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Antibiotika-Resistenz im One-Health-Kontext: Rolle bakterieller Klone und Plasmide

Habilitationsschrift
zur Erlangung der Lehrbefähigung für das Fach

Mikrobiologie

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vorgelegt von

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Inhaltsverzeichnis

1. Einleitung

Der Begriff „Antibiotikum“ wurde 1940 von Waksman und Woodruff eingeführt (1). Als Antibiotikum werden Substanzen bezeichnet, die Bakterien am Wachstum hemmen (bakteriostatische Antibiotika) oder abtöten (bakterizide Antibiotika).

Das erste klinisch angewendete Antibiotikum war Penicillin und wurde zufällig von Sir Alexander Fleming entdeckt (2). Mit dieser Erstbeschreibung eines Antibiotikums war die Menschheit der Ansicht, ein „magic bullet“ für die Behandlung bakterieller Infektionen entdeckt zu haben (3), welches nun ohne Einschränkungen und für immer verwendet werden könnte. Diese Ansicht stellte sich allerdings schnell als Trugschluss heraus. Da alle bis *dato* bekannten Antibiotika einen Selektionsdruck auf Bakterien ausüben, versuchen Bakterien diesem Selektionsdruck auszuweichen und entwickeln als Reaktion darauf Antibiotika-Resistenzen.

Antibiotika-resistente Bakterien können mit den Antibiotika, gegen welche sie resistent sind, nicht mehr behandelt werden. Die Auswahl der Antibiotika zur Behandlung einer Infektion mit diesen Bakterien ist somit eingeschränkt. Wie in Abbildung 1 dargestellt, dauert es mitunter weniger als 10 Jahre nach Einführung eines neuen Antibiotikums in die klinische Anwendung, bevor Bakterien Resistenzen dagegen entwickeln.

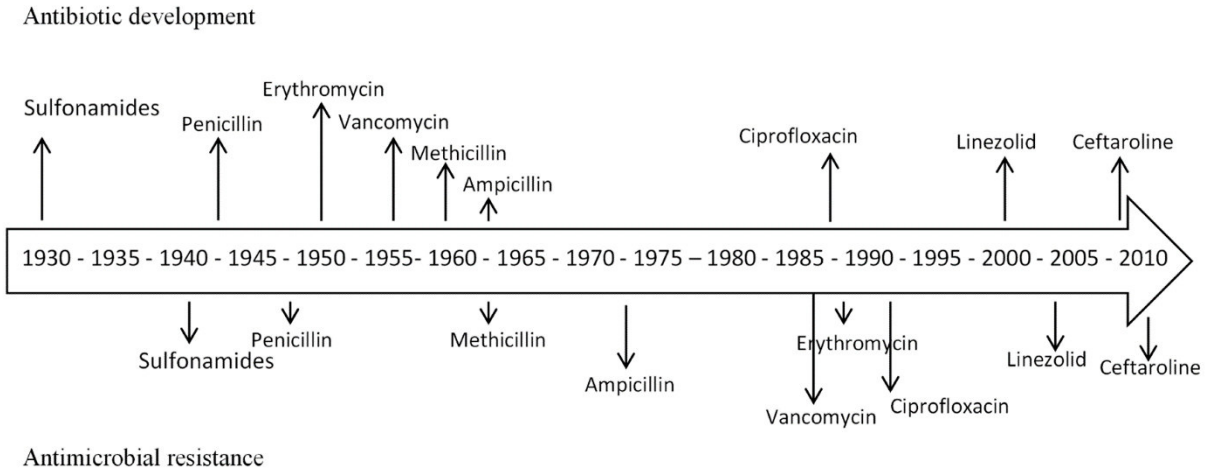


Abbildung 1: Zeitstrahl mit Entwicklung von Antibiotika (obere Zeile) und Antibiotika-Resistenz (untere Zeile). Mit Erlaubnis entnommen aus (4).

Bei der Entdeckung der ersten Antibiotika-Resistenzen waren die Forscher der Meinung, dass deren Entwicklung eine Parallel-Entwicklung mit der klinischen Anwendung von Antibiotika sein müsste. Diese Hypothese wurde allerdings durch die

Detektion von Antibiotika-Resistenzgenen in 30.000 Jahre alten Permafrost-Böden und bei dem vor ca. 3300 Jahren lebenden Mann aus dem Eis „Ötzi“ (5, 6) widerlegt.

1.1. Antibiotika-Resistenz – ein “Emerging“ und One-Health-Problem

Zu dem grundsätzlichen Problem, dass Bakterien unter Antibiose Resistenzen entwickeln, kommt der Aspekt, dass die Anzahl Antibiotika-resistenter Bakterien unter anderem durch die vermehrte Verwendung von Antibiotika seit etwa 20 Jahren stetig zunimmt. Dieser Trend ist sowohl in Europa (Abbildung 2) als auch in der ganzen Welt zu beobachten (7).

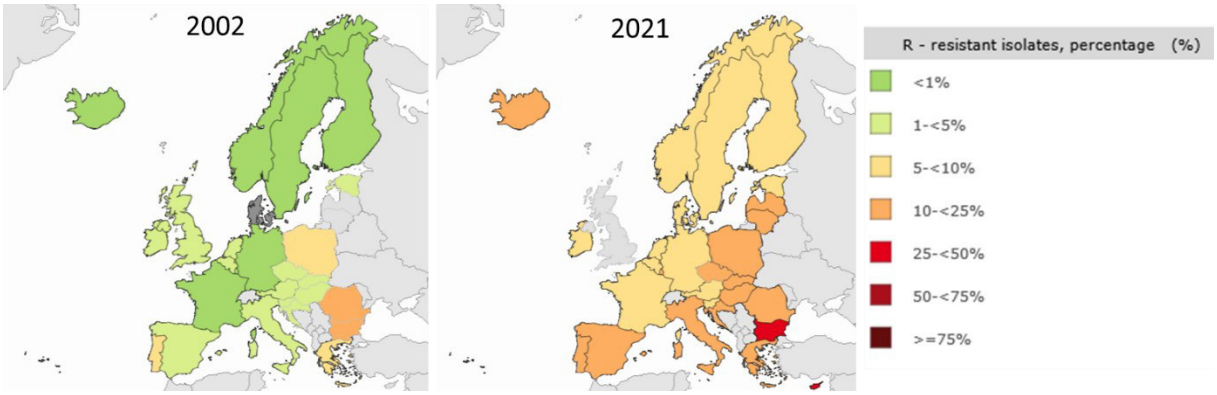


Abbildung 2: Prozentueller Anteil gegen Dritt-Generations-Cephalosporin-resistenter *Escherichia coli* in Europa. Quelle: (8)

International ist das Thema Antibiotika-Resistenz mittlerweile sehr stark präsent. Die Weltgesundheitsorganisation (WHO) hat dieses Problem bereits länger erkannt und einen „Global Action Plan on Antimicrobial Resistance“ in die Welt gerufen. Mit diesem Global Action Plan wurden bestimmte Maßnahmen vorangetrieben, unter anderem das weltweite „Global Antimicrobial Resistance and Use Surveillance System“ (GLASS, (9)), welches eine weltweite Kooperation regionaler Netzwerke zur Surveillance von Antibiotika-Resistenz und Antibiotika-Verbrauch darstellt. Zu diesen regionalen Netzwerken gehören unter anderem das EARS-Net (9), welches Länder der Europäischen Union abdeckt.

Im Rahmen von GLASS wurde unter anderem ermittelt, dass weltweit Antibiotika-resistente Bakterien in Ländern mit hohem Einkommen (high income countries, HICs) weniger häufig zu detektieren sind (Abbildung 3). Als möglicher Grund wird hierfür der

vergleichsmäßig hohe Anstieg des Antibiotika-Verbrauchs in Ländern mit geringerem Einkommen in den letzten Jahren genannt (10).

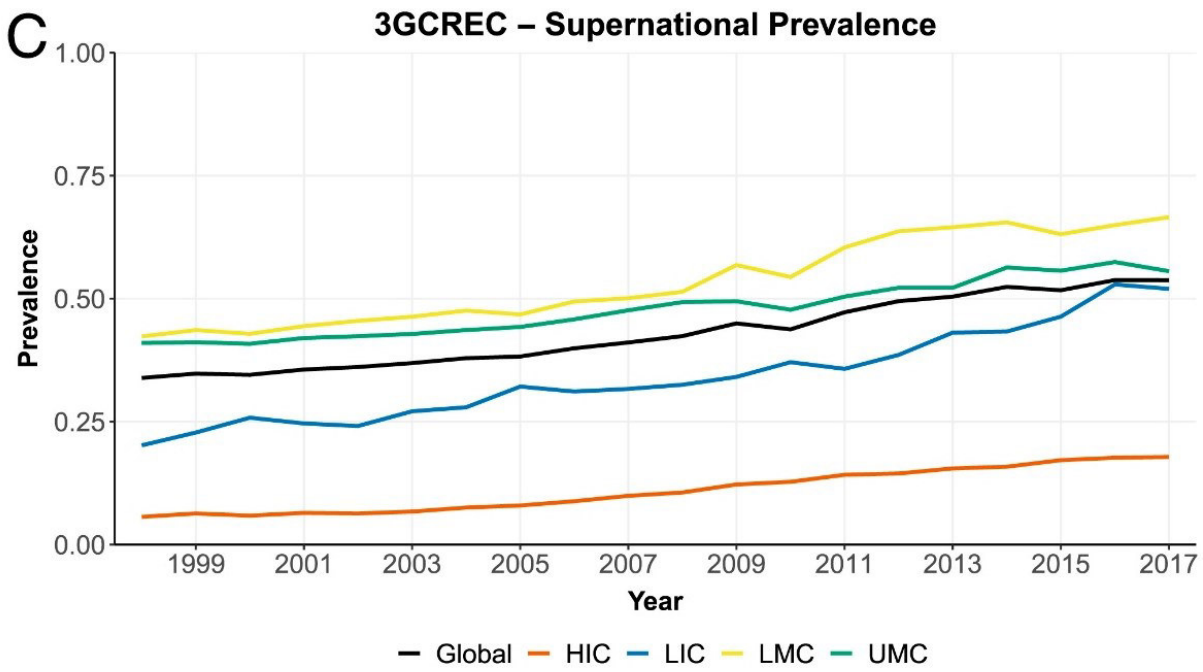


Abbildung 3: Prävalenz von Dritt-Generations-Cephalosporine-resistenten *Escherichia coli* aufgeteilt nach Einkommen. HIC, high income countries, LIC, low income countries, LMC, Lower-Middle Income countries, UMC, Upper middle income countries. Mit Erlaubnis entnommen aus (7).

Antibiotika-Resistenz stellt somit nicht nur ein gesundheitliches Problem dar, sondern auch ein sozialpolitisches. Diese Tatsache wurde 2016 von den Vereinten Nationen wahrgenommen und in einer von 193 Mitgliedsstaaten der Vereinten Nationen unterzeichneten politischen Erklärung zum Thema Antibiotika-Resistenz dokumentiert (11). Diese Erklärung stellte nach den Themen „HIV“ und „nicht-kommunizierbare Krankheiten“ die dritte politische Erklärung der Vereinten Nationen zu einem Gesundheitsthema dar.

In Deutschland wurden als Reaktion auf den rapiden Anstieg der Prävalenz Antibiotika-resistenter Bakterien Anfang der 2000er Jahre Netzwerke für Multiresistente Erreger (MRE-Netzwerke, (12)) und wissenschaftliche Forschungsverbünde mit Fokus auf Antibiotika-Resistenz etabliert (13).

Die Hauptaufgaben der MRE-Netzwerke sind, das Fachpersonal auf das Vorgehen bei Auftreten eines multiresistenten Erregers (MRE) zu schulen und die Aufmerksamkeit

der Allgemeinbevölkerung hinsichtlich Antibiotika-resistenter Bakterien zu erhöhen (14).

Die nationalen Forschungsverbünde sollten, bezogen auf die unterschiedlichen Erregergruppen (z.B. Methicillin-resistente *Staphylococcus aureus*, MRSA, Dritt-Generations-Cephalosporin-resistente *E. coli*, 3GC-EC) ermitteln, wie stark verbreitet Antibiotika-resistente Bakterien in unterschiedlichen Quellen waren (Mensch, Nutztier, Haustier, Wildtier, Umwelt) und ob Lebensmittel ein Risiko für die Übertragung Antibiotika-resistenter Bakterien darstellen könnten. Eines dieser durch BMBF geförderten Forschungsverbünde war das Netzwerk RESET (15). RESET widmete sich der Frage nach dem Vorhandensein von Extended-Spektrum-Beta-Laktamase- (ESBL) und Fluorchinolon-Resistenz-kodierenden Enterobacterales in Tier, Mensch und Umwelt. Entgegen früheren Annahmen wurden im Rahmen der RESET-Studie ESBL-produzierende *Escherichia coli* in allen untersuchten Quellen, also Nutztieren (16), Lebensmitteln (17), Umweltproben (18), Haustieren und Menschen (19) gefunden. Diese Deutschland-spezifischen Ergebnisse waren in Übereinstimmung mit Beobachtungen aus anderen europäischen Ländern und der Welt (20–22). In anderen Studien konnten ESBL-Produzenten in Wildtieren (23–25) und in Nutz- und Abwasserproben detektiert werden (26, 27). Antibiotika-Resistenz ist folglich ein Problem aller Kompartimente, also ein echtes One-Health-Problem. Hierbei sind unterschiedliche Richtungen der Resistenzübertragung denkbar (Abbildung 4).

