*faeGac*	p1713-1 (CP031766.1)	*estb, eltB-Ip*	pUMNK88_Ent (NC_017640.1)
IHIT48351	12/2015	100	ETEC	*faeGac*	p14ODK88 (MG904991.1)	*estb*^{g}*, eltB-Ip*	pUMNK88_Ent (NC_017640.1)
IHIT46534	06/2010	131	ETEC	*fedAac*	p15ODTXV (MG904998.1)	*estb, estap*	p15ODTXV (MG904998.1)
IHIT48340	06/2010	131	ETEC	*fedAac*	p15ODTXV (MG904998.1)	*estb, estap*	p15ODTXV (MG904998.1)
IHIT48343	06/2011	131	ETEC	*fedAac*	p15ODTXV (MG904998.1)	*estb, estap*^{g}	p15ODTXV (MG904998.1)
IHIT52950	07/2012	641	AdhF-*Ec*	*fedA*	pCV839-06-p2 (CP025752.1)	-	-

Strain ID	Date of isolation	ST	Pathotype*	VAG** (adhesin)	Reference plasmid, adhesin (NCBI Reference Sequence)	VAGs** (toxin)	Reference plasmid, for one or several toxin (NCBI Reference Sequence)
IHIT48325	05/2002	710	STEC	-	-	*stx2e*	-
IHIT52947	12/2007	12009	STEC	-	-	*stx2e*	-
Farm 2 (*n* = 44)							
IHIT48329	07/2004	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT48336	08/2005	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT48354	11/2020	1	AdhF-*Ec*	*fedAab*	p1713-1 (CP031766.1)	-	-
IHIT48331	10/2004	10	ETEC-like	-	-	*estb*♯	-
IHIT46535	02/2011	10	ETEC	*fedAac*	p15ODTXV (MG904998.1)	*estb, estap*	p15ODTXV (MG904998.1)
IHIT46540	07/2013	10	ETEC	*fedAac*	p15ODTXV (MG904998.1)	*estb, estap*	p15ODTXV (MG904998.1)
IHIT23335	07/2013	10	ETEC/STEC	*fedAac*	p15ODTXV (MG904998.1)	*stx2e, estb, estap*	p2454 (MG948333.1)
IHIT48348	11/2014	10	ETEC-like	-	-	*estb*	-
IHIT46541	11/2014	10	ETEC	*fedAac*	p15ODTXV (MG904998.1)	*estb, estap*♯	p15ODTXV (MG904998.1)
IHIT46542	01/2015	10	ETEC	*fedAac*	p15ODTXV (MG904998.1)	*estb, estap*	p15ODTXV (MG904998.1)
IHIT46550	02/2018	10	ETEC	*fedAac*	p15ODTXV (MG904998.1)	*estb, estap*	p15ODTXV (MG904998.1)
IHIT48346	12/2012	20	AEEC	*eae*	-	-	-
IHIT48330	07/2004	29	AEEC	*eae*	-	-	-
IHIT48333	12/2004	42	ETEC	*fedAac*	p35K (CP022728.1)	*estb, eltB-Ip*	p35K (CP022728.1)
IHIT48334	12/2004	42	AdhF-*Ec*	*fedAac*	p14ODV (MG904994.1)	-	-
IHIT48342	06/2010	93	AEEC	*eae*♯	-	-	-
IHIT46526	06/2004	100	ETEC	*faeGac*	p14ODK88 (MG904991.1)	*estb, estap, eltB-Ip*	pUMNK88_Ent (NC_017640.1)
IHIT48332	10/2004	100	ETEC	*faeGac*	p15ODTXV (MG904998.1)	*estb, eltB-Ip*	pGMI14-004_1 (CP028195.1)
IHIT48335	03/2005	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-Ip*	pGMI14-004_1 (CP028195.1)
IHIT46529	02/2006	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-Ip*	pUMNK88_Ent (NC_017640.1)
IHIT48338	08/2008	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, eltB-Ip*	pUMNK88_Ent (NC_017640.1)
IHIT46536	11/2011	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-Ip*	pUMNK88_Ent (NC_017640.1)
IHIT46537	01/2012	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap*♯*, eltB-Ip*	pUMNK88_Ent (NC_017640.1)
IHIT45353	07/2013	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-Ip*	pUMNK88_Ent (NC_017640.1)
IHIT46539	07/2013	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-Ip*	pUMNK88_Ent (NC_017640.1)
IHIT25408	03/2014	100	ETEC	*faeGac*	p14ODK88 (MG904991.1)	*estb, estap, eltB-Ip*	pUMNK88_Ent (NC_017640.1)

Strain ID	Date of isolation	ST	Pathotype*	VAG** (adhesin)	Reference plasmid, adhesin (NCBI Reference Sequence)	VAGs** (toxin)	Reference plasmid, for one or several toxin (NCBI Reference Sequence)
IHIT48347	05/2014	100	ETEC	*faeGac*	p14ODK88 (MG904991.1)	*estb, estap, eltB-lp*	pUMNK88_Ent (NC_017640.1)
IHIT27622	10/2014	100	ETEC	*faeGac*	p14ODK88 (MG904991.1)	*estb, estap, eltB-lp*	pGMI14-004_1 (CP028195.1)
IHIT45399	01/2015	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-lp*	pUMNK88_Ent (NC_017640.1)
IHIT48349	07/2015	100	ETEC	*faeGac*	p14ODK88 (MG904991.1)	*estb, estap, eltB-lp*	pUMNK88_Ent (NC_017640.1)
IHIT45401	09/2015	100	ETEC	*faeGac*	p14ODK88 (MG904991.1)	*estb, estap, eltB-lp*	pUMNK88_Ent (NC_017640.1)
IHIT46543	09/2015	100	ETEC	-	p15ODTXV (MG904998.1)	-	
IHIT46546	09/2016	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-lp*	pGMI14-004_1 (CP028195.1)
IHIT45407	08/2017	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-lp*	pUMNK88_Ent (NC_017640.1)
IHIT36144	01/2018	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-lp*	pGMI14-004_1 (CP028195.1)
IHIT36146	01/2018	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-lp*	pGMI14-004_1 (CP028195.1)
IHIT36426	02/2018	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-lp*	pGMI14-004_1 (CP028195.1)
IHIT36427	02/2018	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, estap, eltB-lp*	pGMI14-004_1 (CP028195.1)
IHIT48355	11/2020	641	ETEC-like	-	-	*estb*#	-
IHIT48358	02/2021	641	ETEC-like	-	-	*estb*	-
IHIT32748	09/2016	793	AEEC	*eae*	-	-	-
IHIT48344	12/2011	799	AEEC	*eae*	-	-	-
IHIT48356	11/2020	955	STEC	-	-	*stx2e*	-
IHIT48350	09/2015	2944	ETEC-like	-	-	*estb*	pOX38 (MF370216.1)
Farm 3 (*n* = 16)							
IHIT34769	06/2017	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT48353	06/2017	1	EDEC	*faeGac*	-	*stx2e*	-
IHIT47062	10/2017	1	EDEC	*fedAab*	p15ODTXV (MG904998.1)	*stx2e*	-
IHIT48352	12/2016	10	STEC	-	-	*stx2e*	-
IHIT39537	04/2019	10	ETEC-like	-	-	*estb*	-
IHIT47044	10/2014	86	ETEC/STEC	*fedAac*	p15ODTXV (MG904998.1)	*stx2e, estb, estap*	p15ODTXV (MG904998.1)
IHIT47045	10/2014	86	ETEC/STEC	*fedAac*	p15ODTXV (MG904998.1)	*stx2e, estb, estap*	p15ODTXV (MG904998.1)
IHIT47046	06/2015	86	ETEC/STEC	*fedAac*	p15ODTXV (MG904998.1)	*stx2e, estb, estap*	p15ODTXV (MG904998.1)
IHIT47048	06/2015	86	ETEC/STEC	*fedAac*	p15ODTXV (MG904998.1)	*stx2e, estb, estap*	p15ODTXV (MG904998.1)
IHIT47056	06/2016	86	ETEC/STEC	*fedAac*	p15ODTXV (MG904998.1)	*stx2e, estb, estap*	p15ODTXV (MG904998.1)

Strain ID	Date of isolation	ST	Pathotype*	VAG** (adhesin)	Reference plasmid, adhesin (NCBI Reference Sequence)	VAGs** (toxin)	Reference plasmid, for one or several toxin (NCBI Reference Sequence)
IHIT47057	11/2016	86	ETEC	*fedAac*	p15ODTXV (MG904998.1)	*estb, estap*	p15ODTXV (MG904998.1)
IHIT34315	04/2017	86	ETEC/STEC	*fedAac*	p15ODTXV (MG904998.1)	*stx2e*, *estb, estap*	p15ODTXV (MG904998.1)
IHIT47060	02/2017	90	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, eltB-Ip*	p14ODTX (MG904993.1)
IHIT47047	06/2015	100	ETEC	*faeGac*	pUMNK88_K88 (CP002730.1)	*estb, eltB-Ip*	p14ODTX (MG904993.1)
IHIT47065	07/2018	118	ETEC-like	-	-	*estb*	pOX38 (MF370216.1)
IHIT47072	03/2019	162	ETEC-like	-	-	*estb*	-

*Virulence-associated genes (VAGs) marked with # were positive in the PCR but negative for the respective virulence gene according to whole genome data. **Pathotypes: AdhF-*Ec*, positive for at least one adhesive fimbriae gene (*faeG*, *fanA*, *fasA*, *fedA*, *fimF41a*); AEEC, 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, 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*.