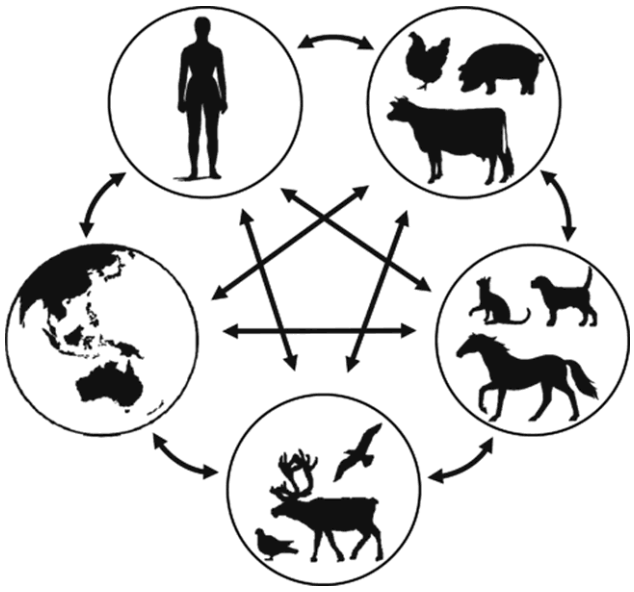


Abbildung 4: Darstellung der möglichen Wege zur Übertragung Antibiotika-resistenter Bakterien. Mit Erlaubnis entnommen aus (28).

Um neue Lösungsansätze für das Problem Antibiotika-Resistenz zu entwickeln, sind zahlreiche nationale und internationale Netzwerke entstanden, die neue Strategien zur Bekämpfung Antibiotika-resistenter Bakterien entwickeln. Zu den nationalen Netzwerken zählt unter anderem das Deutsche Zentrum für Infektionsforschung (DZIF, (29)).

1.2. Bakterielle Antibiotika-Resistenz-Strategien und Resistenz-Übertragungsmechanismen

Bakterien können Resistenzen gegen sehr viele unterschiedliche Antibiotika ausbilden. In Abbildung 5 sind die Ziele verschiedener Antibiotika und die Resistenzstrategien von Bakterien dargestellt.

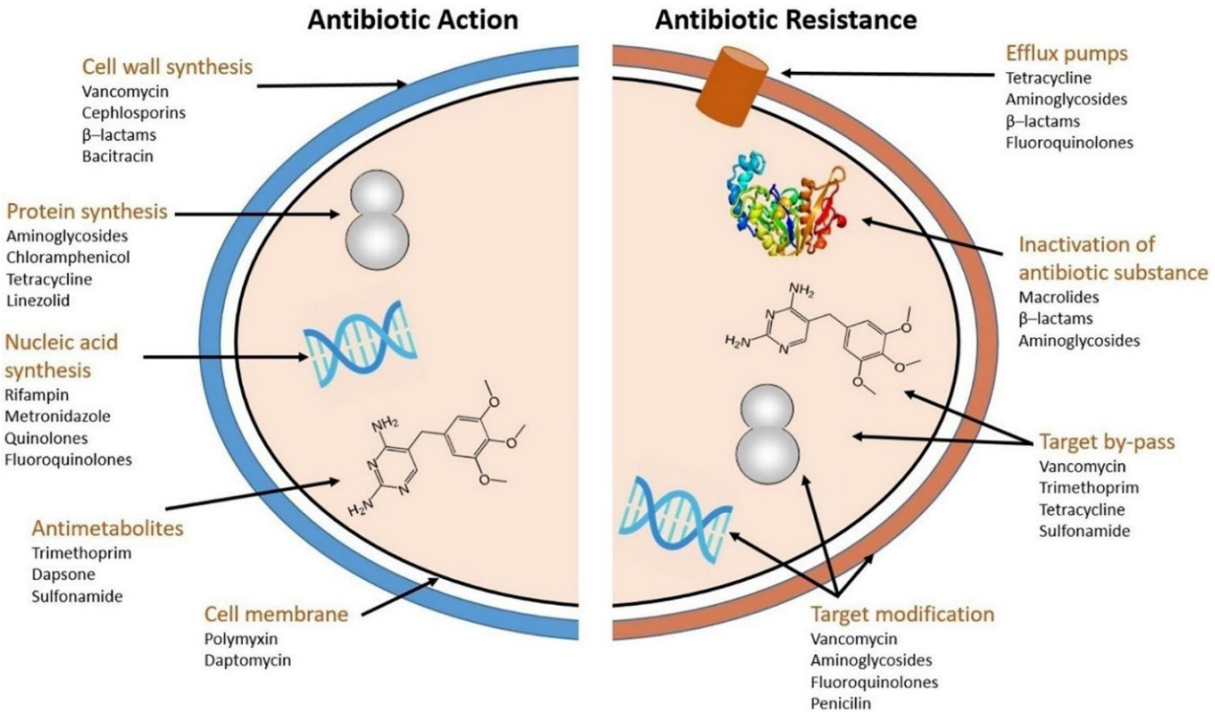


Abbildung 5: Darstellung der Wirkorte der Antibiotika und Strategien zur Antibiotika-Resistenz. Mit Erlaubnis entnommen aus (30).

In Deutschland spielten bis etwa 2010 Methicillin-resistente *Staphylococcus aureus* (MRSA) eine große Rolle in der Humanmedizin wurden danach allerdings von Enterobacterales mit Resistenz gegen 3.-Generations-Cephalosporine (3GC-EC) abgelöst. Aktuell spielen Vancomycin-resistente *Enterococcus faecium/faecalis* (VRE) eine zunehmende Rolle (Abbildung 6).

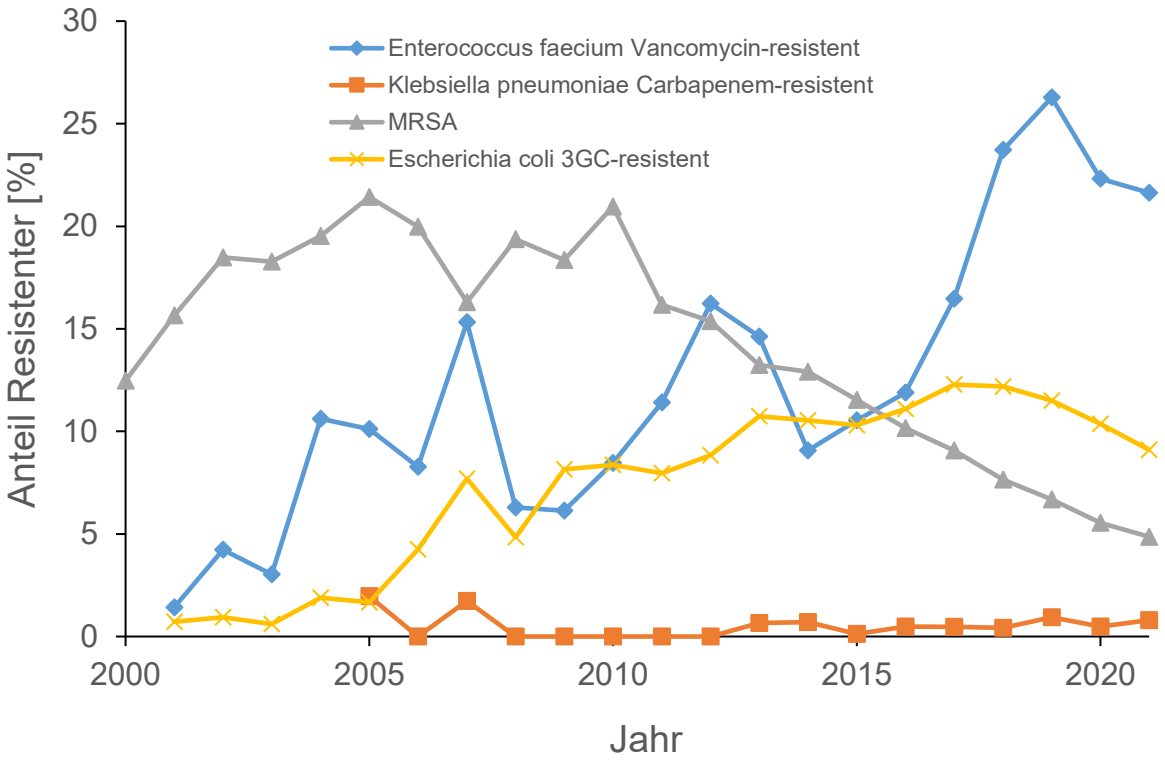


Abbildung 6: Darstellung der Resistenztrends einiger wichtiger Antibiotika-resistenter Spezies in Deutschland. Quelle: (8) Datenstand: 30.01.2023.

Bakterien verwenden unterschiedliche molekulare Strategien, um sich gegen Antibiotika zu schützen (Abbildung 5):

- Effluxpumpen zum Ausschleusen der Antibiotika aus dem Zytoplasma oder periplasmatischen Raum, damit diese nicht an ihrem Wirkort gelangen
- Enzyme zur Modifizierung von Antibiotika, um ihre Wirkung zu inaktivieren
- Verwendung alternativer Pathways, an denen die Antibiotika nicht angreifen können
- Veränderung der Zielstruktur über Einführen von Modifikationen

Diese Strategien können Bakterien auf genetischer Ebene über zwei unterschiedliche Wege umsetzen:

1. Mutation chromosomaler Gene
2. Akquise zusätzlicher genetischer Elemente

Für die Verbreitung von Antibiotika-Resistenzen spielen zwei Mechanismen eine wesentliche Rolle (Abbildung 7):

1. die vertikale Übertragung, auch klonale Übertragung genannt, bei der die Resistenzdeterminanten über Zellteilung von einer Mutter- auf zwei Tochterzellen verbreitet werden.
2. die Übertragung mobiler genetischer Elemente, wie z.B. Plasmide oder Transposons

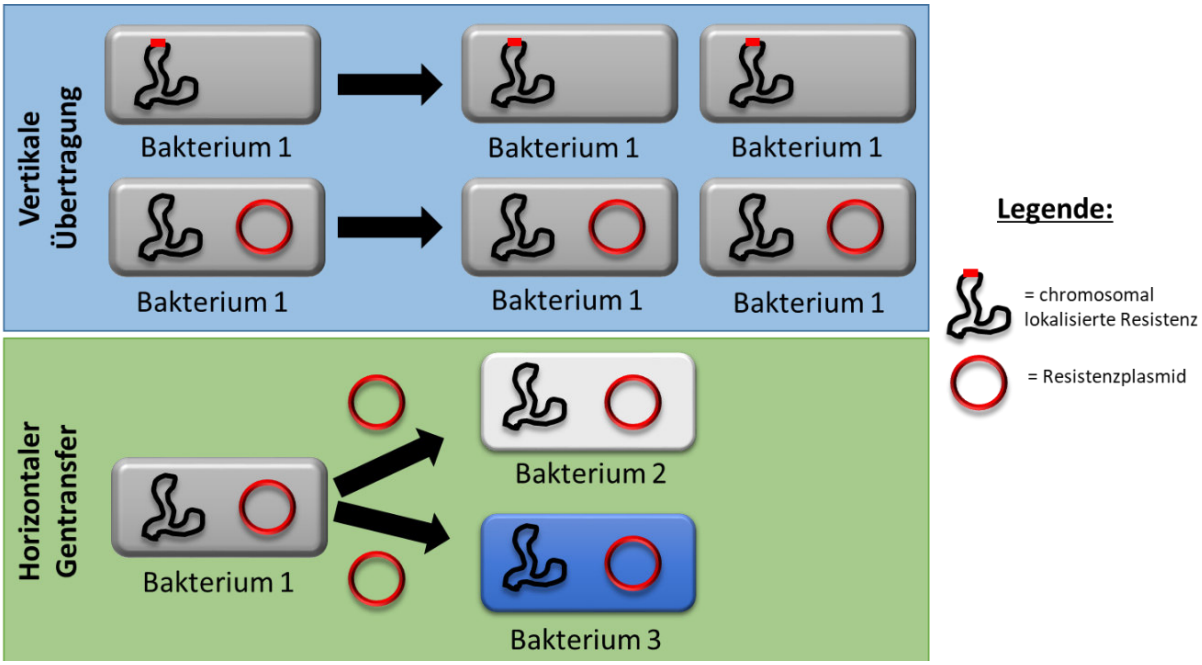


Abbildung 7: Darstellung zweier Wege zur Verbreitung der Antibiotika-Resistenz-Determinanten.

Chromosomal lokalisierte Antibiotika-Resistenzgene, wie z.B. die *mec*-Gene bei Methicillin-resistenten *Staphylococcus aureus* (MRSA, (31)) oder auch *vanB*-Determinanten bei bestimmten Vancomycin-resistenten *Enterococcus faecium*-Isolaten (32) werden üblicherweise vertikal weitergegeben. In sehr seltenen Fällen können auch chromosomal-lokalisierte Resistenzgene mobilisiert werden. In diesem Fall ist das Vorhandensein sogenannter Insertionselemente ausschlaggebend. Insertionselemente kodieren Transposasen, Enzyme, die in der Lage sind, DNA-Stücke aus der DNA auszuschneiden und an anderer Stelle im Chromosom oder auf Plasmiden wieder einzufügen (33). Flankieren ein oder mehrere Insertionselemente Antibiotika-Resistenzgene (Abbildung 8), so können diese mit transferieren werden.

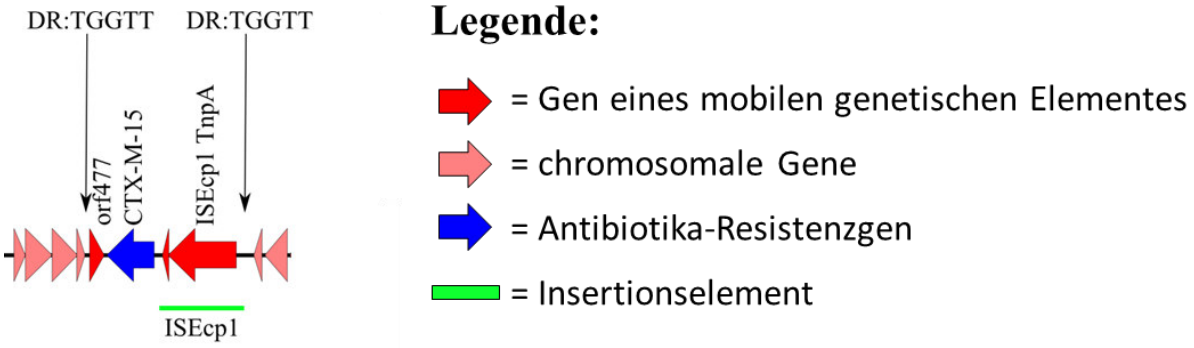


Abbildung 8: Darstellung eines mobilen genetischen Elementes, welches aus dem Chromosom auf ein Plasmid mobilisiert werden könnte (mit Erlaubnis verändert nach (34)).

Ist das Target des Transfers eines solchen Elementes ein konjugatives Plasmid, so wird aus einem bis dahin klonal übertragenen Antibiotika-Resistenzgen ein horizontal übertragenes.

Der zweite wichtige Weg für die Übertragung von Antibiotika-Resistenzgenen ist der horizontale Gentransfer, bei dem DNA-Abschnitte/Elemente ohne Zellteilung von einer bakteriellen Zelle auf eine andere übertragen werden können. Elemente, die über horizontalen Gentransfer übertragen werden, sind unter anderem Plasmide, auf die aufgrund ihrer großen Relevanz im nächsten Kapitel genauer eingegangen wird.

1.3. Plasmide – wichtige Antibiotika-Resistenz-Vehikel

Antibiotika-Resistenzplasmide stellen ein wichtiges Vehikel zur Übertragung von Antibiotika-Resistenzen mittels horizontalem Gentransfer dar (35). Plasmide sind zirkuläre DNAs und für die bakterielle Zelle nicht essentiell, d.h. sie tragen lediglich Gene, die für das Überleben der Bakterien nicht zwingend notwendig sind. Wie in Abbildung 9 dargestellt, tragen sie Gene zur unabhängigen Replikation und Transfer.

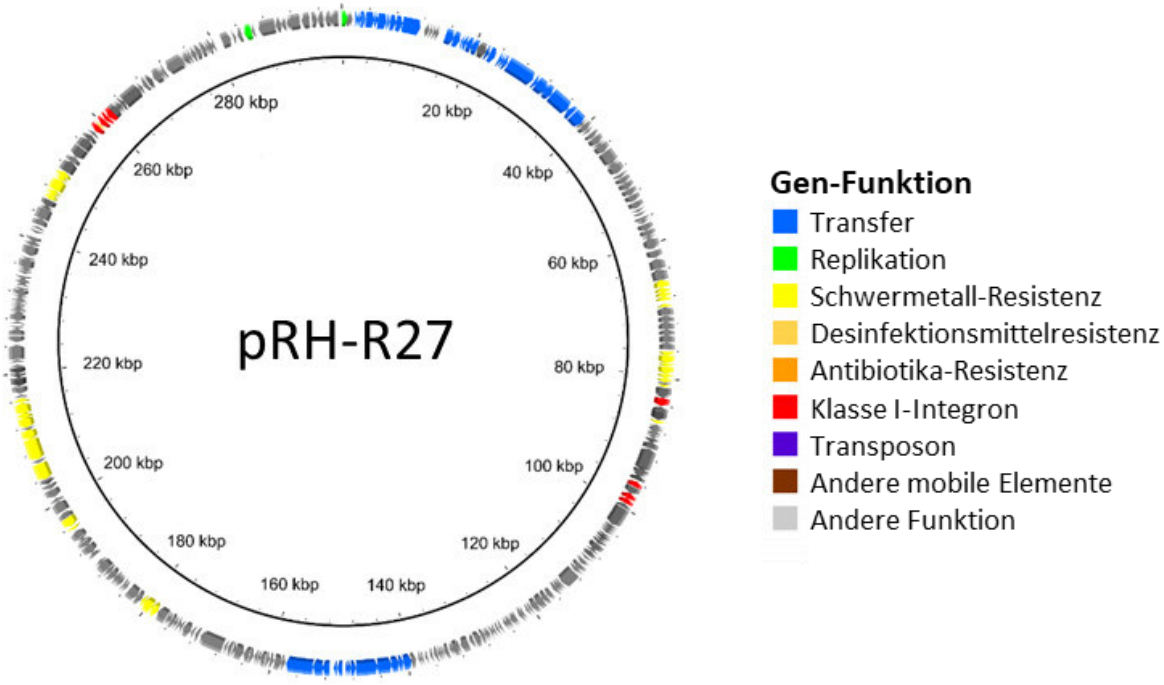


Abbildung 9: Beispiel eines Antibiotika-Resistenzplasmides. Abbildung mit Erlaubnis verändert nach: (36).

Eine Plasmid-Übertragung von einer Bakterienzelle auf eine andere kann entweder über Konjugation oder über eine Mobilisierung stattfinden. Bei einer Konjugation können Plasmide entweder innerhalb einer Spezies (z.B. *E. coli* auf *E. coli*) oder auch auf eine andere Spezies (z.B. *E. coli* auf *K. pneumoniae*) übertragen werden. Eine Übertragung von Plasmiden über Konjugation ist im Labor innerhalb weniger Stunden (>4h bei dem IncX4-Plasmid pV163M) abgeschlossen. Die Konjugation ist ein komplexer Prozess und bedarf sowohl Plasmid-kodierter (36) als auch chromosomal lokalisierter Gene (37).

Plasmide tragen in der Regel mindestens ein Toxin-Antitoxin-System (38). Diese Systeme dienen der Stabilisierung der Plasmide innerhalb der Bakterienzelle, auch wenn kein Antibiotika-Selektionsdruck mehr vorhanden ist.

Zusätzlich zu diesen Elementen tragen Plasmide eine beliebige Anzahl an Antibiotika-Resistenzgenen. Einige Plasmide weisen zusätzlich zu diesen Genen auch Resistenzen gegen andere Substanzen auf, wie z.B. Schwermetalle oder Desinfektionsmittel (36).

Plasmide werden in Gruppen eingeteilt, die sogenannten Inkompatibilitätsgruppen (39). Ein Beispiel hiervon sind IncX4 Plasmide (40). Zwei Plasmide einer identischen Inkompatibilitätsgruppe können nicht in einem Bakterium koexistieren (41).

Antibiotika-Resistenzplasmide tragen erheblich zur Entstehung multiresistenter Bakterien bei, da Bakterien eine Vielzahl unterschiedlicher Antibiotika-Resistenzplasmide aufnehmen können (Abbildung 10).

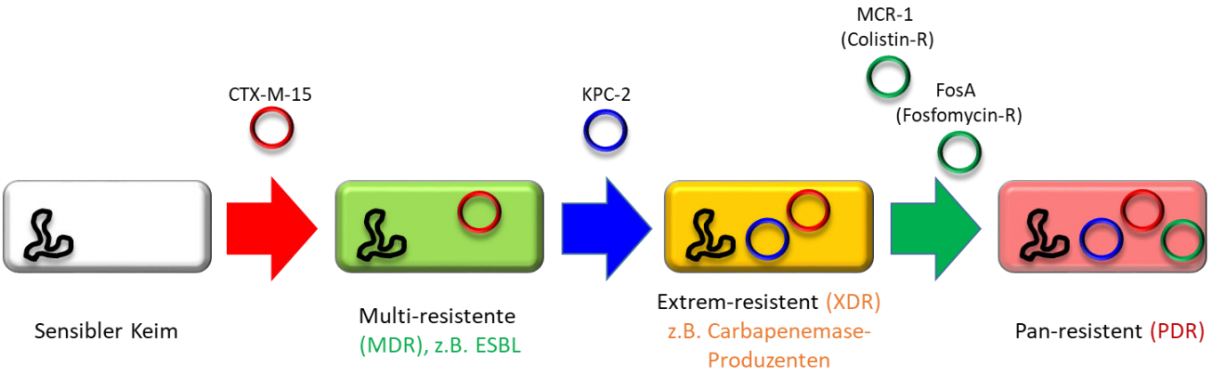


Abbildung 10: Darstellung der Plasmid-vermittelten Resistenzproblematik am Beispiel der *Enterobacterales*.

Durch die Aufnahme unterschiedlicher Antibiotika-Resistenzplasmide könnte ein pan-resistentes Bakterium entstehen, welches nicht mehr mit gängigen Antibiotika behandelbar ist. In einem solchen Fall wären die Behandlungsmöglichkeiten einer bakteriellen Infektion ähnlich limitiert wie in der präantibiotischen Ära. Derzeit als ungefährlich betrachtete Infektionen könnten einen fatalen Ausgang nehmen. Eine der größten Befürchtungen in Bezug auf Antibiotika-Resistenzen ist es, dass ein sehr virulentes Bakterium pan-resistent wird, es dadurch das Immunsystem des Patienten unterlaufen kann und dieser somit wenig Chancen auf Überleben hat.

1.4. Methoden zur Charakterisierung/Typisierung Antibiotika-resistenter Bakterien

Wie in Abschnitt 1.2 beschrieben, können Resistenzen über unterschiedliche Mechanismen verbreitet werden. Diese Mechanismen führen dazu, dass entweder identische Bakterien (= bakterielle Klone) oder identische Plasmide verbreitet werden. Der Nachweis solcher identischen Bakterien und Plasmide ist insbesondere dann wichtig, wenn ein Ausbruch vermutet wird. Solche Ausbrüche spielen sowohl in der Human- als auch Veterinärmedizin eine Rolle.

1.4.1. Klassische Methoden zur Charakterisierung von Bakterien

Eine wichtige Frage in der Antibiotika-Resistenzforschung ist, welche genetischen Eigenschaften Antibiotika-resistente Bakterien tragen. Diese genetischen Eigenschaften können über die Amplifikation bestimmter Gene mittels PCR abgefragt werden. So kann zum Beispiel das Vorhandensein bestimmter Antibiotika-Resistenzgene (19) oder auch bestimmter Plasmidtypen (42) abgefragt werden. Der Nachteil dieser Methode ist, dass nur gesuchte Zielgene gefunden werden können. Gene, für welche keine PCR durchgeführt wird, werden nicht detektiert.

1.4.2. Klassische Methoden zur Typisierung von Bakterien

Für den Nachweis identischer Bakterien bzw. Plasmide wurden in der Vergangenheit Methoden wie die Multilokus-Sequenztypisierung (43) oder die Pulsfeld-Gelelektrophorese (PFGE, (44)) verwendet.

Bei der Multilokus-Sequenztypisierung werden in der Regel sieben *housekeeping* Gene (Haushaltsgene), auch Allele genannt, über PCR amplifiziert (Abbildung 11).

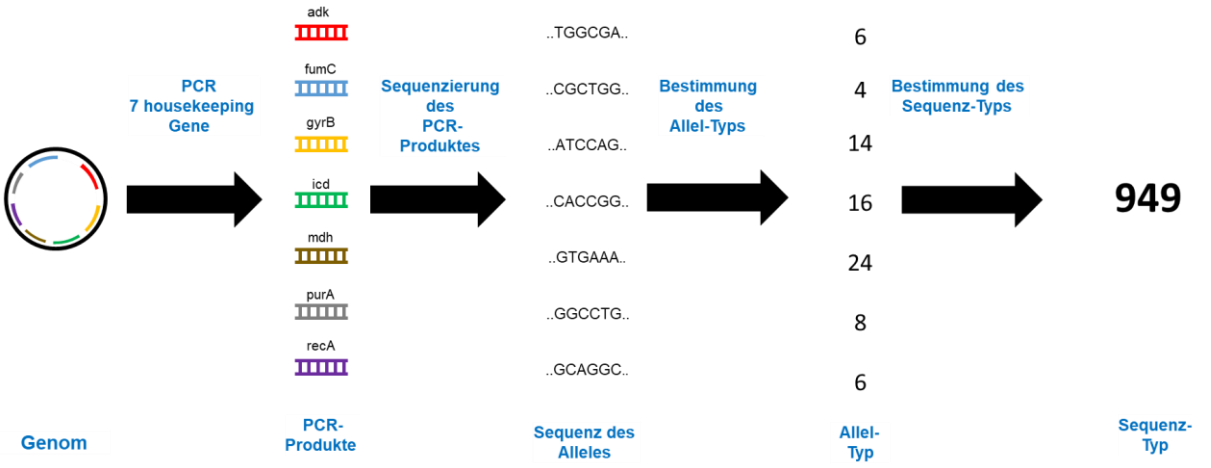


Abbildung 11: Schematische Darstellung des Ablaufes der MLST-Typisierung am Beispiel der *E. coli* MLST-Typisierung.

Im Anschluss darauf wird mittels DNA-Sequenzierung die Sequenz dieser Allele bestimmt. Die ermittelten Einzel-Allel-Sequenzen werden mit einer Datenbank (z.B. PubMLST, (45)) verglichen, die Allel-Typen bestimmt, die Kombination der Allel-Typen zu einer Zahl kombiniert und dadurch einem bestimmten Sequenz-Typ zugeordnet. Bakterien, die einen identischen Sequenz-Typ aufweisen, sind nah verwandt.

Bei der PFGE wird hochmolekulare DNA aus Bakterien in Agaroseblöckchen mittels Restriktionsenzymen (= Gesamt-DNA) oder S1-Nuklease (= Plasmidprofil) verdaut (Abbildung 12).

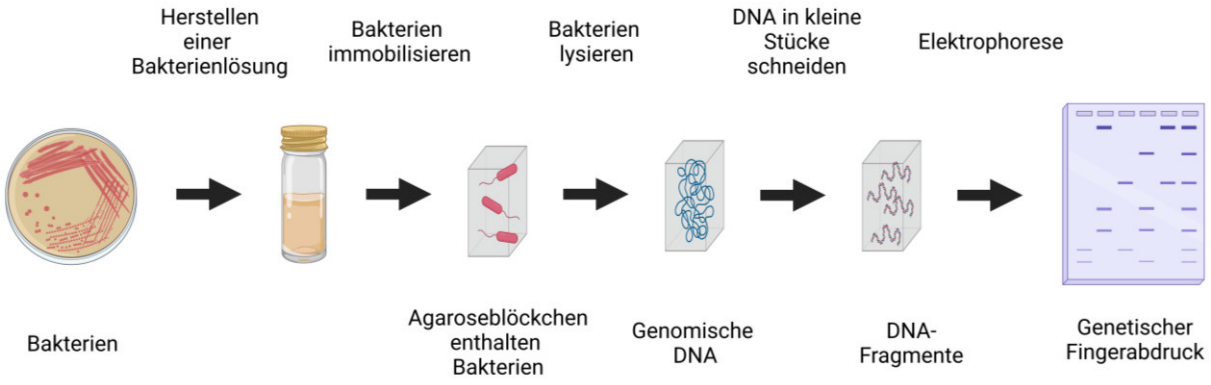


Abbildung 12: Schematische Darstellung des Ablaufes einer PFGE. *Created with Biorender.*

Über eine besondere Elektrophorese-Art werden die Fragmente aufgetrennt und dann das Muster analysiert. Weisen Bakterien ein identisches Chromosom/Plasmidprofil auf, ist von einer nahen Verwandtschaft auszugehen.