Table S2: Distribution of virulence-associated genes among 87 *E. coli* isolates from swine, sorted by farms, sequence types (ST), and date of isolation. Only

				Category	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion
Farm	Strain	Isolation date	ST	Gene	*air*	*csgA*	*eae-b01a-beta*	*eae-b04-delta*	*efa1*	*eilA*	*faeB*	*faeC*	*faeD*	*faeE*	*faeF*	*faeGac*	*faeH*	*faeI*	*faeJ*	*fdeC*	*fedAab*	*fedAac*	*fedAnt*	*fedB*
1	IHIT46527	06/2005	1			1				1										1	1			1
1	IHIT48337	12/2005	1			1				1										1	1			1
1	IHIT46528	01/2006	1			1				1										1	1			1
1	IHIT46530	07/2009	1			1				1										1	1			1
1	IHIT45339	08/2009	1			1				1										1	1			1
1	IHIT46531	12/2009	1			1				1										1	1			1
1	IHIT46532	01/2010	1			1				1										1	1			1
1	IHIT46533	03/2010	1			1				1										1	1			1
1	IHIT48339	04/2010	1			1				1										1	1			1
1	IHIT32406	06/2010	1			1				1										1	1			1
1	IHIT48341	06/2010	1			1														1	1			1
1	IHIT45342	07/2011	1			1				1										1	1			1
1	IHIT46538	09/2012	1			1				1										1	1			1
1	IHIT46553	10/2019	1			1				1										1	1			1
1	IHIT52949	06/2011	23			1														1		1		1
1	IHIT48327	04/2004	42			1																1		1
1	IHIT48328	07/2004	42		1	1				1										1		1		1
1	IHIT48326	05/2002	100			1					1	1	1	1	1	1	1	1	1	1				
1	IHIT52948	02/2008	100			1					1	1	1	1	1	1	1	1	1	1				
1	IHIT45341	07/2011	100			1				1	1	1	1	1	1	1	1	1	1	1		1		1
1	IHIT48351	12/2015	100			1					1	1	1	1	1	1	1	1	1	1				
1	IHIT46534	06/2010	131			1														1		1		1
1	IHIT48340	06/2010	131			1														1		1		1
1	IHIT48343	06/2011	131			1														1		1		1
1	IHIT52950	07/2012	641			1														1			1	1
1	IHIT48325	05/2002	710			1														1				
1	IHIT52947	12/2007	12009																	1				
2	IHIT48329	07/2004	1			1				1										1	1			1
2	IHIT48336	08/2005	1			1				1										1	1			1
2	IHIT48354	11/2020	1			1				1										1	1			1
2	IHIT48331	10/2004	10			1														1				
2	IHIT46535	02/2011	10			1																1		1
2	IHIT46540	07/2013	10			1																1		1
2	IHIT23335	07/2013	10			1																1		1
2	IHIT46541	11/2014	10			1																1		1
2	IHIT48348	11/2014	10			1														1				
2	IHIT46542	01/2015	10			1																1		1
2	IHIT46550	02/2018	10			1																1		1
2	IHIT48346	12/2012	20			1	1													1				
2	IHIT48330	07/2004	29			1	1		1											1				
2	IHIT48333	12/2004	42		1	1				1										1		1		1
2	IHIT48334	12/2004	42			1				1										1		1		1
2	IHIT48342	06/2010	93			1														1				
2	IHIT46526	06/2004	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT48332	10/2004	100			1						1	1	1	1	1	1	1	1	1				
2	IHIT48335	03/2005	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT46529	02/2006	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT48338	08/2008	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT46536	11/2011	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT46537	01/2012	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT45353	07/2013	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT46539	07/2013	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT25408	03/2014	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT48347	05/2014	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT27622	10/2014	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT45399	01/2015	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT48349	07/2015	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT45401	09/2015	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT46543	09/2015	100			1					1	1	1		1	1	1	1	1	1				
2	IHIT46546	09/2016	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT45407	08/2017	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT36144	01/2018	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT36146	01/2018	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT36426	02/2018	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT36427	02/2018	100			1					1	1	1	1	1	1	1	1	1	1				
2	IHIT48355	11/2020	641			1														1				
2	IHIT48358	02/2021	641			1														1				
2	IHIT32748	09/2016	793			1		1												1				
2	IHIT48344	12/2011	799		1	1	1			1										1	1			1
2	IHIT48356	11/2020	955			1														1				
2	IHIT48350	09/2015	2944		1	1				1										1				
3	IHIT34769	06/2017	1			1														1	1			1
3	IHIT48353	06/2017	1			1					1	1	1	1	1	1	1	1	1	1				
3	IHIT47062	10/2017	1			1				1										1	1			1
3	IHIT48352	12/2016	10			1														1				
3	IHIT39537	04/2019	10			1														1				
3	IHIT47044	10/2014	86			1														1		1		1
3	IHIT47045	10/2014	86			1														1		1		1
3	IHIT47046	06/2015	86			1														1		1		1
3	IHIT47048	06/2015	86			1														1		1		1
3	IHIT47056	06/2016	86			1														1		1		1
3	IHIT47057	11/2016	86			1														1		1		1
3	IHIT34315	04/2017	86			1														1		1		1
3	IHIT47060	02/2017	90			1						1	1	1	1	1	1	1	1					
3	IHIT47047	06/2015	100			1						1	1	1	1	1	1	1	1	1				
3	IHIT47065	07/2018	118		1	1				1										1				
3	IHIT47072	03/2019	162			1														1				

genes, that were at least present in one of the isolates with an identity of >90% (VirulenceFinder 2.0 and BacWGSTdb), are listed.

				adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion
Farm	Strain	Isolation date	ST	*fedC*	*fedE*	*fedF*	*fes*	*fimA*	*fimB*	*fimC*	*fimD*	*fimE*	*fimF*	*fimG*	*fimH*	*fimI*	*hra*	*lpfA*	*paa*	*papA_F13*	*papA_F20*	*papA_F48*	*papB*	*papC*	*papD*
1	IHIT46527	06/2005	1		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT48337	12/2005	1		1	1	1	1	1	1	1	1	1	1	1	1		1							
1	IHIT46528	01/2006	1		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT46530	07/2009	1		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT45339	08/2009	1		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT46531	12/2009	1		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT46532	01/2010	1		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT46533	03/2010	1		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT48339	04/2010	1		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT32406	06/2010	1		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT48341	06/2010	1		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT45342	07/2011	1		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT46538	09/2012	1		1	1	1		1	1	1	1	1	1	1			1							
1	IHIT46553	10/2019	1	1	1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT52949	06/2011	23		1	1	1		1	1	1	1	1	1	1	1		1							
1	IHIT48327	04/2004	42		1	1	1	1	1	1	1	1	1	1	1	1	1	1							
1	IHIT48328	07/2004	42		1	1	1	1	1	1	1	1	1	1	1	1	1	1							
1	IHIT48326	05/2002	100				1																		
1	IHIT52948	02/2008	100				1																		
1	IHIT45341	07/2011	100		1	1	1	1	1	1	1	1	1	1	1	1	1	1							
1	IHIT48351	12/2015	100				1																		
1	IHIT46534	06/2010	131		1	1	1	1	1	1	1	1	1	1	1	1	1					1	1	1	1
1	IHIT48340	06/2010	131		1	1	1	1	1	1	1	1	1	1	1	1	1					1	1	1	1
1	IHIT48343	06/2011	131		1	1	1	1	1	1	1	1	1	1	1	1	1					1	1	1	1
1	IHIT52950	07/2012	641	1	1	1	1			1	1		1	1	1	1		1	1						
1	IHIT48325	05/2002	710				1		1	1	1	1	1	1	1	1									
1	IHIT52947	12/2007	12009		1		1	1	1	1	1	1	1	1	1	1									
2	IHIT48329	07/2004	1	1	1	1	1		1	1	1	1	1	1	1			1							
2	IHIT48336	08/2005	1	1	1	1	1		1	1	1	1	1	1	1			1							
2	IHIT48354	11/2020	1	1	1	1	1		1	1	1	1	1	1	1		1	1							
2	IHIT48331	10/2004	10				1		1	1	1	1	1	1	1										
2	IHIT46535	02/2011	10		1	1	1	1	1	1	1	1	1	1	1										
2	IHIT46540	07/2013	10		1	1	1	1	1	1	1	1	1	1	1										
2	IHIT23335	07/2013	10		1	1	1	1	1	1	1	1	1	1	1										
2	IHIT46541	11/2014	10		1	1	1	1	1	1	1	1	1	1	1										
2	IHIT48348	11/2014	10				1		1	1	1	1	1	1	1										
2	IHIT46542	01/2015	10		1	1	1	1	1	1	1	1	1	1	1										
2	IHIT46550	02/2018	10		1	1	1	1	1	1	1	1	1	1	1										
2	IHIT48346	12/2012	20				1		1	1	1	1	1	1	1										
2	IHIT48330	07/2004	29				1		1	1	1	1	1	1	1			1	1						
2	IHIT48333	12/2004	42		1	1	1	1	1	1	1	1	1	1	1		1	1							
2	IHIT48334	12/2004	42		1	1	1	1	1	1	1	1	1	1	1		1	1							
2	IHIT48342	06/2010	93				1										1			1			1	1	1
2	IHIT46526	06/2004	100				1											1							
2	IHIT48332	10/2004	100				1																		
2	IHIT48335	03/2005	100				1											1							
2	IHIT46529	02/2006	100				1											1							
2	IHIT48338	08/2008	100				1																		
2	IHIT46536	11/2011	100				1											1							
2	IHIT46537	01/2012	100				1											1							
2	IHIT45353	07/2013	100				1											1							
2	IHIT46539	07/2013	100				1											1							
2	IHIT25408	03/2014	100				1											1							
2	IHIT48347	05/2014	100				1											1							
2	IHIT27622	10/2014	100				1											1							
2	IHIT45399	01/2015	100				1											1							
2	IHIT48349	07/2015	100				1											1							
2	IHIT45401	09/2015	100				1											1							
2	IHIT46543	09/2015	100				1	1	1	1	1	1	1	1	1	1									
2	IHIT46546	09/2016	100				1											1							
2	IHIT45407	08/2017	100				1											1							
2	IHIT36144	01/2018	100				1											1							
2	IHIT36146	01/2018	100				1											1							
2	IHIT36426	02/2018	100				1											1							
2	IHIT36427	02/2018	100				1											1							
2	IHIT48355	11/2020	641				1		1	1	1	1	1	1	1	1		1							
2	IHIT48358	02/2021	641		1	1	1		1	1	1	1	1	1	1	1		1							
2	IHIT32748	09/2016	793				1	1	1	1		1	1	1	1	1									
2	IHIT48344	12/2011	799	1	1	1	1		1	1	1	1	1	1	1	1		1	1						
2	IHIT48356	11/2020	955		1		1	1	1	1	1	1	1	1	1	1		1							
2	IHIT48350	09/2015	2944				1	1	1	1	1	1	1	1	1	1		1							
3	IHIT34769	06/2017	1	1	1	1	1		1	1	1	1	1	1	1	1		1							
3	IHIT48353	06/2017	1				1		1	1	1	1	1	1	1	1		1							
3	IHIT47062	10/2017	1	1	1	1	1		1	1	1	1	1	1	1	1		1							
3	IHIT48352	12/2016	10				1			1	1		1	1	1	1									
3	IHIT39537	04/2019	10				1		1	1	1	1	1	1	1	1									
3	IHIT47044	10/2014	86		1	1	1	1	1	1	1	1	1	1	1	1		1			1		1	1	1
3	IHIT47045	10/2014	86		1	1	1	1	1	1	1	1	1	1	1	1		1			1		1	1	1
3	IHIT47046	06/2015	86		1	1	1	1	1	1	1	1	1	1	1	1		1			1		1	1	1
3	IHIT47048	06/2015	86		1	1	1	1	1	1	1	1	1	1	1	1		1			1		1	1	1
3	IHIT47056	06/2016	86		1	1	1	1	1	1	1	1	1	1	1	1		1			1		1	1	1
3	IHIT47057	11/2016	86		1	1	1	1	1	1	1	1	1	1	1	1		1			1		1	1	1
3	IHIT34315	04/2017	86		1	1	1	1	1	1	1	1	1	1	1	1		1			1		1	1	1
3	IHIT47060	02/2017	90				1		1	1	1	1	1	1	1	1		1							
3	IHIT47047	06/2015	100				1																		
3	IHIT47065	07/2018	118				1	1	1	1	1	1	1	1	1	1									
3	IHIT47072	03/2019	162				1	1	1	1	1	1	1	1	1	1		1							

				adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	adhesion	hemolysin/ toxin genes	hemolysin/ toxin genes	hemolysin/ toxin genes	hemolysin/ toxin genes	hemolysin/ toxin genes	hemolysin/ toxin genes	hemolysin/ toxin genes	hemolysin/ toxin genes	hemolysin/ toxin genes
Farm	Strain	Isolation date	ST	papE	papF	papG	papH	papI	papJ	papK	papX	sfaC	sfaX	tir	yfcV	astA	ehxA	eltIAB-4	estap-Sta1	estb-STb1	hlyA	hlyB	hlyC	hlyD	hlyE
1	IHIT46527	06/2005	1																		1	1	1	1	1
1	IHIT48337	12/2005	1																		1	1	1	1	1
1	IHIT46528	01/2006	1																		1	1	1	1	1
1	IHIT46530	07/2009	1																		1	1	1	1	1
1	IHIT45339	08/2009	1																		1	1	1	1	1
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1	IHIT46533	03/2010	1																		1	1	1	1	1
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1	IHIT32406	06/2010	1																		1	1	1	1	1
1	IHIT48341	06/2010	1																		1	1	1	1	1
1	IHIT45342	07/2011	1																		1	1	1	1	1
1	IHIT46538	09/2012	1																		1	1	1	1	1
1	IHIT46553	10/2019	1																		1	1	1	1	1
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2	IHIT46541	11/2014	10																	1	1	1	1	1	1
2	IHIT48348	11/2014	10													1				1					1
2	IHIT46542	01/2015	10																1	1	1	1	1	1	1
2	IHIT46550	02/2018	10																1	1	1	1	1	1	1
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2	IHIT48332	10/2004	100													1		1		1	1	1	1	1	1
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2	IHIT48358	02/2021	641													1				1					1
2	IHIT32748	09/2016	793											1		1									
2	IHIT48344	12/2011	799											1			1				1			1	1
2	IHIT48356	11/2020	955																		1	1		1	1
2	IHIT48350	09/2015	2944													1				1					1
3	IHIT34769	06/2017	1																		1	1	1	1	1
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3	IHIT47062	10/2017	1																		1	1	1	1	1
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3	IHIT39537	04/2019	10													1				1					1
3	IHIT47044	10/2014	86	1	1	1	1	1	1	1									1	1	1	1	1	1	1
3	IHIT47045	10/2014	86	1	1	1	1	1	1	1									1	1	1	1	1	1	1
3	IHIT47046	06/2015	86	1	1	1	1	1	1	1									1	1	1	1	1	1	1
3	IHIT47048	06/2015	86	1	1	1	1	1	1	1									1	1	1	1	1	1	1
3	IHIT47056	06/2016	86	1	1	1	1	1	1	1									1	1	1	1	1	1	1
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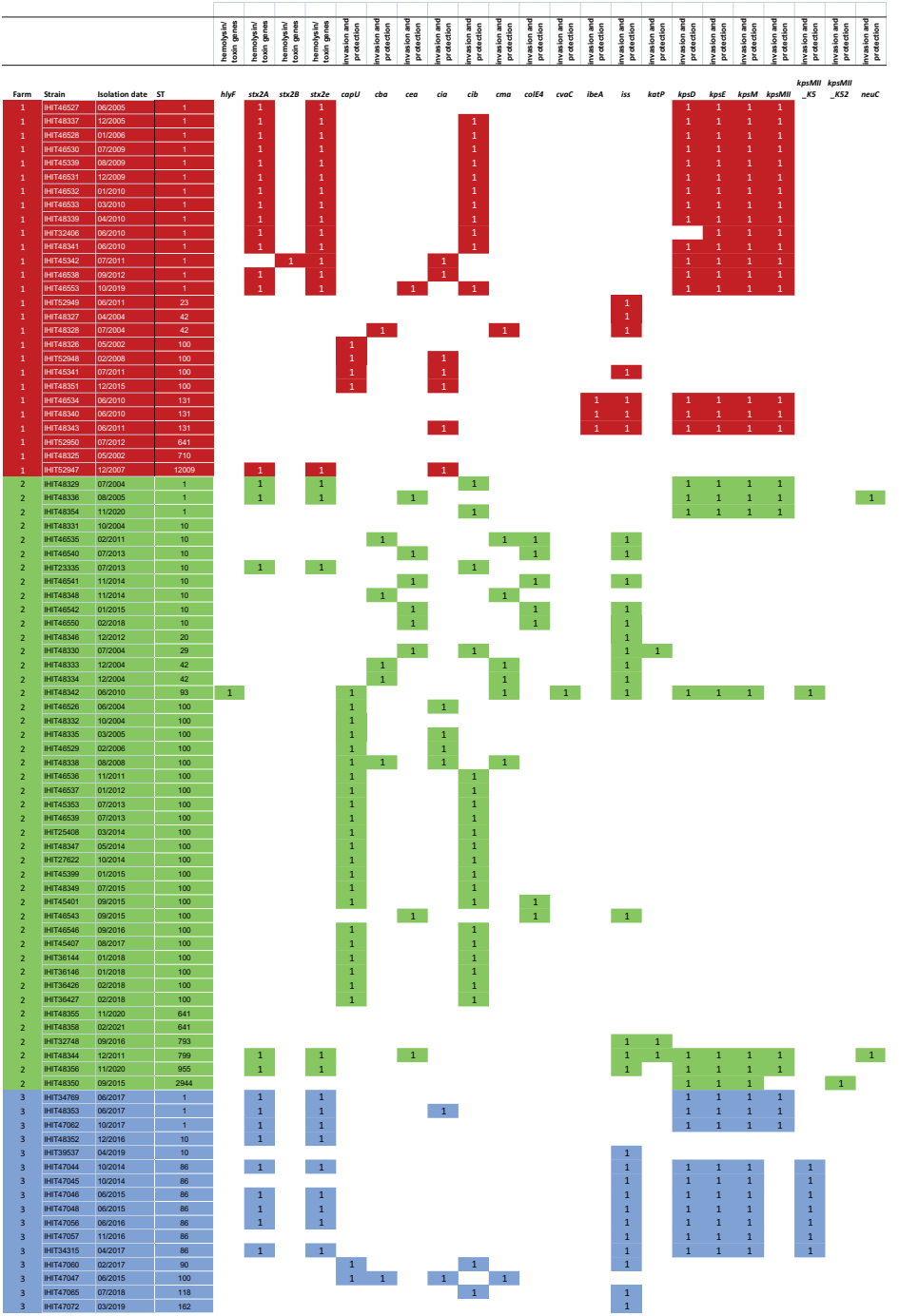
3	IHIT47060	02/2017	90													1		1		1	1	1	1	1	1
3	IHIT47047	06/2015	100													1		1		1	1	1	1	1	1
3	IHIT47065	07/2018	118													1				1					1
3	IHIT47072	03/2019	162													1				1					1


hemolysin/ toxin genes
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invasion and protection
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Farm
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colE4
cvaC
ibeA
iss
katP
kpsD
kpsE
kpsM
kpsMII
kpsMII _K5
kpsMII _K52
neuC