Beide der oben genannten Typisierungs-Methoden haben zwei Eigenschaften gemeinsam: Sie können lediglich für einen groben Vergleich von Bakterien angewendet werden und sind methodisch und zeitlich sehr aufwendig.

1.4.3. Ganzgenom-basierte Methoden

Für einen hochauflösenden Vergleich bakterieller Isolate wird mittlerweile die sogenannte Ganz-Genom-Sequenzierung und nachfolgende Genom-basierte Analysen verwendet. Bei dieser Methode wird die DNA-Sequenz des gesamten Genoms der Bakterien (beinhaltet Chromosom, Plasmide, (Pro)phagen etc.) bestimmt. Bei klinisch relevanten Bakterien beinhaltet die Sequenz zwischen drei (z.B. *Enterococcus faecium*) und sechs Millionen (z.B. *Pseudomonas aeruginosa*) Nukleotiden. Der schematische Ablauf einer Ganz-Genom-Sequenzierung ist in Abbildung 13 dargestellt.

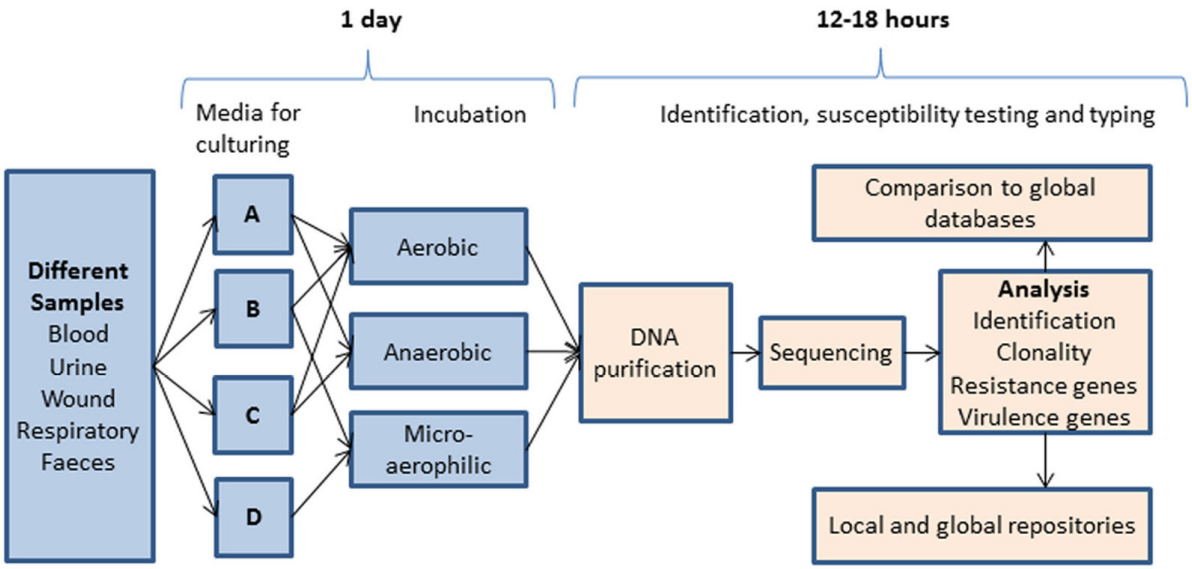


Abbildung 13: Schematische Darstellung des Ablaufes einer Ganz-Genom-Sequenzierung. Mit Erlaubnis verändert nach (46).

Für die Ganz-Genom-Sequenzierung wird zunächst ein einzelnes bakterielles Isolat aus einer Probe gewonnen. Hierfür wird die Probe auf spezielle Nährmedien ausgestrichen und über Nacht bei 37°C inkubiert. Die dort gewachsenen Kolonien werden dann vereinzelt (Abbildung 14).

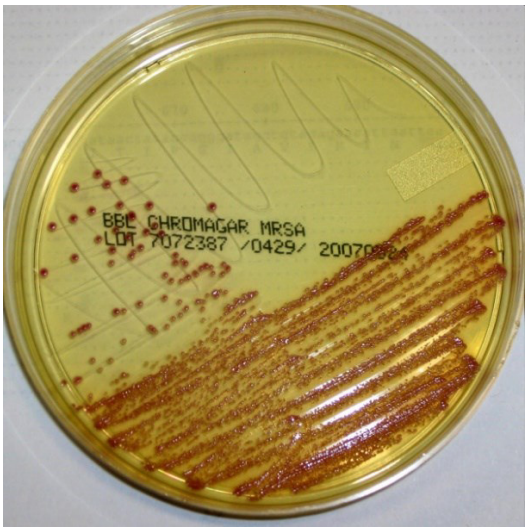


Abbildung 14: Vereinzelte Kolonien eines MRSA auf einem MRSA Chromagar.

Nach der Vereinzelung folgt die Spezies-Identifizierung über MALDI-TOF (47) und die Bestimmung der Minimalen Hemmkonzentration (MHK) gegenüber einer Auswahl bestimmter Antibiotika. Nach dieser Vorcharakterisierung wird genomische DNA aus einer Übernachtkultur des Isolates aufgereinigt, diese entweder über Short-read (z.B.

Illumina) oder Long-read (z.B. Nanopore, Pacific Biosciences)-Technologien sequenziert und danach mit bioinformatischen Tools analysiert.

1.5. Bioinformatische Tools zur Analyse bakterieller Genomdaten

Aus der DNA-Sequenz können viele bakterielle Eigenschaften extrahiert werden (Abbildung 15).

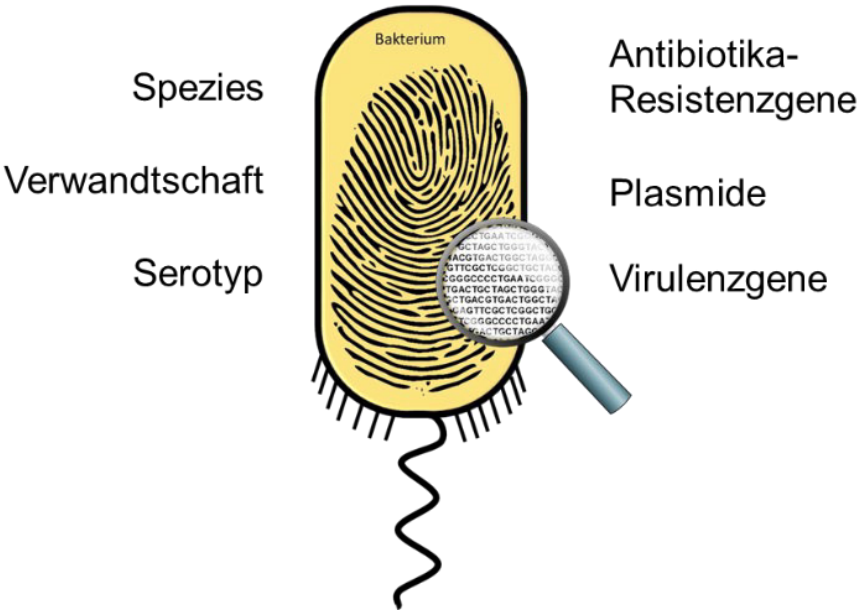


Abbildung 15: Beispiele genetischer Eigenschaften, die aus einer DNA-Sequenz ermittelt werden können.

Ein besonderer Vorteil Genom-basierter Analysen ist, dass eine „backward compatibility“ mit vielen der klassischen Typisierungsmethoden gewährleistet ist. Zusätzlich zu der Detektion von Antibiotika-Resistenz und Virulenzgenen kann auch eine MLST-Typisierung durchgeführt werden (45). Die klassische 7-Gen-MLST-Typisierung kann aber auch zu einer Core-Genom-Sequenztypisierung ausgeweitet werden, dem sogenannten cgMLST, welches bei z.B. *Enterococcus faecium* auf der vergleichenden Sequenzanalyse von 1423 Genen basiert (Abbildung 16, (48)).

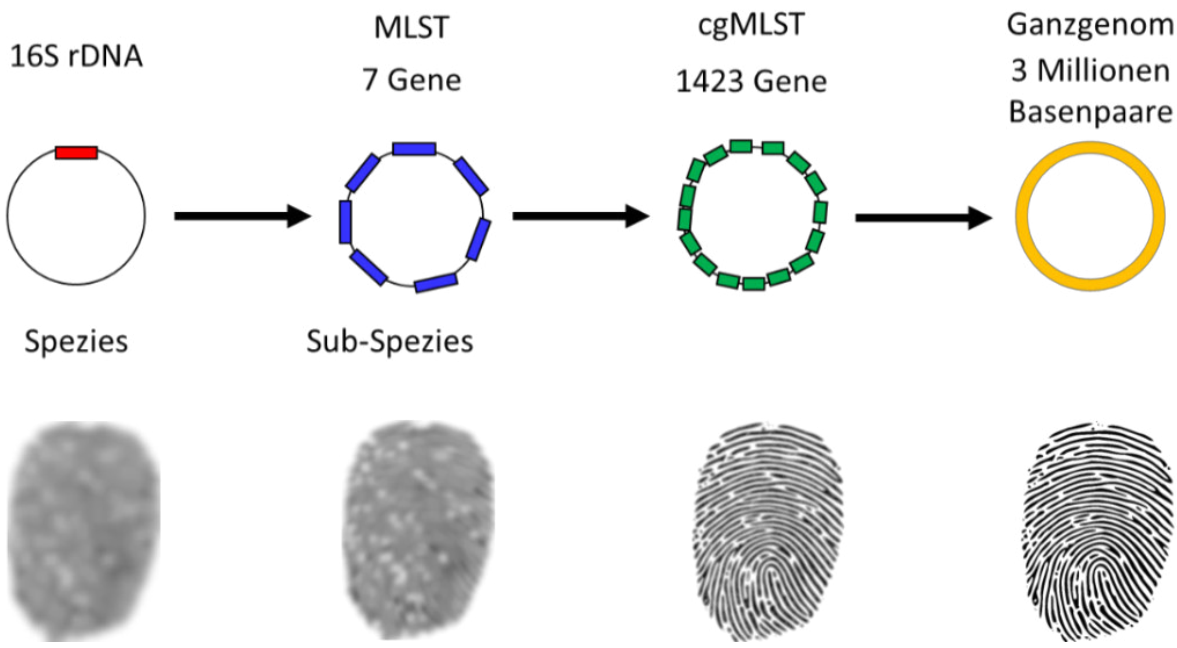


Abbildung 16: Der Weg zum hochauflösenden genetischen Fingerabdruck am Beispiel des *E. faecium.*

Zwei *E. faecium* mit einem identischen 7-Gen-MLST (z.B. ST-117) und einem identischen cgMLST (z.B. CT-71) werden als klonal, d.h. von einem gleichen Vorgänger abstammend, angesehen.

Eine weit verbreitete Analyse der Ganz-Genom-Sequenzen beinhaltet den Nachweis bestimmter Eigenschaften über einen Sequenz-basierten Vergleich mit Datenbanken. Es gibt Datenbanken für die Detektion zahlreicher bakterieller Eigenschaften, wie z.B. Antibiotika-Resistenzgenen (49) oder Virulenzgenen (50). Zusätzlich kann die Verwandtschaft der Bakterien über das Vorhandensein sogenannter Einzelnukleotid-Austausche (SNPs) im gesamten Genom des Bakteriums ermittelt werden. Die bei der bioinformatischen Analyse ermittelten Eigenschaften werden zu einem genetischen Fingerabdruck zusammengefasst (Abbildung 17).

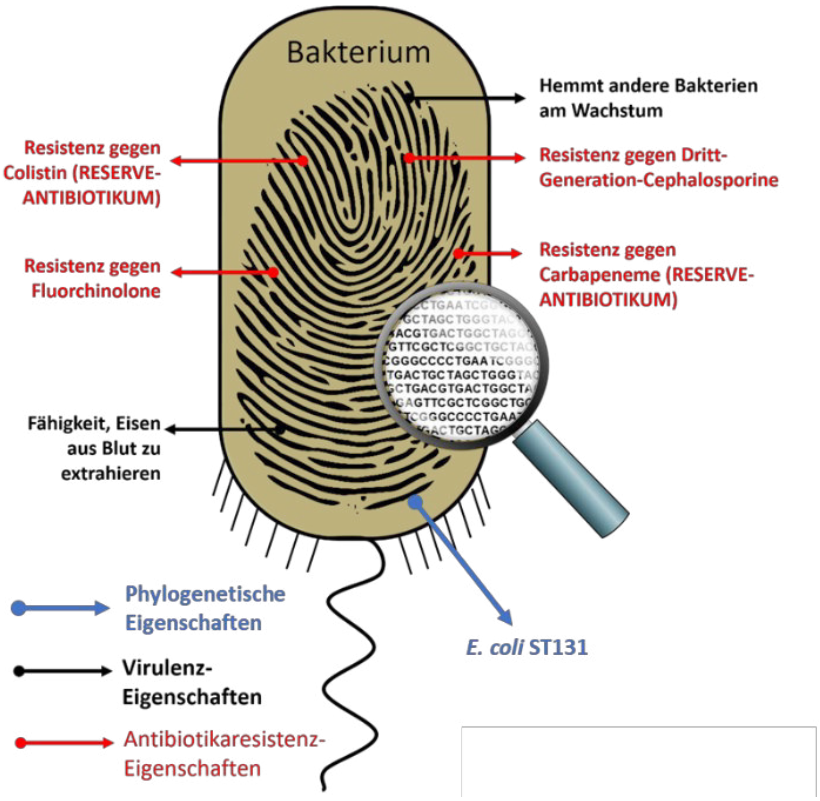


Abbildung 17: Beispiel eines genetischen Fingerabdruckes. Mit Erlaubnis verändert nach (51).

Genetische Fingerabdrücke mehrerer Bakterien können mittels komparativer genomischer Analysen miteinander verglichen werden. Sind genetische Fingerabdrücke identisch, so liegt eine klonale Übertragung der Bakterien vor. Im klinischen Zusammenhang bedeutet dies, dass ein Ausbruch vorliegt, und somit zusätzliche hygienische Maßnahmen erfolgen müssen.

Seit etwa 2012 kommen Ganz-Genom-Sequenzier-Plattformen zum Einsatz, die eine kostengünstige Ganzgenomsequenzierung möglich machen (51). Der Rückgang der Kosten für die Sequenzierung eines bakteriellen Genoms führte dazu, dass heutzutage sehr viele Bakterien sequenziert werden können. Mit neuen Sequenziergeräten (z.B. Illumina NextSeq 500) ist die Sequenzierung mehrerer hundert Genome in einem einzigen Sequenzierlauf möglich. Dieser Trend eröffnete schnell ein ganz anderes Problem: Eine händische Bearbeitung dieser großen Anzahl an Genomen wurde unmöglich – es mussten bioinformatische Plattformen/Pipelines generiert werden, die eine automatische Bearbeitung mehrerer Genome auf einmal erlaubten. Zum Vorreiter auf diesem Gebiet gehört das Center for Genomic Epidemiology (http://www.genomicepidemiology.org/, (52)). Das dort vorhandene Online-Tool „Resfinder“ ist für die Analyse multiresistenter Bakterien von großer Bedeutung (53).

Mit diesem Tool können, nach Upload der Sequenz in unterschiedlichen Formaten (.fastq, .fasta), Antibiotika-Resistenzgene und mit Antibiotika-Resistenz-assoziierte Mutationen chromosomaler Gene (z.B. *gyrB* und Fluorchinolon-Resistenz bei *E. coli*) detektiert werden. Das Output der Analyse ist eine statische html-Seite (Abbildung 18).

A

ResFinder-4.1 Server - Results

Input Files: *NRZ14408_complete.fas*

Warning:
One or more resistance genes does not exist in the phenotype database. The Summary table does not take this into account.

escherichia coli | complete

Antimicrobial	Class	WGS-predicted phenotype	Genetic background
amikacin	aminoglycoside	Resistant	aac(6')-Ib-cr (aac(6')-Ib-cr_EF636461)
tigecycline	tetracycline	No resistance	
tobramycin	aminoglycoside	Resistant	aac(6')-Ib-cr (aac(6')-Ib-cr_EF636461), aac(3)-IIa (aac(3)-IIa_X51534), aac(3)-IId (aac(3)-IId_EU022314)
cefepime	beta-lactam	Resistant	blaKPC-2 (blaKPC-2_AY034847), blaOXA-1 (blaOXA-1_HQ170510)
chloramphenicol	amphenicol	Resistant	catB3 (catB3_U13880), cmlA1 (cmlA1_M64556), catA1 (catA1_V00622)
piperacillin+tazobactam	beta-lactam	Resistant	blaKPC-2 (blaKPC-2_AY034847), blaOXA-1 (blaOXA-1_HQ170510)
cefoxitin	beta-lactam	Resistant	blaKPC-2 (blaKPC-2_AY034847)
ampicillin	beta-lactam	Resistant	blaTEM-1A (blaTEM-1A_HM749966), blaKPC-2 (blaKPC-2_AY034847), blaOXA-1 (blaOXA-1_HQ170510), blaTEM-1B (blaTEM-1B_AY458016)
ampicillin+clavulanic acid	beta-lactam	Resistant	blaKPC-2 (blaKPC-2_AY034847), blaOXA-1 (blaOXA-1_HQ170510)
cefotaxime	beta-lactam	Resistant	blaKPC-2 (blaKPC-2_AY034847)
ciprofloxacin	quinolone	Resistant	aac(6')-Ib-cr (aac(6')-Ib-cr_EF636461), qnrB2 (qnrB2_DQ351242) gyrA (p.S83L)
colistin	polymyxin	Resistant	mcr-1.1 (mcr-1.1_KP347127)

B

Beta-lactam									
Resistance gene	Identity	Alignment Length/Gene Length	Position in reference	Contig or Depth	Position in contig	Phenotype	PMID	Accession no.	Notes
blaOXA-1	100.0	831/831	1..831	LT599827.1 Escherichia coli isolate E. coli NRZ14408 genome assembly, plasmid: p14408_2	57566..58396	amoxicillin,amoxicillin+clavulanic acid,ampicillin,ampicillin+clavulanic acid,cefepime,piperacillin,piperacillin+tazobactam	10898672, 16735436	HQ170510	Class D;OXA-1-like;;Alternative name blaOXA-30;
blaKPC-2	100.0	882/882	1..882	LT599827.1 Escherichia coli isolate E. coli NRZ14408 genome assembly, plasmid: p14408_2	68332..69213	amoxicillin,amoxicillin+clavulanic acid,ampicillin,ampicillin+clavulanic acid,aztreonam,cefepime,cefotaxime,cefoxitin,ceftazidime,ertapenem,imipenem,meropenem,piperacillin,piperacillin+tazobactam,ticarcillin,ticarcillin+clavulanic acid	12615876, 11257029	AY034847	Class A;Group 2f;Alternative name blaKPC-1

Abbildung 18: Darstellung der bei einer Analyse mit ResFinder 4.1 generierten Ergebnisse am Beispiel des Isolates NRZ14408. A. Extrapolation der phänotypischen Resistenz aus den Antibiotika-Resistenzgenen (Auszug) B. Darstellung von Resistenzgenen (Auszug).

Der Nachteil dieses und auch ähnlicher Tools ist, dass sie nicht lokal auf einem Computer oder Cluster installiert werden können, sondern ein Online-Upload-Portal verwendet werden muss. Aus diesem Grund wurde die lokale ASA^{3}P-Pipeline (*Automatic Sequence Assembly, Annotation and Analyses Pipeline/Platform*) entwickelt (54). ASA^{3}P beinhaltet vier Module, die aufeinander aufbauen (Abbildung 19).

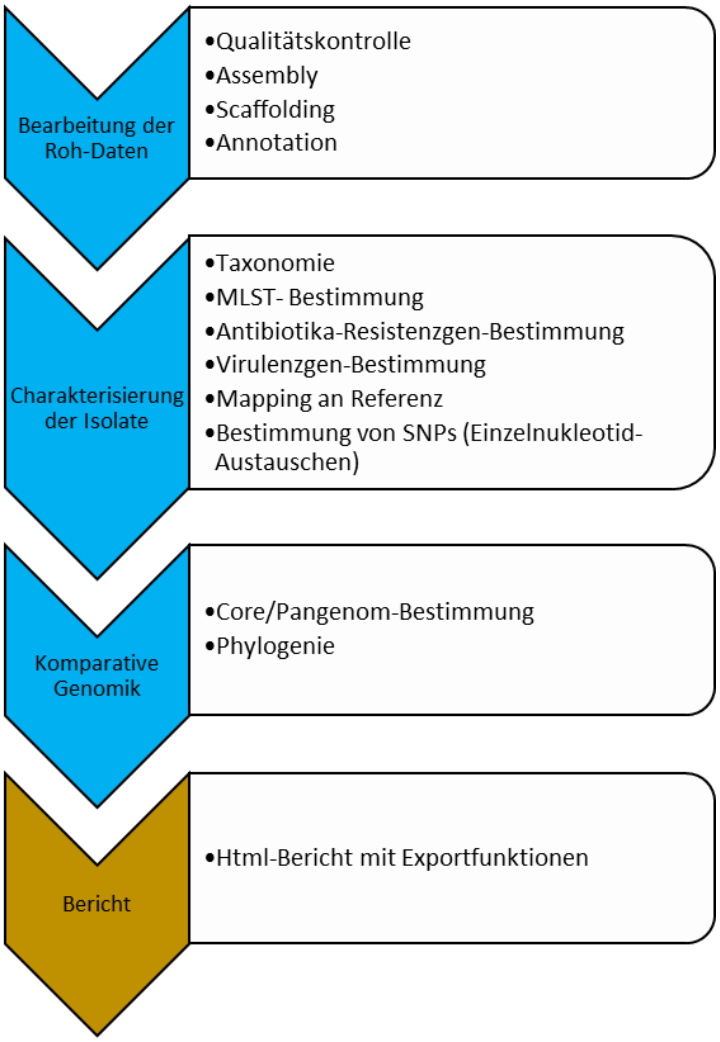


Abbildung 19: Schema des Ablaufes bei ASA^{3}P. Mit Erlaubnis verändert nach (54).

Im Modul „Bearbeitung der Roh-Daten“ wird mit der Qualitätskontrolle überprüft, ob die Roh-Daten für die weiteren Analysen ausreichend sind. Sie werden im Nachhinein zu contigs assembliert, an eine Referenz angeordnet und zu einem Scaffold verarbeitet (=Scaffolding). Bei der Annotation werden Gene definiert.

Im Modul „Charakterisierung“ werden bestimmte Eigenschaften des Bakteriums bestimmt, darunter die Spezies, der Multilokus-Sequenztyp (falls für die Spezies ein solches vorhanden ist), die Antibiotika-Resistenzgene und die Virulenzgene. Liegt ein Verdacht auf einen Ausbruch vor, kann dies mithilfe der Bestimmung von Einzelnukleotid-Austauschen (SNPs) verifiziert werden.

Im Modul „komparative Genomik“ wird das Core/Pan-Genom definiert und aufbauend auf den SNPs eine Phylogenie (Verwandtschaftsanalyse) durchgeführt, die in der Erstellung eines Stammbaumes mündet.

Das letzte Modul von ASA^{3}P ist das Modul „Bericht“. Hier werden die Ergebnisse der Pipeline in einem statischen html-File zusammengefasst.

Ein weiteres Tool, welches entwickelt worden ist, ist das Plasmid-Detektions-Tool „Platon“ (55). Mit diesem Tool ist es möglich, die von ASA^{3}P assemblierten *contigs* auf ihre Zugehörigkeit zum Chromosom bzw. zu Plasmiden hin zu überprüfen.

1.6. Ziele der Arbeit

Wie in den Abschnitten zuvor beschrieben, ist Antibiotika-Resistenz ein sehr komplexes und dringendes Problem. Um dieses vielschichtige Problem angehen zu können, müssen die Antibiotika-resistenten Bakterien so gut wie möglich charakterisiert werden. Dabei ist es nötig, Bakterien aus unterschiedlichen Kompartimenten zu untersuchen – also einen One-Health-Ansatz zu verfolgen.

In dieser Arbeit wurde folgenden Fragestellungen nachgegangen:

1. In welchen der untersuchten Kompartimente (Tier, Mensch, Umwelt) und der untersuchten Länder sind Antibiotika-resistente Bakterien vorhanden?
2. Sind in unterschiedlichen Kompartimenten nachgewiesene Antibiotika-resistente Bakterien identisch oder unterschiedlich?
3. Gibt es national oder sogar international verbreitete Klone?
4. Gibt es Antibiotika-Resistenz-Plasmide, die national oder international verbreitet sind (=epidemische Plasmide)?

2. Ergebnisse und Diskussion

Um eine Aussage darüber treffen zu können, ob klonale Antibiotika-resistente Bakterien zwischen einzelnen Kompartimenten übertragen werden können, wurden zunächst die unterschiedlichen Kompartimente auf das Vorhandensein solcher Bakterien überprüft. Es wurden Studien zur Detektion Antibiotika-resistenter Bakterien in fünf Kompartimenten (Mensch, Nutztier, Umwelt, Lebensmittel, Haustier) und in fünf unterschiedlichen Ländern durchgeführt (Abbildung 20).

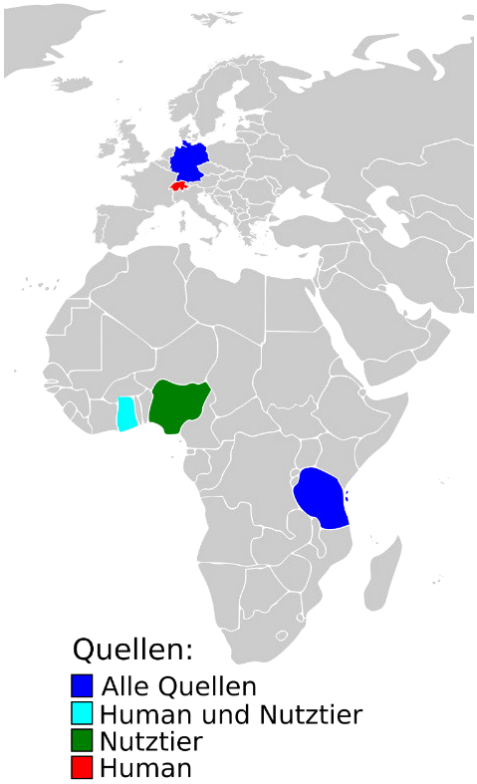


Abbildung 20: Darstellung der in den diskutierten Studien beteiligten Länder. Karte mit Erlaubnis verändert nach (56).

Der Focus der Studien lag auf ESBL-produzierenden *Enterobacterales* und Vancomycin-resistenten *E. faecium* (VREfm).

ESBL-produzierende *Enterobacterales* wurden in allen untersuchten Kompartimenten und Ländern gefunden. In humanen Proben wurden ESBL-Produzenten in Deutschland, Ghana und Tansania nachgewiesen (19, 57, 58). In Haustieren wurden ESBL-Produzenten in Deutschland und Tansania detektiert (19, 21). Die zusammenhängenden Kompartimente Nutztier und Lebensmittel wiesen ebenfalls ESBL-Produzenten auf (Nutztier: (17, 42, 57); Lebensmittel: (17, 42, 59, 60). Auch in

den bis *dato* seltener behandelten Kompartimenten, wie der Umwelt, wurden insbesondere in Wasserproben (61) und in Staubproben (62) ESBL-Produzenten nachgewiesen. VREfm wurden in humanen Proben aus Deutschland nachgewiesen (32, 63, 64).