				Invasion and protection	Invasion and protection	Invasion and protection	Invasion and protection	Invasion and protection	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition	Iron aquisition
Farm	Strain	Isolation date	ST	ompA	ompT	tia	traI	traT	chuA	chuS	chuT	chuU	chuV	chuW	chuX	chuY	entA	entB	entC	entD	entE	entF	entS	fepA	fepB
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1	IHIT48337	12/2005	1	1	1				1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT46528	01/2006	1	1	1				1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT46530	07/2009	1	1	1			1	1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT45339	08/2009	1	1	1			1	1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT46531	12/2009	1	1	1		1	1	1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT46532	01/2010	1	1	1			1	1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT46533	03/2010	1	1	1		1	1	1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT48339	04/2010	1	1	1		1	1	1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT32406	06/2010	1	1	1		1	1	1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT48341	06/2010	1	1	1		1	1	1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT45342	07/2011	1	1	1		1	1	1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT46538	09/2012	1	1	1		1	1	1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT46553	10/2019	1	1	1			1	1	1		1		1				1	1	1	1	1	1	1	1
1	IHIT52949	06/2011	23	1	1	1		1										1	1	1	1	1	1	1	1
1	IHIT48327	04/2004	42	1	1			1	1	1				1		1	1	1	1	1	1	1	1	1	1
1	IHIT48328	07/2004	42	1	1			1	1	1				1		1		1	1	1	1	1	1	1	1
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1	IHIT52948	02/2008	100	1				1										1	1	1	1	1	1	1	1
1	IHIT45341	07/2011	100	1	1			1	1	1				1		1		1	1	1	1		1	1	1
1	IHIT48351	12/2015	100	1				1										1	1	1	1	1	1	1	1
1	IHIT46534	06/2010	131	1	1			1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1
1	IHIT48340	06/2010	131	1	1			1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1
1	IHIT48343	06/2011	131	1	1			1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1
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1	IHIT48325	05/2002	710	1	1	1	1	1										1	1	1	1	1	1	1	1
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2	IHIT48329	07/2004	1	1	1				1	1		1		1				1	1	1	1	1	1	1	1
2	IHIT48336	08/2005	1	1	1			1	1	1		1		1				1	1	1	1	1	1	1	1
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2	IHIT48331	10/2004	10	1			1	1										1	1	1	1	1	1	1	1
2	IHIT46535	02/2011	10	1	1		1	1	1	1				1		1		1	1	1	1	1	1	1	1
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2	IHIT23335	07/2013	10	1				1										1	1	1	1	1	1	1	1
2	IHIT46541	11/2014	10	1	1			1	1	1				1		1		1	1	1	1	1	1	1	1
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2	IHIT48346	12/2012	20	1	1			1										1	1	1	1	1	1	1	1
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2	IHIT46543	09/2015	100	1	1			1	1	1				1		1		1	1	1	1	1	1	1	1
2	IHIT46546	09/2016	100	1			1	1										1	1	1	1	1	1	1	1
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2	IHIT36144	01/2018	100	1			1	1										1	1	1	1	1	1	1	1
2	IHIT36146	01/2018	100	1			1	1										1	1	1	1	1	1	1	1
2	IHIT36426	02/2018	100	1			1	1										1	1	1	1	1	1	1	1
2	IHIT36427	02/2018	100	1			1	1										1	1	1	1	1	1	1	1
2	IHIT48355	11/2020	641	1			1	1										1	1	1	1	1	1	1	1
2	IHIT48358	02/2021	641	1	1		1	1					1					1	1	1	1	1	1	1	1
2	IHIT32748	09/2016	793	1	1			1										1	1	1	1	1	1	1	1
2	IHIT48344	12/2011	799	1	1			1	1					1				1	1	1	1	1	1	1	1
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3	IHIT34315	04/2017	86	1		1		1										1	1	1	1	1	1	1	1
3	IHIT47060	02/2017	90	1														1	1	1	1	1	1		
3	IHIT47047	06/2015	100	1				1										1	1	1	1	1	1	1	1
3	IHIT47065	07/2018	118	1	1		1	1	1			1		1		1		1	1	1	1	1	1	1	1
3	IHIT47072	03/2019	162	1			1	1										1	1	1	1	1	1	1	1

				iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition
Farm	**Strain**	**Isolation date**	**ST**	***fepC***	***fepD***	***fepE***	***fepG***	***fyuA***	***iha***	***ireA***	***iroB***	***iroC***	***iroD***	***iroE***	***iroN***	***irp1***	***irp2***	***iucA***	***iucB***	***iucC***	***iucD***	***iutA***	***sitA***	***ybtA***	***ybtE***
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1	IHIT48337	12/2005	1	1	1		1																		
1	IHIT46528	01/2006	1	1	1		1																		
1	IHIT46530	07/2009	1	1	1		1																		
1	IHIT45339	08/2009	1	1	1		1																		
1	IHIT46531	12/2009	1	1	1		1																		
1	IHIT46532	01/2010	1	1	1		1																		
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1	IHIT48339	04/2010	1	1	1		1																		
1	IHIT32406	06/2010	1	1	1		1																		
1	IHIT48341	06/2010	1	1	1		1																		
1	IHIT45342	07/2011	1	1	1		1																		
1	IHIT46538	09/2012	1	1	1		1																		
1	IHIT46553	10/2019	1	1	1		1																		
1	IHIT52949	06/2011	23	1	1		1	1								1	1							1	1
1	IHIT48327	04/2004	42	1	1		1		1																
1	IHIT48328	07/2004	42	1	1		1		1																
1	IHIT48326	05/2002	100	1	1		1		1																
1	IHIT52948	02/2008	100	1	1		1		1																
1	IHIT45341	07/2011	100	1	1		1		1																
1	IHIT48351	12/2015	100	1	1		1		1																
1	IHIT46534	06/2010	131	1	1		1	1								1	1						1	1	1
1	IHIT48340	06/2010	131	1	1		1	1								1	1						1	1	1
1	IHIT48343	06/2011	131	1	1		1	1								1	1						1	1	1
1	IHIT52950	07/2012	641	1	1		1																		
1	IHIT48325	05/2002	710	1	1		1																		
1	IHIT52947	12/2007	12009	1	1		1																		
2	IHIT48329	07/2004	1	1	1		1																		
2	IHIT48336	08/2005	1	1	1		1																1		
2	IHIT48354	11/2020	1	1	1		1	1	1															1	
2	IHIT48331	10/2004	10	1	1		1																		
2	IHIT46535	02/2011	10	1	1		1		1																
2	IHIT46540	07/2013	10	1	1		1																		
2	IHIT23335	07/2013	10	1	1		1		1																
2	IHIT46541	11/2014	10	1	1		1																		
2	IHIT48348	11/2014	10	1	1		1																		
2	IHIT46542	01/2015	10	1	1		1																		
2	IHIT46550	02/2018	10	1	1		1																		
2	IHIT48346	12/2012	20	1	1		1	1								1	1							1	1
2	IHIT48330	07/2004	29	1	1		1		1									1	1	1	1	1			
2	IHIT48333	12/2004	42	1	1		1		1																
2	IHIT48334	12/2004	42	1	1		1		1																
2	IHIT48342	06/2010	93	1	1		1	1			1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	IHIT46526	06/2004	100	1	1		1		1																
2	IHIT48332	10/2004	100	1	1		1		1																
2	IHIT48335	03/2005	100	1	1		1		1																
2	IHIT46529	02/2006	100	1	1		1		1																
2	IHIT48338	08/2008	100	1	1		1		1																
2	IHIT46536	11/2011	100	1	1		1		1																
2	IHIT46537	01/2012	100	1	1		1		1																
2	IHIT45353	07/2013	100	1	1		1		1																
2	IHIT46539	07/2013	100	1	1		1		1																
2	IHIT25408	03/2014	100	1	1		1		1																
2	IHIT48347	05/2014	100	1	1		1		1																
2	IHIT27622	10/2014	100	1	1		1		1																
2	IHIT45399	01/2015	100	1	1	1			1																
2	IHIT48349	07/2015	100	1	1		1		1																
2	IHIT45401	09/2015	100	1	1		1		1																
2	IHIT46543	09/2015	100	1	1		1																		
2	IHIT46546	09/2016	100	1	1		1		1																
2	IHIT45407	08/2017	100	1	1		1		1																
2	IHIT36144	01/2018	100	1	1		1		1																
2	IHIT36146	01/2018	100	1	1		1		1																
2	IHIT36426	02/2018	100	1	1		1		1																
2	IHIT36427	02/2018	100	1	1		1		1																
2	IHIT48355	11/2020	641	1	1		1	1								1	1							1	1
2	IHIT48358	02/2021	641	1	1		1																		
2	IHIT32748	09/2016	793	1	1		1	1	1							1	1	1	1	1	1	1		1	1
2	IHIT48344	12/2011	799	1	1		1																1		
2	IHIT48356	11/2020	955	1	1		1																		
2	IHIT48350	09/2015	2944	1	1		1																1		
3	IHIT34769	06/2017	1	1	1		1																		
3	IHIT48353	06/2017	1	1	1		1		1																
3	IHIT47062	10/2017	1	1	1		1																		
3	IHIT48352	12/2016	10	1	1		1																		
3	IHIT39537	04/2019	10	1	1		1																1		
3	IHIT47044	10/2014	86	1	1		1		1	1															
3	IHIT47045	10/2014	86	1	1		1		1	1															
3	IHIT47046	06/2015	86	1	1		1		1	1															
3	IHIT47048	06/2015	86	1	1		1		1	1															
3	IHIT47056	06/2016	86	1	1		1		1	1															
3	IHIT47057	11/2016	86	1	1		1		1	1															
3	IHIT34315	04/2017	86	1	1		1		1	1															
3	IHIT47060	02/2017	90					1								1	1							1	1
3	IHIT47047	06/2015	100	1	1		1		1																
3	IHIT47065	07/2018	118	1	1		1																		
3	IHIT47072	03/2019	162	1	1		1																		

				iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	iron aquisition	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system
Farm	**Strain**	**Isolation date**	**ST**	***ybtP***	***ybtQ***	***ybtS***	***ybtT***	***ybtU***	***ybtX***	***cesD2***	***cesD***	***cesL***	***cesT***	***cif***	***escC***	***escD***	***escE***	***escF***	***escG***	***escI***	***escJ***	***escL***	***escN***	***escO***	***escP***
1	IHIT46527	06/2005	1																						
1	IHIT48337	12/2005	1																						
1	IHIT46528	01/2006	1																						
1	IHIT46530	07/2009	1																						
1	IHIT45339	08/2009	1																						
1	IHIT46531	12/2009	1																						
1	IHIT46532	01/2010	1																						
1	IHIT46533	03/2010	1																						
1	IHIT48339	04/2010	1																						
1	IHIT32406	06/2010	1																						
1	IHIT48341	06/2010	1																						
1	IHIT45342	07/2011	1																						
1	IHIT46538	09/2012	1																						
1	IHIT46553	10/2019	1																						
1	IHIT52949	06/2011	23	1	1	1	1	1	1																
1	IHIT48327	04/2004	42																						
1	IHIT48328	07/2004	42																						
1	IHIT48326	05/2002	100																						
1	IHIT52948	02/2008	100																						
1	IHIT45341	07/2011	100																						
1	IHIT48351	12/2015	100																						
1	IHIT46534	06/2010	131	1	1	1	1	1	1																
1	IHIT48340	06/2010	131	1	1	1	1	1	1																
1	IHIT48343	06/2011	131	1	1	1	1	1	1																
1	IHIT52950	07/2012	641																						
1	IHIT48325	05/2002	710																						
1	IHIT52947	12/2007	12009																						
2	IHIT48329	07/2004	1																						
2	IHIT48336	08/2005	1							1				1											
2	IHIT48354	11/2020	1				1																		
2	IHIT48331	10/2004	10																						
2	IHIT46535	02/2011	10																						
2	IHIT46540	07/2013	10																						
2	IHIT23335	07/2013	10																						
2	IHIT46541	11/2014	10																						
2	IHIT48348	11/2014	10																						
2	IHIT46542	01/2015	10																						
2	IHIT46550	02/2018	10																						
2	IHIT48346	12/2012	20	1	1	1	1	1	1	1	1	1	1	1		1	1	1	1	1	1	1	1	1	
2	IHIT48330	07/2004	29							1	1			1		1	1	1		1	1		1	1	
2	IHIT48333	12/2004	42																						
2	IHIT48334	12/2004	42																						
2	IHIT48342	06/2010	93	1	1	1	1	1	1																
2	IHIT46526	06/2004	100																						
2	IHIT48332	10/2004	100																						
2	IHIT48335	03/2005	100																						
2	IHIT46529	02/2006	100																						
2	IHIT48338	08/2008	100																						
2	IHIT46536	11/2011	100																						
2	IHIT46537	01/2012	100																						
2	IHIT45353	07/2013	100																						
2	IHIT46539	07/2013	100																						
2	IHIT25408	03/2014	100																						
2	IHIT48347	05/2014	100																						
2	IHIT27622	10/2014	100																						
2	IHIT45399	01/2015	100																						
2	IHIT48349	07/2015	100																						
2	IHIT45401	09/2015	100																						
2	IHIT46543	09/2015	100																						
2	IHIT46546	09/2016	100																						
2	IHIT45407	08/2017	100																						
2	IHIT36144	01/2018	100																						
2	IHIT36146	01/2018	100																						
2	IHIT36426	02/2018	100																						
2	IHIT36427	02/2018	100																						
2	IHIT48355	11/2020	641	1	1	1	1	1	1																
2	IHIT48358	02/2021	641																						
2	IHIT32748	09/2016	793	1	1	1	1	1	1	1	1		1	1			1	1	1	1	1		1	1	1
2	IHIT48344	12/2011	799							1	1			1	1		1	1	1	1	1		1	1	
2	IHIT48356	11/2020	955																						
2	IHIT48350	09/2015	2944																						
3	IHIT34769	06/2017	1																						
3	IHIT48353	06/2017	1																						
3	IHIT47062	10/2017	1																						
3	IHIT48352	12/2016	10																						
3	IHIT39537	04/2019	10																						
3	IHIT47044	10/2014	86																						
3	IHIT47045	10/2014	86																						
3	IHIT47046	06/2015	86																						
3	IHIT47048	06/2015	86																						
3	IHIT47056	06/2016	86																						
3	IHIT47057	11/2016	86																						
3	IHIT34315	04/2017	86																						
3	IHIT47060	02/2017	90	1	1	1	1	1	1																
3	IHIT47047	06/2015	100																						
3	IHIT47065	07/2018	118																						
3	IHIT47072	03/2019	162																						

				secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system
Farm	**Strain**	**Isolation date**	**ST**	***escS***	***escV***	***espA***	***espB***	***espD***	***espF***	***espG***	***espI***	***espL1***	***espL4***	***espM1***	***espM2***	***espR1***	***espR3***	***espR4***	***espW***	***espX1***	***espX2***	***espX4***	***espX5***	***espY1***	***espY2***				
1	IHIT46527	06/2005	1									1	1			1				1	1	1	1	1	1				
1	IHIT48337	12/2005	1									1	1											1	1				
1	IHIT46528	01/2006	1									1	1							1	1		1	1	1				
1	IHIT46530	07/2009	1									1	1			1				1	1		1	1	1				
1	IHIT45339	08/2009	1									1	1			1				1	1	1	1	1	1				
1	IHIT46531	12/2009	1									1	1			1				1		1		1	1				
1	IHIT46532	01/2010	1									1	1			1				1		1		1	1				
1	IHIT46533	03/2010	1									1	1								1	1	1	1	1				
1	IHIT48339	04/2010	1									1									1			1	1				
1	IHIT32406	06/2010	1									1	1			1				1	1	1	1	1	1				
1	IHIT48341	06/2010	1									1	1											1	1				
1	IHIT45342	07/2011	1									1	1			1				1	1	1	1	1	1				
1	IHIT46538	09/2012	1									1	1							1	1	1		1	1				
1	IHIT46553	10/2019	1									1	1			1				1			1		1				
1	IHIT52949	06/2011	23									1				1				1		1	1						
1	IHIT48327	04/2004	42									1	1											1	1				
1	IHIT48328	07/2004	42									1												1	1				
1	IHIT48326	05/2002	100									1																	
1	IHIT52948	02/2008	100									1				1													
1	IHIT45341	07/2011	100									1				1				1		1		1	1				
1	IHIT48351	12/2015	100									1																	
1	IHIT46534	06/2010	131																										
1	IHIT48340	06/2010	131																										
1	IHIT48343	06/2011	131																										
1	IHIT52950	07/2012	641									1				1						1	1						
1	IHIT48325	05/2002	710									1										1	1						
1	IHIT52947	12/2007	12009									1				1				1									
2	IHIT48329	07/2004	1									1	1								1		1	1	1				
2	IHIT48336	08/2005	1	1				1				1	1								1			1	1				
2	IHIT48354	11/2020	1									1	1										1	1	1				
2	IHIT48331	10/2004	10									1	1																
2	IHIT46535	02/2011	10									1	1			1							1	1					
2	IHIT46540	07/2013	10									1	1			1				1			1	1					
2	IHIT23335	07/2013	10									1	1			1				1		1	1	1					
2	IHIT46541	11/2014	10									1	1									1		1					
2	IHIT48348	11/2014	10									1	1											1					
2	IHIT46542	01/2015	10									1	1											1					
2	IHIT46550	02/2018	10									1	1							1				1					
2	IHIT48346	12/2012	20	1			1	1		1	1	1			1				1										
2	IHIT48330	07/2004	29	1	1		1	1	1	1	1			1	1														
2	IHIT48333	12/2004	42									1	1								1			1	1				
2	IHIT48334	12/2004	42									1	1			1					1			1	1				
2	IHIT48342	06/2010	93									1										1							
2	IHIT46526	06/2004	100																	1									
2	IHIT48332	10/2004	100									1																	
2	IHIT48335	03/2005	100									1										1							
2	IHIT46529	02/2006	100									1				1				1		1	1						
2	IHIT48338	08/2008	100									1								1									
2	IHIT46536	11/2011	100									1										1							
2	IHIT46537	01/2012	100									1								1		1							
2	IHIT45353	07/2013	100									1				1				1		1	1						
2	IHIT46539	07/2013	100									1				1				1		1							
2	IHIT25408	03/2014	100									1																	
2	IHIT48347	05/2014	100									1																	
2	IHIT27622	10/2014	100									1										1							
2	IHIT45399	01/2015	100									1																	
2	IHIT48349	07/2015	100									1																	
2	IHIT45401	09/2015	100									1																	
2	IHIT46543	09/2015	100									1	1							1			1	1					
2	IHIT46546	09/2016	100									1																	
2	IHIT45407	08/2017	100									1																	
2	IHIT36144	01/2018	100									1				1				1		1	1						
2	IHIT36146	01/2018	100									1				1				1		1	1						
2	IHIT36426	02/2018	100									1				1				1		1	1						
2	IHIT36427	02/2018	100									1				1				1		1	1						
2	IHIT48355	11/2020	641									1																	
2	IHIT48358	02/2021	641									1											1						
2	IHIT32748	09/2016	793	1		1	1		1	1	1	1			1									1					
2	IHIT48344	12/2011	799	1	1		1	1	1	1	1	1	1		1		1							1	1				
2	IHIT48356	11/2020	955									1	1								1			1	1				
2	IHIT48350	09/2015	2944									1	1				1				1		1		1				
3	IHIT34769	06/2017	1									1	1								1				1				
3	IHIT48353	06/2017	1									1	1												1				
3	IHIT47062	10/2017	1									1	1										1		1				
3	IHIT48352	12/2016	10									1	1											1					
3	IHIT39537	04/2019	10									1	1							1				1					
3	IHIT47044	10/2014	86									1																	
3	IHIT47045	10/2014	86									1																	
3	IHIT47046	06/2015	86									1				1													
3	IHIT47048	06/2015	86									1				1													
3	IHIT47056	06/2016	86									1																	
3	IHIT47057	11/2016	86									1											1						
3	IHIT34315	04/2017	86									1																	
3	IHIT47060	02/2017	90									1				1				1			1						
3	IHIT47047	06/2015	100									1																	
3	IHIT47065	07/2018	118									1	1								1			1	1				
3	IHIT47072	03/2019	162									1																	

				secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system	secretion system
Farm	Strain	Isolation date	ST	*espY3*	*espY4*	*etpD*	*gspC*	*gspD*	*gspE*	*gspF*	*gspG*	*gspH*	*gspI*	*gspJ*	*gspK*	*gspL*	*gspM*	*nleA*	*nleC*	*nleD*	*nleG7*	*nleH1*	*nleH2*	*sepA*	*sepD*
1	IHIT46527	06/2005	1	1			1	1	1	1	1	1	1	1	1	1	1								
1	IHIT48337	12/2005	1				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT46528	01/2006	1				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT46530	07/2009	1	1			1	1	1	1	1	1	1	1	1	1	1							1	
1	IHIT45339	08/2009	1	1	1		1	1	1	1	1	1	1	1	1	1	1							1	
1	IHIT46531	12/2009	1				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT46532	01/2010	1	1			1	1	1	1	1	1	1	1	1	1	1								
1	IHIT46533	03/2010	1				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT48339	04/2010	1				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT32406	06/2010	1	1	1		1	1	1	1	1	1	1	1	1	1	1								
1	IHIT48341	06/2010	1				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT45342	07/2011	1	1	1		1	1	1	1	1	1	1	1	1	1	1								
1	IHIT46538	09/2012	1	1			1	1	1	1	1	1	1	1	1	1	1								
1	IHIT46553	10/2019	1	1			1	1	1	1	1	1	1	1	1	1	1								
1	IHIT52949	06/2011	23				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT48327	04/2004	42				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT48328	07/2004	42				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT48326	05/2002	100						1	1	1	1	1	1		1									
1	IHIT52948	02/2008	100					1		1	1	1	1	1	1	1									
1	IHIT45341	07/2011	100				1				1	1	1	1	1	1	1								
1	IHIT48351	12/2015	100							1	1		1	1	1	1	1								
1	IHIT46534	06/2010	131				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT48340	06/2010	131				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT48343	06/2011	131				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT52950	07/2012	641				1	1	1	1	1	1	1	1	1	1	1								
1	IHIT48325	05/2002	710				1									1	1							1	
1	IHIT52947	12/2007	12009				1	1	1	1	1	1	1	1	1	1	1							1	
2	IHIT48329	07/2004	1				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT48336	08/2005	1	1			1	1	1	1	1	1	1	1	1	1	1								
2	IHIT48354	11/2020	1				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT48331	10/2004	10				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT46535	02/2011	10				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT46540	07/2013	10				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT23335	07/2013	10				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT46541	11/2014	10				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT48348	11/2014	10				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT46542	01/2015	10				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT46550	02/2018	10				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT48346	12/2012	20				1	1	1	1	1	1	1	1	1	1	1	1	1			1			
2	IHIT48330	07/2004	29				1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
2	IHIT48333	12/2004	42				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT48334	12/2004	42	1			1	1	1	1	1	1	1	1	1	1	1								
2	IHIT48342	06/2010	93				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT46526	06/2004	100				1				1	1		1	1	1								1	
2	IHIT48332	10/2004	100				1	1	1	1	1	1	1	1	1		1								
2	IHIT48335	03/2005	100							1	1	1		1	1		1							1	
2	IHIT46529	02/2006	100				1				1	1			1	1	1							1	
2	IHIT48338	08/2008	100							1	1	1		1	1	1									
2	IHIT46536	11/2011	100				1				1	1			1	1	1							1	
2	IHIT46537	01/2012	100				1				1	1			1	1	1							1	
2	IHIT45353	07/2013	100				1				1	1		1	1	1	1							1	
2	IHIT46539	07/2013	100				1				1	1			1	1	1							1	
2	IHIT25408	03/2014	100				1				1	1	1	1	1	1	1							1	
2	IHIT48347	05/2014	100							1	1	1	1	1	1		1							1	
2	IHIT27622	10/2014	100				1				1	1			1	1	1							1	
2	IHIT45399	01/2015	100				1				1	1		1	1	1	1							1	
2	IHIT48349	07/2015	100							1	1	1		1	1		1							1	
2	IHIT45401	09/2015	100				1				1	1		1	1	1	1							1	
2	IHIT46543	09/2015	100				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT46546	09/2016	100				1				1	1			1	1	1							1	
2	IHIT45407	08/2017	100				1				1	1		1	1	1	1							1	
2	IHIT36144	01/2018	100				1			1	1	1	1	1	1		1							1	
2	IHIT36146	01/2018	100				1			1	1	1	1	1	1		1							1	
2	IHIT36426	02/2018	100				1			1	1	1	1	1	1		1							1	
2	IHIT36427	02/2018	100				1			1	1	1	1	1	1		1							1	
2	IHIT48355	11/2020	641				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT48358	02/2021	641				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT32748	09/2016	793			1	1	1	1	1	1	1	1	1	1	1	1	1				1	1		
2	IHIT48344	12/2011	799			1	1	1	1	1	1	1	1	1	1	1	1	1			1	1			
2	IHIT48356	11/2020	955				1	1	1	1	1	1	1	1	1	1	1								
2	IHIT48350	09/2015	2944				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT34769	06/2017	1	1			1	1	1	1	1	1	1	1	1	1	1								
3	IHIT48353	06/2017	1				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT47062	10/2017	1				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT48352	12/2016	10				1	1	1	1	1	1	1	1	1	1	1							1	
3	IHIT39537	04/2019	10				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT47044	10/2014	86				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT47045	10/2014	86				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT47046	06/2015	86				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT47048	06/2015	86				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT47056	06/2016	86				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT47057	11/2016	86				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT34315	04/2017	86				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT47060	02/2017	90				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT47047	06/2015	100				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT47065	07/2018	118				1	1	1	1	1	1	1	1	1	1	1								
3	IHIT47072	03/2019	162				1	1	1	1	1	1	1	1	1	1	1								

				secretion system	secretion system	secretion system
Farm	Strain	Isolation date	ST	*sepQ/ escQ*	*ssaS*	*usp*
1	IHIT46527	06/2005	1			
1	IHIT48337	12/2005	1			
1	IHIT46528	01/2006	1			
1	IHIT46530	07/2009	1			
1	IHIT45339	08/2009	1			
1	IHIT46531	12/2009	1			
1	IHIT46532	01/2010	1			
1	IHIT46533	03/2010	1			
1	IHIT48339	04/2010	1			
1	IHIT32406	06/2010	1			
1	IHIT48341	06/2010	1			
1	IHIT45342	07/2011	1			
1	IHIT46538	09/2012	1			
1	IHIT46553	10/2019	1			
1	IHIT52949	06/2011	23			
1	IHIT48327	04/2004	42			
1	IHIT48328	07/2004	42			
1	IHIT48326	05/2002	100			
1	IHIT52948	02/2008	100			
1	IHIT45341	07/2011	100			
1	IHIT48351	12/2015	100			
1	IHIT46534	06/2010	131			1
1	IHIT48340	06/2010	131			1
1	IHIT48343	06/2011	131			1
1	IHIT52950	07/2012	641			
1	IHIT48325	05/2002	710			
1	IHIT52947	12/2007	12009			
2	IHIT48329	07/2004	1			
2	IHIT48336	08/2005	1			
2	IHIT48354	11/2020	1			
2	IHIT48331	10/2004	10			
2	IHIT46535	02/2011	10			
2	IHIT46540	07/2013	10			
2	IHIT23335	07/2013	10			
2	IHIT46541	11/2014	10			
2	IHIT48348	11/2014	10			
2	IHIT46542	01/2015	10			
2	IHIT46550	02/2018	10			
2	IHIT48346	12/2012	20			
2	IHIT48330	07/2004	29	1		
2	IHIT48333	12/2004	42			
2	IHIT48334	12/2004	42			
2	IHIT48342	06/2010	93			
2	IHIT46526	06/2004	100			
2	IHIT48332	10/2004	100			
2	IHIT48335	03/2005	100			
2	IHIT46529	02/2006	100			
2	IHIT48338	08/2008	100			
2	IHIT46536	11/2011	100			
2	IHIT46537	01/2012	100			
2	IHIT45353	07/2013	100			
2	IHIT46539	07/2013	100			
2	IHIT25408	03/2014	100			
2	IHIT48347	05/2014	100			
2	IHIT27622	10/2014	100			
2	IHIT45399	01/2015	100			
2	IHIT48349	07/2015	100			
2	IHIT45401	09/2015	100			
2	IHIT46543	09/2015	100			
2	IHIT46546	09/2016	100			
2	IHIT45407	08/2017	100			
2	IHIT36144	01/2018	100			
2	IHIT36146	01/2018	100			
2	IHIT36426	02/2018	100			
2	IHIT36427	02/2018	100			
2	IHIT48355	11/2020	641			
2	IHIT48358	02/2021	641			
2	IHIT32748	09/2016	793	1	1	
2	IHIT48344	12/2011	799			
2	IHIT48356	11/2020	955			
2	IHIT48350	09/2015	2944			
3	IHIT34769	06/2017	1			
3	IHIT48353	06/2017	1			
3	IHIT47062	10/2017	1			
3	IHIT48352	12/2016	10			
3	IHIT39537	04/2019	10			
3	IHIT47044	10/2014	86			
3	IHIT47045	10/2014	86			
3	IHIT47046	06/2015	86			
3	IHIT47048	06/2015	86			
3	IHIT47056	06/2016	86			
3	IHIT47057	11/2016	86			
3	IHIT34315	04/2017	86			
3	IHIT47060	02/2017	90			
3	IHIT47047	06/2015	100			
3	IHIT47065	07/2018	118			
3	IHIT47072	03/2019	162			