Mit der Detektion Antibiotika-resistenter Bakterien in unterschiedlichen Kompartimenten stellte sich die Frage der Phylogenie, d.h. wie verwandt diese Bakterien sind und ob es innerhalb der unterschiedlichen Kompartimente zu einem Austausch von Plasmiden oder auch Klonen kommt. Um dieser Frage nachzugehen, wurde eine Ganzgenom-basierte Analyse der Antibiotika-resistenten Bakterien durchgeführt. Alle in den weiteren Kapiteln vorgestellten Studien und Ergebnisse basieren auf Ganzgenomdaten. Wie in den Abschnitten 1.4.3 und 1.5 beschrieben, können Ganzgenomsequenzen verwendet werden, um genetische Fingerabdrücke zu generieren und zu vergleichen. Ein Ziel dieser Arbeit war es daher, zu ermitteln, ob es spezifische Klone (= vorhanden in einem Kompartiment) oder multi-kompartimentelle Klone (= vorhanden in mindestens zwei Kompartimenten) innerhalb der untersuchten Bakterien gibt.

Ein Klon wurde wie folgt definiert:

1. identischer Multilokus-Sequenztyp
2. geringe Anzahl an Einzelnukleotid-Austauschen (≤100) oder identischer cgMLST-Typ
3. identische Resistenz (bei chromosomaler Insertion des Antibiotika-Resistenzgenes auch identische Integrationsstelle)

Basierend auf diesen Kriterien wurden die Genomsequenzen von mehr als 2000 Isolaten überprüft. Im Zuge der Arbeiten wurden Antibiotika-resistente Klone detektiert, die nur in einzelnen Kompartimenten vorkamen:

1. ESBL-Klone in deutschen Wasserproben (Abschnitt 2.1, Anhang A)
2. ESBL-Klone in Hähnchen in Nigeria (Abschnitt 2.2, Anhang B)
3. VREfm-Klone in Humanproben aus dem Rhein-Main-Gebiet (Abschnitt 2.3, Anhänge C, D)
4. Carbapenemase-Produzenten-Klone in Humanproben aus Deutschland und der Schweiz (Abschnitt 2.4, Anhang E)

Entgegen allen Erwartungen wurde auch ein Klon detektiert, der in vielen unterschiedlichen Kompartimenten vorkam:

- ESBL-Klon ST-410 in Mensch, Tier, Umwelt, Haustierproben (Abschnitt 2.5, Anhang F-H)

Dass Plasmide eine große Rolle bei der Übertragung von Antibiotika-Resistenzen spielen, ist in der Einleitung beschrieben worden. Überraschenderweise gibt es auch identische Plasmide, die weltweit vorkommen. Im Rahmen dieser Arbeiten wurde ein solches ebenfalls gefunden:

- IncX4-Plasmid kodierend für das neuartige Colistin-Resistenzgen *mcr-1* (Abschnitt 2.6, Anhang H-M)

2.1. ESBL-Klone in deutschen Wasserproben (Anhang A)

Wie anfangs erwähnt, sind Genom-basierte Studien zu ESBL-Produzenten aus Wasser sehr rar. Aus diesem Grund wurde 2018 eine Studie in Mittelhessen durchgeführt, um die molekulare Epidemiologie der ESBL-Erreger in Wasser zu untersuchen (61). Es wurde ein Fluss und einige Badeseen auf das Vorhandensein von ESBL-Produzenten beprobt. Im Gegensatz zu anderen Studien wurde in der mittelhessischen Studie lediglich ESBL-*E. coli* detektiert. Dieses Ergebnis war unerwartet, da in anderen Studien auch andere ESBL-Produzenten (z.B. *Klebsiella pneumoniae*, *Enterobacter* spp., *Citrobacter* spp. (65, 66)) gefunden wurden.

Bei der initialen Untersuchung stellte sich heraus, dass überwiegend ein Sequenztyp vorhanden war – ST-949 (Abbildung 21).

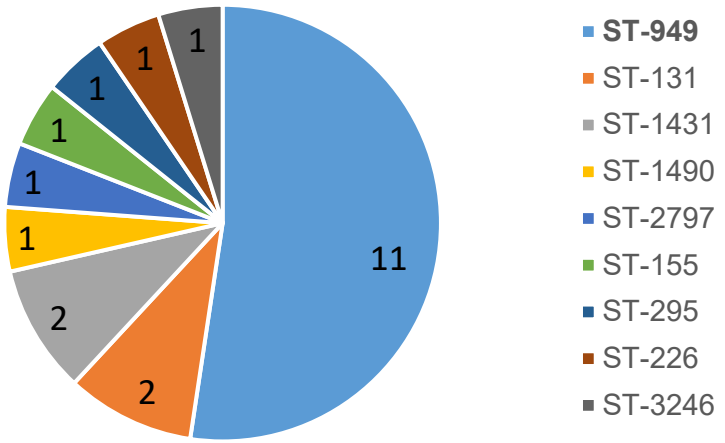


Abbildung 21: In Wasserproben aus Mittelhessen identifizierte MLST-Typen. Basierend auf den Daten von (61).

Eine nähere Betrachtung der ST-949-Isolate zeigte, dass sie einen identischen cgMLST-Typ (CT-114289), ein identisches ESBL-Gen (*bla*$_{\text{CTX-M-15}}$) und eine identische Insertionsstelle dieses ESBL-Gens im Chromosom aufwiesen. Alle diese Ergebnisse waren ein eindeutiger Hinweis darauf, dass es sich um einen Klon handelt.

Eine Suche in der Enterobase-Datenbank (67), ergab ein erstaunliches Ergebnis: Der Sequenztyp ST-949 ist sehr selten zu finden (n=41, im Gegensatz zum häufigen Sequenztyp ST-131, n=9202). In der Literatur ist er lediglich in einer geringen Anzahl an Publikationen erwähnt (68–72). Aus diesem Grund wurde eine Analyse aller in der Datenbank verfügbaren ST-949-Isolate durchgeführt, um die Verwandtschaft der mittelhessischen Isolate mit den internationalen Isolaten zu ermitteln (Abbildung 22).

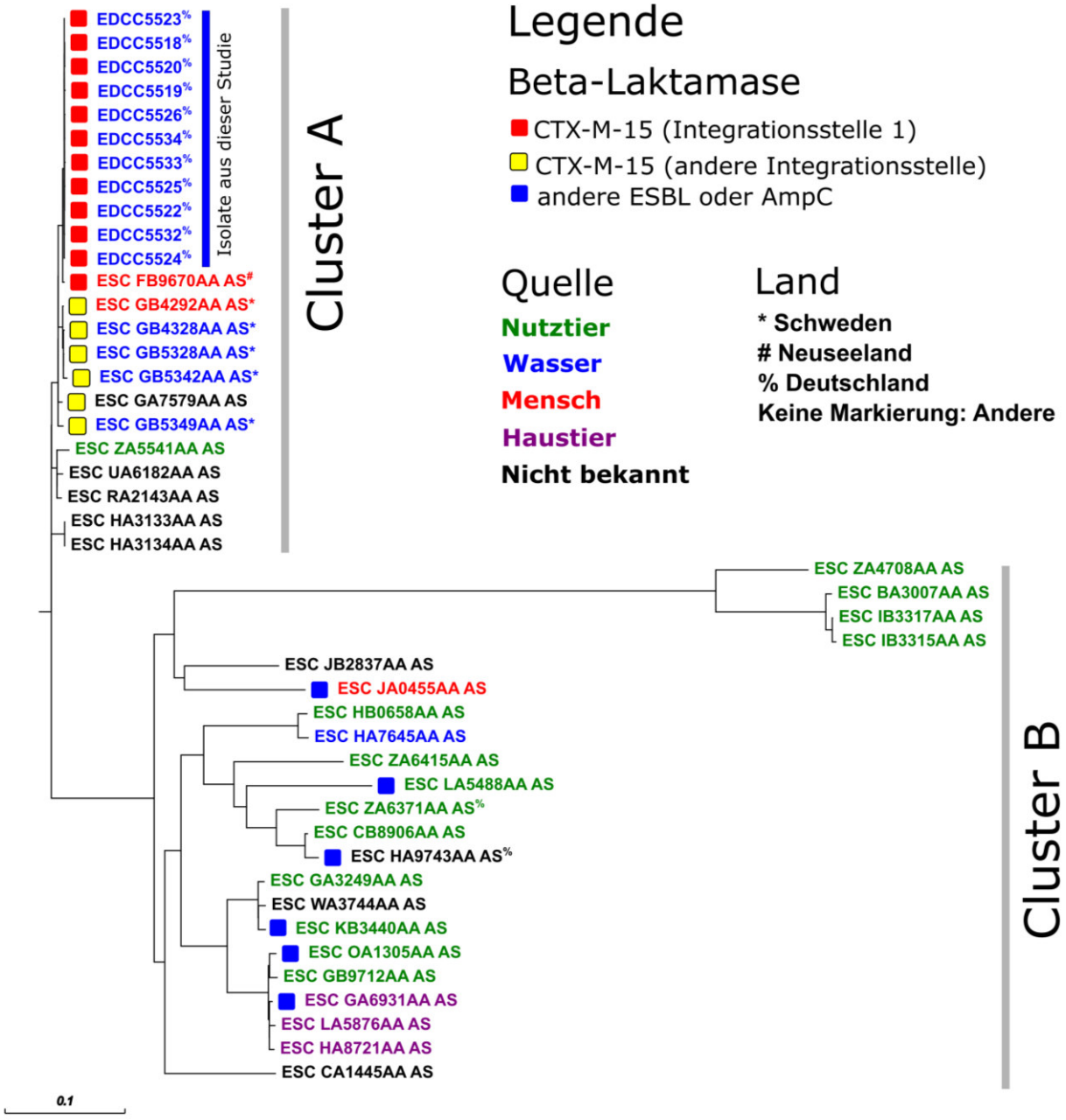


Abbildung 22: Phylogenetischer Baum aller am 11.08.2020 in Enterobase verfügbaren *E. coli* ST-949-Isolate. Die Isolate aus dieser Studie sind mit einem blauen Balken markiert. Mit Erlaubnis verändert nach (61).

Es stellte sich heraus, dass ein CTX-M-15-produzierendes Isolat aus Neuseeland eine hohe Identität mit den mittelhessischen Isolaten aufwies Er hatte allerdings einen anderen cgMLST-Typ. Somit handelt es sich bei den hessischen Isolaten um einen einzigartigen Cluster. Die Frage, warum diese klonalen Isolate so weit verbreitet sind und ob sie in humanen Proben ebenfalls vorkommen, ist derzeit Gegenstand der Forschung. Es gibt allerdings erste Hinweise darauf aus laufenden Analysen, dass sie auch in deutschen Humanproben vorkommen.

2.2. ESBL-Klone in Nigeria (Anhang B)

Im Rahmen einer Zusammenarbeit mit Kolleg/innen aus Nigeria wurde überprüft, ob ESBL-kodierende *E. coli* in nigerianischen Hähnchenfarmen zu finden sind. Dabei wurden Kot-Proben aus elf Hähnchen-Farmen in fünf Bundesländern untersucht (73). In 90% der Farmen (10/11) wurden ESBL-positive *E. coli* (n=52) detektiert. Diese *E. coli* wurden mittels Ganzgenomsequenzierung sequenziert und bioinformatisch analysiert. Bei der Analyse der Multilokus-Sequenztypen wurden sieben unterschiedliche Sequenztypen detektiert (Abbildung 23).

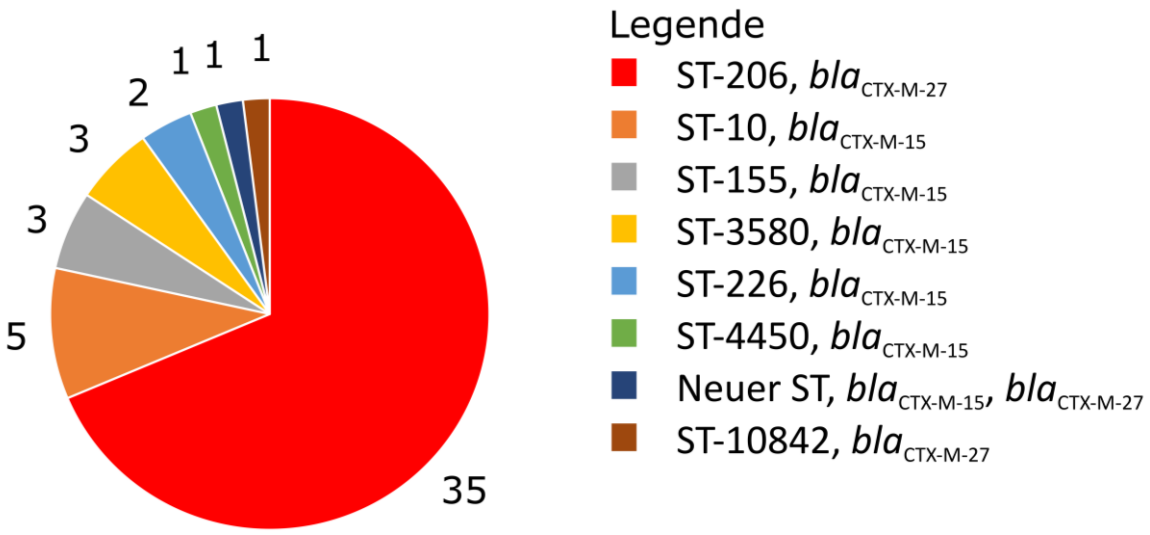


Abbildung 23: In der Studie aus Nigeria detektierte Sequenztypen mit Angabe der darin gefundenen ESBL-Gene (mit Erlaubnis verändert nach (73))

Einer dieser Sequenztypen (ST-206) war nicht nur dominant (68.6%) sondern wurde in neun der zehn ESBL-positiven Farmen (Abbildung 24) und in allen fünf betroffenen nigerianischen Bundesländern detektiert.

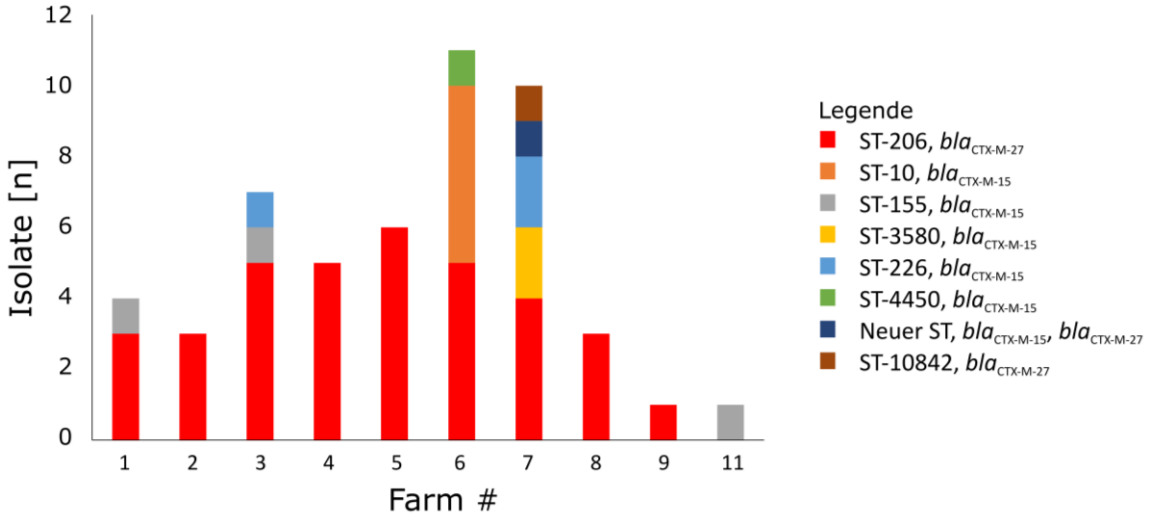


Abbildung 24: Darstellung der Farmen, auf denen die unterschiedlichen ST-Typen detektiert wurden (mit Erlaubnis verändert nach (73)).

Alle *E. coli* ST-206 enthielten das ESBL-Gen *bla*$_{\text{CTX-M-27}}$. CTX-M-27 ist eine Variante von CTX-M-14 und wird in den letzten Jahren zunehmend detektiert (74). Die spezielle ESBL-Kassette war in allen ST-206-Isolaten an einer identischen Stelle im Chromosom inseriert (Abbildung 25).

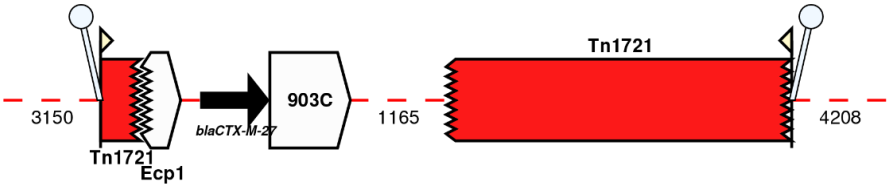


Abbildung 25: ESBL-Kassette in ST-206 *E. coli* (mit Erlaubnis verändert nach (73).

Die SNP-Anzahl innerhalb der nigerianischen *E. coli* ST-206 CTX-M-27-Isolate war gering (durchschnittlich 36 SNPs) – ein zusätzlicher Hinweis darauf, dass es sich tatsächlich um einen Klon handelt. Ein Vergleich mit anderen ST-206-Isolaten aus Enterobase (67) zeigte, dass es sich um einen einzigartigen Klon handelt, der nur in Nigeria vorkommt (73). Weltweit war dies die Erstbeschreibung dieses Klons.

Es bleiben einige unbeantwortete Fragen: Zum einen konnte aufgrund des Studienaufbaus die Quelle dieses Klons nicht ermittelt werden. Aufgrund der weiten Verbreitung dieses Klons in fünf nigerianischen Bundesländern ist zu vermuten, dass eine Brüterei die Quelle dieses Klons sein könnte. Zum anderen wurden in dieser Studie keine Menschen beprobt, daher ist bislang unbekannt, ob und wie weit dieser Klon in Nigeria auch in Menschen verbreitet ist.

2.3. VREfm-Klon ST-117/CT-71/*vanB* (Anhänge C, D)

Weitere Antibiotika-resistente Bakterien, die in letzter Zeit an Wichtigkeit zugenommen haben, sind Vancomycin-resistente *Enterococcus faecium* (VREfm). Seit 2007 wurde in Deutschland ein übermäßiger Anstieg von VREfm verzeichnet (75). Besonders in den ersten Studien zu VREfm in Deutschland wurde ein sogenannter „VRE-Gürtel" beobachtet, eine Häufung von VREfm in der geographischen Mitte von Deutschland. Basierend auf dieser Beobachtung stellte sich die Frage, ob dieser extreme Anstieg mit dem Anstieg eines besonderen VREfm-Sequenztyps verbunden sein könnte.

Um dieser Frage nachzugehen, wurden im Rhein-Main-Gebiet zwei Studien zu VREfm durchgeführt: Eine über zweieinhalb Jahre laufende Studie in einer neurologischen Reha-Klinik, um eine mögliche Veränderung der STs zu beobachten (64), und eine Bestandsaufnahme im Jahr 2017/2018, welche in unterschiedlichen Kliniken im Rhein-Main-Gebiet durchgeführt worden ist (32). Bei beiden Studien wurde eine Genomsequenzierung der VREfm-Isolate durchgeführt und im Anschluss der cgMLST, die Antibiotika-Resistenzgene und deren Lokalisierung bestimmt.

In der neurologischen Reha-Klinik-Studie stellte sich heraus, dass sich die Epidemiologie der VREfm im Laufe der Jahre komplett wandelte (Abbildung 26, (64)).

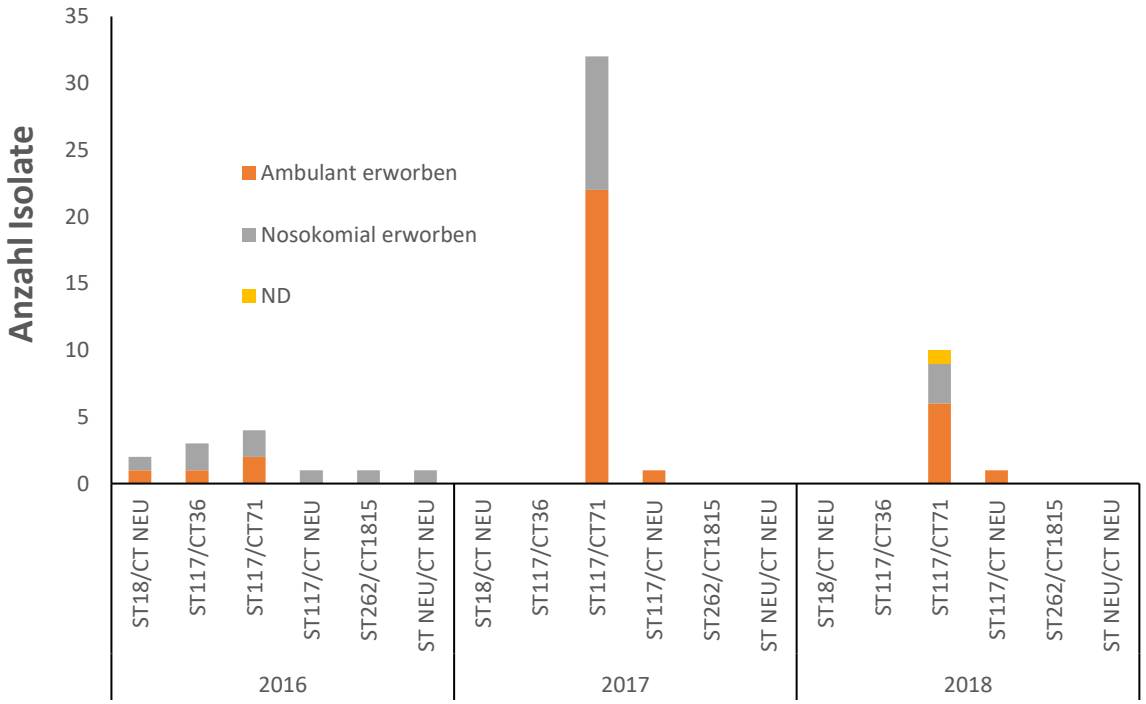


Abbildung 26: Darstellung der zeitlichen Entwicklung der VREfm-ST/CT-Typen (mit Erlaubnis verändert nach: (64))

Im Jahr 2016 waren noch sehr viele unterschiedliche ST-Typen zu finden, im Jahr 2017 und 2018 überwiegte allerdings nur noch ein Sequenztyp (ST-117, mit einem cgMLST-Typ (CT-71) und einem bestimmten *van*-Gen (*vanB*) – im nachfolgenden ST-117/CT-71/*vanB*-Typ genannt.

Bei der weiterführenden Studie im Rhein-Main-Gebiet stellte sich heraus, dass der ST-117/CT-71/*vanB*-Typ am häufigsten unter den isolierten VREfm vertreten war. Von 78 ST-117/CT-71/*vanB*-Typ-Isolaten fielen dabei 74 in ein einziges cgMLST Cluster (Cluster 1, 10 cgMLST Allel-*Cut-off* für die Cluster-Definition). Isolate dieses Clusters wurden an neun unterschiedlichen Standorten (Abbildung 27) gefunden, und wiesen so gut wie keine (bekannte) epidemiologische Verknüpfung untereinander auf.

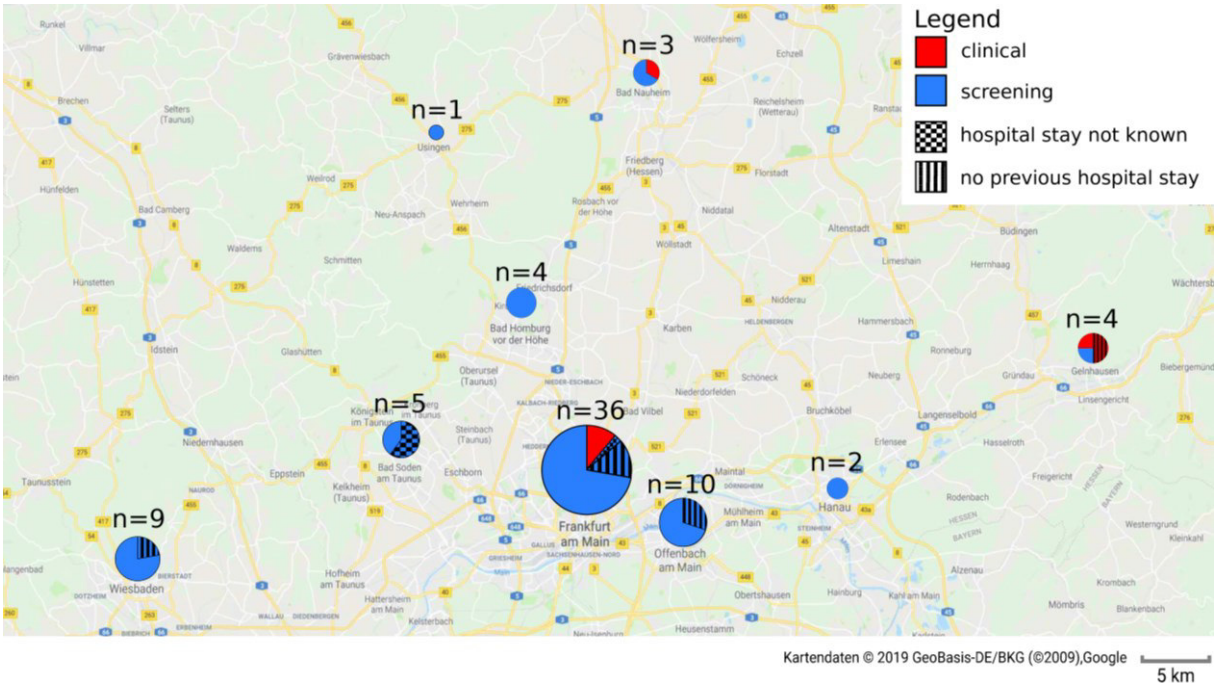


Abbildung 27: Darstellung der Orte, an denen Cluster 1 ST-117/CT-71/*vanB*-Isolate im Rhein-Main-Gebiet detektiert worden sind mit Angabe, ob es klinische/Screening-Isolate waren und ob die Patienten einen vorherigen Krankenhausaufenthalt hatten oder nicht (mit Erlaubnis übernommen aus (32)).

Vergleicht man dieses Ergebnis mit anderen deutschen Studien, so stellt es sich heraus, dass VREfm ST-117/CT-71/*vanB*-Isolate insbesondere in der geographischen Mitte Deutschlands und im Norden verbreitet sind (Abbildung 28, (63, 76, 77)). Im Süden Deutschlands sind dies zwar auch ST-117-Isolate, aber eher solche, die einen anderen cgMLST-Typ aufweisen (CT-36, CT-469, (78, 79)).

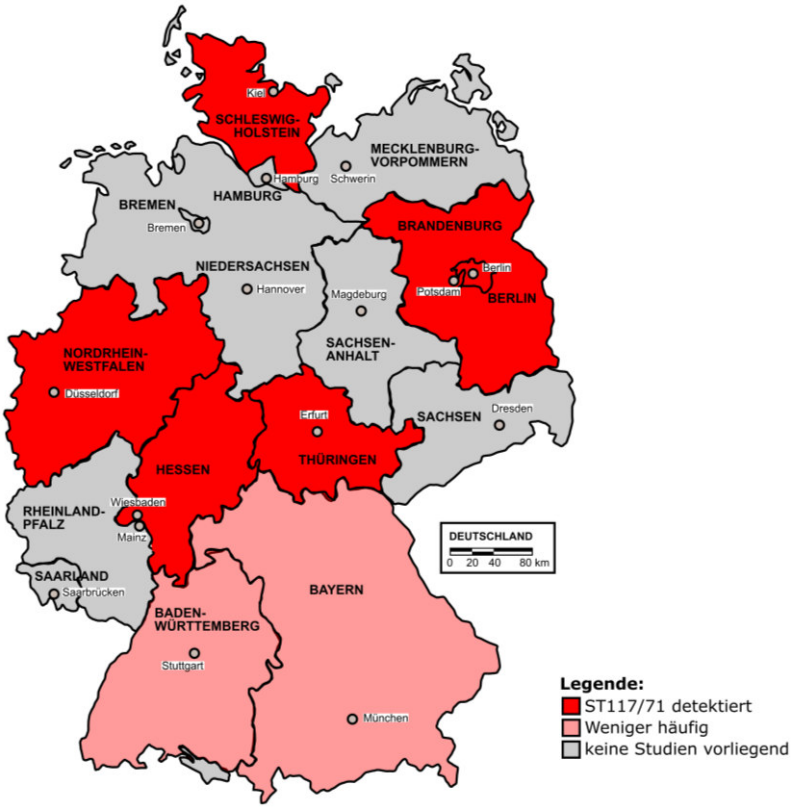


Abbildung 28: Darstellung der Verbreitung von VREfm ST-117/CT-71-Isolaten in Deutschland. Zusammenfassung der Daten aus: (32, 63, 64, 76, 78), Karte mit Erlaubnis verändert nach: (80).