Table S3: Pathotypes, sequence types (STs), resistance genes and chromosomal point mutations related to antimicrobial resistance of 87 representative whole genome sequenced *E. coli* isolates, sorted by farms, STs, and date of isolation.

Strain ID	Date of isolation	Pathotype*	ST	*mcr* gene	Further resistance genes	Chromosomal point mutation
Farm 1 (*n* = 27)	05/2002 - 10/2019	EDEC (51.9%), ETEC (37.0%), STEC (7.4%), AdhF-*Ec* (3.7%)	ST1 (51.9%), ST100 (14.8%), ST131 (11.1%), ST42 (7.4%), ST23 (3.7%), ST641 (3.7%), ST710 (3.7%), ST12009 (3.7%)	*mcr-1.1* (51.9%)	*sul2* (85.2%), *aph(3``)-Ib* (77.8%), *aph(6)-Id* (77.8%), *bla*TEM-1B (77.8%), *tet(A)* (74.1%), *catA1* (33.3%), *aadA2b* (29.6%), *sul3* (29.6%), *tet(C)* (29.6%), *aac(3)-IV* (25.9%), *aph(4)-Ia* (25.9%), *dfrA1* (18.5%), *aadA1* (14.8%), *dfrA14* (14.8%), *sul1* (14.8%), *tet(B)* (11.1%), *aadA24* (7.4%), *aadA13* (3.7%), *bla*TEM-1A (3.7%), *cmlA1* (3.7%)	*parE* I529L (11.1%), *pmrB* V161G (7.4%), *gyrA* S83L (3.7%)
IHIT46527	06/2005	EDEC	1	-	-	-
IHIT48337	12/2005	EDEC	1	-	*aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *sul2, tet(A)*	-
IHIT46528	01/2006	EDEC	1	-	*aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *sul2, tet(A)*	-
IHIT46530	07/2009	EDEC	1	*mcr-1.1*	*aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *sul2, tet(A), tet(C)*	-
IHIT45339	08/2009	EDEC	1	*mcr-1.1*	*aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *sul2, tet(A), tet(C)*	-
IHIT46531	12/2009	EDEC	1	*mcr-1.1*	*aac(3)-IV, aadA2b, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*TEM-1B, *catA1, sul2, sul3, tet(A), tet(C)*	-
IHIT46532	01/2010	EDEC	1	*mcr-1.1*	*aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *sul2, tet(A), tet(C)*	-
IHIT46533	03/2010	EDEC	1	*mcr-1.1*	*aac(3)-IV, aadA2b, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*TEM-1B, *catA1, sul2, sul3, tet(A), tet(C)*	-
IHIT48339	04/2010	EDEC	1	*mcr-1.1*	*aac(3)-IV, aadA2b, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*TEM-1B, *catA1, sul2, sul3, tet(A), tet(C)*	-
IHIT32406	06/2010	EDEC	1	*mcr-1.1*	*aac(3)-IV, aadA2b, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*TEM-1B, *catA1, sul2, sul3, tet(A), tet(C)*	-
IHIT48341	06/2010	EDEC	1	*mcr-1.1*	*aac(3)-IV, aadA2b, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*TEM-1B, *catA1, sul2, sul3, tet(A), tet(C)*	-
IHIT45342	07/2011	EDEC	1	*mcr-1.1*	*aac(3)-IV, aadA2b, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*TEM-1B, *catA1, sul2, sul3, tet(A)*	-
IHIT46538	09/2012	EDEC	1	*mcr-1.1*	*aac(3)-IV, aadA2b, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*TEM-1B, *catA1, sul2, sul3, tet(A)*	-
IHIT46553	10/2019	EDEC	1	-	*bla*TEM-1B, *dfrA1, sul2*	-
IHIT52949	06/2011	ETEC	23	-	*aadA1, bla*TEM-1A, *dfrA1, sul2, tet(B)*	-
IHIT48327	04/2004	ETEC	42	-	*aph(3``)-Ib, aph(6)-Id, sul2*	*pmrB* V161G
IHIT48328	07/2004	ETEC	42	-	*aadA1*	*pmrB* V161G, *gyrA* S83L
IHIT48326	05/2002	ETEC	100	-	*aadA1, catA1, sul1*	-
IHIT52948	02/2008	ETEC	100	-	*aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *dfrA14, sul2, tet(A)*	-
IHIT45341	07/2011	ETEC	100	*mcr-1.1*	*aadA1, aadA2b, aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *cmlA1, catA1, sul2, sul3, dfrA1, dfrA14, tet(A)*	-

Strain ID	Date of isolation	Pathotype*	ST	*mcr* gene	Further resistance genes	Chromosomal point mutation
IHIT48351	12/2015	ETEC	100	-	*aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *dfrA14, sul2, tet(A)*	-
IHIT46534	06/2010	ETEC	131	*mcr-1.1*	*aadA24, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *dfrA1, sul1, sul2, tet(A)*	*parE* I529L
IHIT48340	06/2010	ETEC	131	*mcr-1.1*	*aadA24, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *dfrA1, sul1, sul2, tet(A)*	*parE* I529L
IHIT48343	06/2011	ETEC	131	*mcr-1.1*	*aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *sul2, tet(A)*	*parE* I529L
IHIT52950	07/2012	AdhF-*Ec*	641	-	*tet(B)*	-
IHIT48325	05/2002	STEC	710	-	*aadA13, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *sul1, sul2, tet(A)*	-
IHIT52947	12/2007	STEC	12009	-	*aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *dfrA14, sul2, tet(A), tet(B)*	-
Farm 2 (*n* = 44)	06/2004-02/2021	ETEC (63.6%), AEEC (11.4%), ETEC-like (11.4%), AdhF-*Ec* (4.6%), EDEC (4.6%), ETEC/STEC (2.3%), STEC (2.3%)	ST100 (50%), ST10 (18.2%), ST1 (6.8%), ST42 (4.6%), ST641 (4.6%), ST20 (2.3%), ST29 (2.3%), ST93 (2.3%), ST793 (2.3%), ST799 (2.3%), ST955 (2.3%), ST2944 (2.3%)	*mcr-1.1* (38.6%)	*tet(A)* (79.6%), *bla*$_{TEM-1B}$ (72.7%), *sul2* (68.2%), *aadA1* (45.5%), *aph(3``)-Ib* (43.2%), *aph(6)-Id* (43.2%), *sul1* (29.6%), *dfrA1* (20.5%), *aadA5* (18.2%), *dfrA14* (15.9%), *catB3* (13.6%), *tet(B)* (13.6%), *bla*$_{TEM-135}$ (11.4%), *catA1* (11.4%), *mph(A)* (9.1%), *aac(3)-IV* (4.6%), *aph(4)-Ia* (4.6%), *bla*$_{TEM-1A}$ (4.6%), *dfrA17* (4.6%), *sul3* (4.6%), *aadA2* (2.3%), *aadA24* (2.3%), *bla*$_{CTX-M-1}$ (2.3%), *cmlA* (2.3%), *dfrA8* (2.3%), *dfrA12* (2.3%), *qnrS1* (2.3%)	*pmrB* V161G (4.6%), *gyrA* S83L (4.6%), *parC* A56T (2.3%), *parE* I355T (2.3%)
IHIT48329	07/2004	EDEC	1	-	*aadA1, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *catA1, sul1, sul2, tet(A)*	-
IHIT48336	08/2005	EDEC	1	-	*aadA1, bla*$_{TEM-1A}$, *dfrA1, sul2, tet(B)*	-
IHIT48354	11/2020	AdhF-*Ec*	1	-	*aadA1, bla*$_{TEM-1B}$, *sul1, tet(A), tet(B)*	-
IHIT48331	10/2004	ETEC-like	10	-	*aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *dfrA14, sul2, tet(A)*	-
IHIT46535	02/2011	ETEC	10	-	*aadA1, aph(3``)-Ib, aph(6)-Id, bla*$_{CTX-M-1}$, *bla*$_{TEM-135}$, *sul2, tet(A)*	-
IHIT46540	07/2013	ETEC	10	-	*aadA1, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-135}$, *sul2, tet(A)*	-
IHIT23335	07/2013	ETEC/STEC	10	-	*aadA1, aadA24, aph(3')-Ia, bla*$_{TEM-1B}$, *sul3, tet(A)*	-
IHIT48348	11/2014	ETEC-like	10	-	*aadA1, aadA2, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$, *cmlA, dfrA12, mph(A), sul3, tet(B)*	-
IHIT46541	11/2014	ETEC	10	*mcr-1.1*	*aadA1, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-135}$, *sul2, tet(A)*	-
IHIT46542	01/2015	ETEC	10	*mcr-1.1*	*aadA1, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-135}$, *sul2, tet(A)*	-
IHIT46550	02/2018	ETEC	10	*mcr-1.1*	*aadA1, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-135}$, *sul2, tet(A)*	-
IHIT48346	12/2012	AEEC	20	-	*aadA1, dfrA1, sul2, tet(B)*	-
IHIT48330	07/2004	AEEC	29	-	*aadA1, aadA5, bla*$_{TEM-1B}$, *mph(A), sul1, dfrA17, tet(A)*	-
IHIT48333	12/2004	ETEC	42	-	*aadA1*	*pmrB* V161G, *gyrA* S83L

Strain ID	Date of isolation	Pathotype*	ST	*mcr* gene	Further resistance genes	Chromosomal point mutation
IHIT48334	12/2004	AdhF-*Ec*	42	-	*aadA1*	*pmrB* V161G, *gyrA* S83L
IHIT48342	06/2010	AEEC	93	-	*aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, dfrA8, sul2, tet(A)*	-
IHIT46526	06/2004	ETEC	100	-	*aadA1, bla*$_{TEM-1B}$*, catA1, sul1, tet(A)*	-
IHIT48332	10/2004	ETEC	100	-	*aac(3)-IV, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT48335	03/2005	ETEC	100	-	*aadA1, bla*$_{TEM-1B}$*, catA1, sul1, tet(A),*	-
IHIT46529	02/2006	ETEC	100	-	*aadA1, aadA5, aac(3)-IId, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, dfrA17, catA1, mph(A), sul1, tet(A)*	-
IHIT48338	08/2008	ETEC	100	-	*aadA1, aac(3)-IV, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*$_{TEM-1B}$*, catA1, mph(A), tet(A)*	-
IHIT46536	11/2011	ETEC	100	-	*aadA5, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, catB3, dfrA1, dfrA14, sul1, sul2, tet(A)*	-
IHIT46537	01/2012	ETEC	100	-	*aadA5, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, catB3, dfrA1, dfrA14, sul1, sul2, tet(A)*	-
IHIT45353	07/2013	ETEC	100	*mcr-1.1*	*aadA5, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, catB3, dfrA1, dfrA14, sul1, sul2, tet(A)*	-
IHIT46539	07/2013	ETEC	100	-	*aadA5, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, catB3, dfrA1, dfrA14, sul1, sul2, tet(A)*	-
IHIT25408	03/2014	ETEC	100	*mcr-1.1*	*aadA5, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, catB3, dfrA1, dfrA14, sul1, sul2, tet(A)*	-
IHIT48347	05/2014	ETEC	100	*mcr-1.1*	*aadA5, aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, catB3, dfrA1, dfrA14, sul1, sul2, tet(A)*	-
IHIT27622	10/2014	ETEC	100	*mcr-1.1*	*bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT45399	01/2015	ETEC	100	*mcr-1.1*	*bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT48349	07/2015	ETEC	100	*mcr-1.1*	*bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT45401	09/2015	ETEC	100	*mcr-1.1*	*bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT46543	09/2015	ETEC	100	*mcr-1.1*	*bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT46546	09/2016	ETEC	100	*mcr-1.1*	*bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT45407	08/2017	ETEC	100	*mcr-1.1*	*bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT36144	01/2018	ETEC	100	*mcr-1.1*	*bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT36146	01/2018	ETEC	100	*mcr-1.1*	*bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT36426	02/2018	ETEC	100	*mcr-1.1*	*bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT36427	02/2018	ETEC	100	*mcr-1.1*	*bla*$_{TEM-1B}$*, sul2, tet(A)*	-
IHIT48355	11/2020	ETEC-like	641	-	*tet(B)*	-
IHIT48358	02/2021	ETEC-like	641	-	*bla*$_{TEM-1B}$	-
IHIT32748	09/2016	AEEC	793	-	*aadA1, bla*$_{TEM-1B}$*, qnrS1, sul1, tet(A)*	*parC* A56T
IHIT48344	12/2011	AEEC	799	-	*aadA1, bla*$_{TEM-1A}$*, dfrA1, sul2, tet(A)*	-
IHIT48356	11/2020	STEC	955	-	*aph(3``)-Ib, aph(6)-Id, bla*$_{TEM-1B}$*, sul2, tet(B)*	-

Strain ID	Date of isolation	Pathotype*	ST	*mcr* gene	Further resistance genes	Chromosomal point mutation
IHIT48350	09/2015	ETEC-like	2944	-	-	*parE* I355T
Farm 3 (*n* = 16)	10/2014 – 04/2019	ETEC/STEC (37.5%), ETEC (18.8%), ETEC-like (18.8%), EDEC (12.5%), STEC (12.5%)	ST86 (43.8), ST1 (18.8%), ST10 (12.5%), ST90 (6.3%), ST100 (6.3%), ST118 (6.3%), ST162 (6.3%)	*mcr-1.1* (50%), *mcr-4.8* (6.3%)	*dfrA1* (75%), *aph(3``)-Ib* (68.8%), *aph(6)-Id* (68.8%), *bla*TEM-1B (68.8%), *sul1* (68.8%), *sul2* (68.8%), *aadA1* (62.5%), *tet(A)* (62.5%), *tet(B)* (56.3%), *sul3* (50%), *aadA13* (43.8%), *catA1* (43.8%), *aph(3`)-Ia* (37.5%), *aac(3)-IV* (18.8%), *aph(4)-Ia* (18.8%), *floR-like* (18.8%), *bla*TEM-1A (12.5%), *aadA5* (6.3%), *ant(3``)-Ia* (6.3%), *catB3* (6.3%), *dfrA14* (6.3%) *mph(B)* (6.3%), *mph(G)* (6.3%), *mef(C)* (6.3%)	*gyrA* S83L (50%), *parE* I355T (43.8%), *ampC*-prom 42C>T (18.8%)
IHIT34769	06/2017	EDEC	1	*mcr-1.1*	-	-
IHIT48353	06/2017	STEC	1	*mcr-1.1*	*aadA5, aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *catB3, dfrA1, dfrA14, sul1, sul2, tet(A)*	-
IHIT47062	10/2017	EDEC	1	-	-	-
IHIT48352	12/2016	STEC	10	*mcr-1.1*	*aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *dfrA1, sul1, sul2, tet(B)*	-
IHIT39537	04/2019	ETEC-like	10	*mcr-4.8*	*bla*TEM-1A	-
IHIT47044	10/2014	ETEC/STEC	86	*mcr-1.1*	*aac(3)-IV, aadA1, aadA13, aph(3`)-Ia, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*TEM-1B, *catA1, dfrA1, floR-like, sul1, sul2, sul3, tet(A), tet(B)*	*gyrA* S83L, *parE* I355T
IHIT47045	10/2014	ETEC/STEC	86	-	*aac(3)-IV, aadA1, aadA13, aph(3`)-Ia, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*TEM-1B, *catA1, dfrA1, floR-like, sul1, sul2, sul3, tet(A), tet(B)*	*gyrA* S83L, *parE* I355T
IHIT47046	06/2015	ETEC/STEC	86	*mcr-1.1*	*aac(3)-IV, aadA1, aadA13, aph(3`)-Ia, aph(3``)-Ib, aph(4)-Ia, aph(6)-Id, bla*TEM-1B, *catA1, dfrA1, floR-like, sul1, sul2, sul3, tet(A), tet(B)*	*gyrA* S83L, *parE* I355T
IHIT47048	06/2015	ETEC/STEC	86	-	*aadA1, aadA13, aph(3`)-Ia, aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *catA1, dfrA1, sul1, sul2, sul3, tet(A), tet(B)*	*gyrA* S83L, *parE* I355T
IHIT47056	06/2016	ETEC/STEC	86	*mcr-1.1*	*aadA1, aadA13, ant(3``)-Ia, aph(3`)-Ia, aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *catA1, dfrA1, sul1, sul2, sul3, tet(A), tet(B)*	*gyrA* S83L, *parE* I355T, *ampC*-prom 42C>T
IHIT47057	11/2016	ETEC	86	-	*aadA1, aadA13, aph(3`)-Ia, aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *catA1, dfrA1, sul1, sul2, sul3, tet(A), tet(B)*	*gyrA* S83L, *parE* I355T, *ampC*-prom 42C>T
IHIT34315	04/2017	ETEC/STEC	86	*mcr-1.1*	*aadA1, aadA13, aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *catA1, dfrA1, sul1, sul2, sul3, tet(A), tet(B)*	*gyrA* S83L, *parE* I355T, *ampC*-prom 42C>T
IHIT47060	02/2017	ETEC	90	-	*aadA1, aph(3``)-Ib, aph(6)-Id, bla*TEM-1B, *dfrA1, mph(B), mph(G), mef(C), sul1, sul2, tet(B)*	-
IHIT47047	06/2015	ETEC	100	*mcr-1.1*	*aadA1, bla*TEM-1A, *dfrA1, sul3, tet(A)*	-
IHIT47065	07/2018	ETEC-like	118	-	*aph(3``)-Ib, aph(6)-Id, aadA1, bla*TEM-1B, *dfrA1, sul1, sul2, tet(A)*	-

Strain ID	Date of isolation	Pathotype*	ST	*mcr* gene	Further resistance genes	Chromosomal point mutation
IHIT47072	03/2019	ETEC-like	162	-	-	*gyrA* S83L

* Pathotypes: AdhF-*Ec*, positive for at least one adhesive fimbriae gene (*faeG, fanA, fasA, fedA, fimF41a*); AEEC, 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, 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*.

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