International sind VREfm anderer Sequenztypen oder auch ST-117 anderer cgMLST-Typen zu beobachten (81). Ob die Expansion von ST-117/CT-71 für die schnelle Ausbreitung der VREfm in Deutschland verantwortlich ist, ist zwar zu vermuten, muss aber durch weitergehende molekulare Studien geklärt werden. Ebenso müssten auch Isolate aus Tier und Umwelt charakterisiert werden, um deren Rolle an der Ausbreitung zu identifizieren.

2.4. Detektion Carbapenemase-Produzenten-Klone in Deutschland und der Schweiz (Anhang E)

In den oben genannten Fällen wurden lokale ESBL/VREfm-Klone beschrieben. Im Zuge der Globalisierung kann die Hypothese, dass eine Übertragung Antibiotika-resistenter Klone innerhalb unterschiedlicher Länder stattfinden kann, nicht von der Hand gewiesen werden. Nachweise solcher Klone sind bis *dato* selten und häufig mit Lebensmitteln assoziiert. Im Laufe der Arbeiten wurden *E. coli* aus zwei Netzwerken zur Charakterisierung Carbapenem-resistenter Isolate in Deutschland (Surveillance Carbapenem-resistenter Erreger in Hessen, SurvCARE Hessen) und der Schweiz (Nationales Referenzlaboratorium zur Früherkennung und Überwachung neuartiger Antibiotikaresistenzen, NARA) genauer untersucht. Der Fokus lag auf *E. coli*, welche die seit kurzer Zeit neu aufgetretene Carbapenemase OXA-244 – ein Derivat von OXA-48 – trugen. Es wurden zehn unterschiedliche Sequenztypen identifiziert, von denen drei Sequenztypen (ST-38, ST-69, ST-10) in beiden Ländern zu finden waren (Abbildung 29).

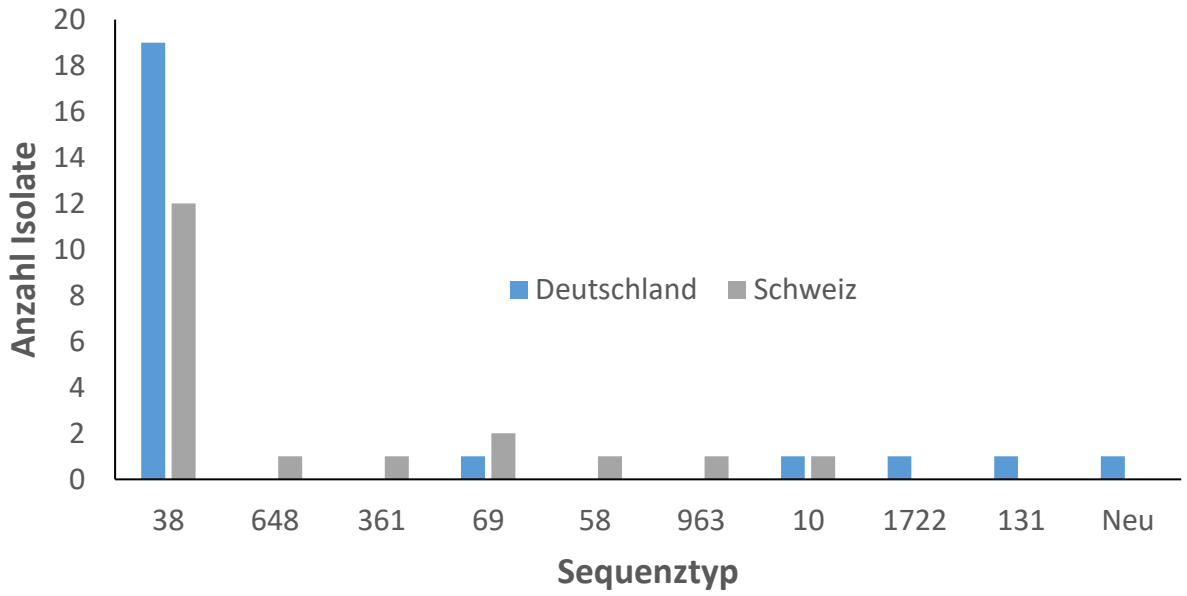


Abbildung 29: Detektierte Sequenztypen in OXA-244-tragenden *E. coli* aus Deutschland und der Schweiz (Abbildung erstellt nach Daten aus (82))

ST-38 war der am häufigsten vorkommende Sequenztyp (n=31). Daher wurden Isolate mit diesem Sequenztyp genauer analysiert (82). Es stellte sich heraus, dass klonale ST-38/OXA-244-*E. coli* in beiden Ländern vorkamen (Abbildung 30).

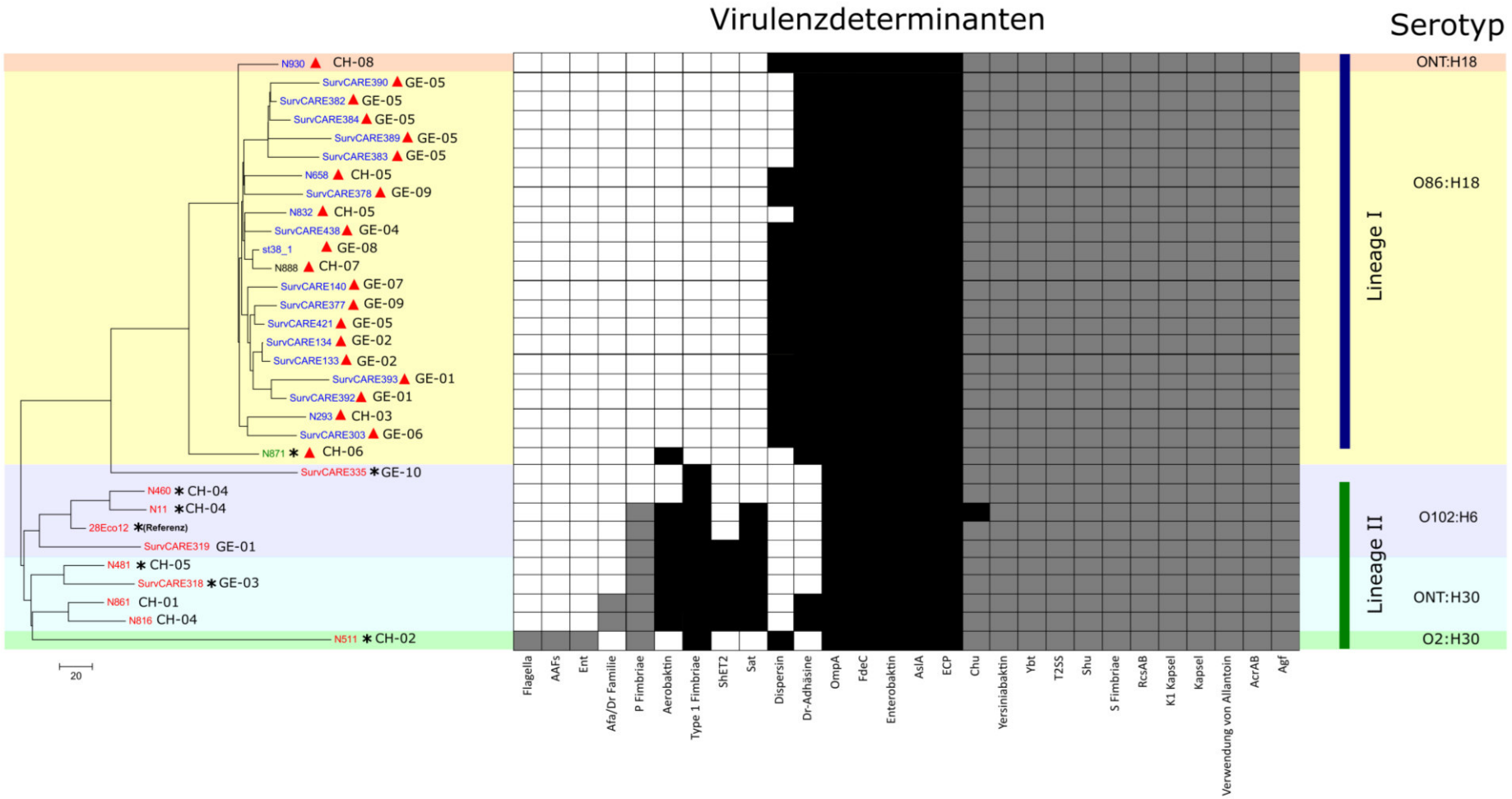


Abbildung 30: Core-Genom-basierter Stammbaum und Eigenschaften der *E. coli* ST-38-Isolate, welche in Deutschland und der Schweiz gefunden wurden. Mit Erlaubnis verändert nach (82).

Die meisten Isolate trugen den Serotyp O86:H18 und gehörten dem sogenannten Diffusely adherent *E. coli* (DAEC) Pathotyp an - sie kodieren sogenannte DR-Adhäsine. Mithilfe dieser Adhäsine sind diese ST-38-Isolate in der Lage, an eukaryotische Zellen (HeLa) anzuheften (Abbildung 31).

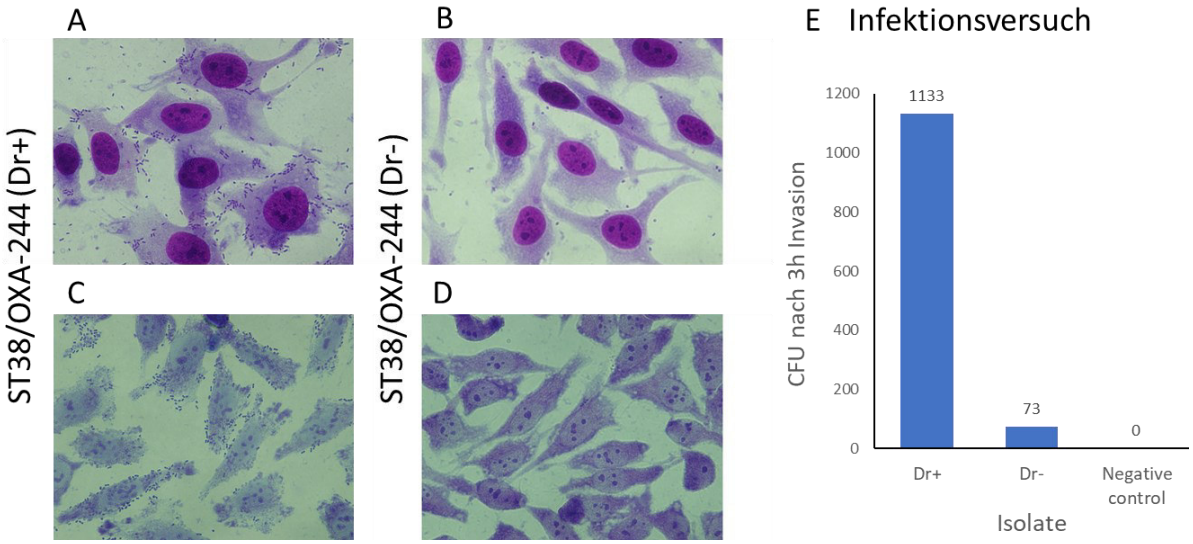


Abbildung 31: Überprüfung der Anheftung und der Infektion bei ST38-Isolaten mit (A/C/E) und ohne (B/D/E)-Dr-Adhäsine, mit Erlaubnis verändert nach (82).

Zusätzlich zum Vorhandensein dieser Virulenzfaktoren fehlen bei diesem Serotyp Typ I-Fimbrien, welche vom Immunsystem erkannt werden. Somit kann die Hypothese aufgestellt werden, dass die Detektion dieser Bakterien durch das Immunsystem erschwert ist, diese aber trotzdem über die DR-Fimbrien an humanen Zellen andocken können.

Parallel zu der Detektion identischer *bla*$_{\text{OXA-244}}$-kodierender *E. coli* ST-38-Isolate in Deutschland und der Schweiz wurde eine Expansion dieses STs auch in anderen europäischen Ländern festgestellt (83, 84). Der Grund für diese Expansion könnte in den oben genannten Eigenschaften der ST-38/OXA-244-*E. coli* liegen, ist aber weiter zu untersuchen.

2.5. Detektion eines multi-kompartimentären *E. coli* Klons (Anhänge F-H)

Die in den vorangegangenen Kapiteln dargestellte Tatsache, dass identische Klone in identischen Kompartimenten zu finden sind, ist insbesondere bei lokalen Geschehen (ein Staat, ein Bundesland) noch verständlich, da hierbei die Arten/Kompartiments-Grenze nicht überschritten wird. Das Vorhandensein identischer Klone über die Art/Kompartiment-Grenze hinweg ist als etwas unwahrscheinlicher zu deuten.
Im Rahmen der RESET-Studie wurden unterschiedliche Kompartimente auf das Vorhandensein ESBL-produzierender *E. coli* untersucht. Isolate, die ein identisches ESBL-Gen trugen, wurden mittels Ganzgenomsequenzierung näher charakterisiert. Bei der Untersuchung der CTX-M-15-Produzenten zeigte sich die in Abbildung 32 dargestellte Situation.

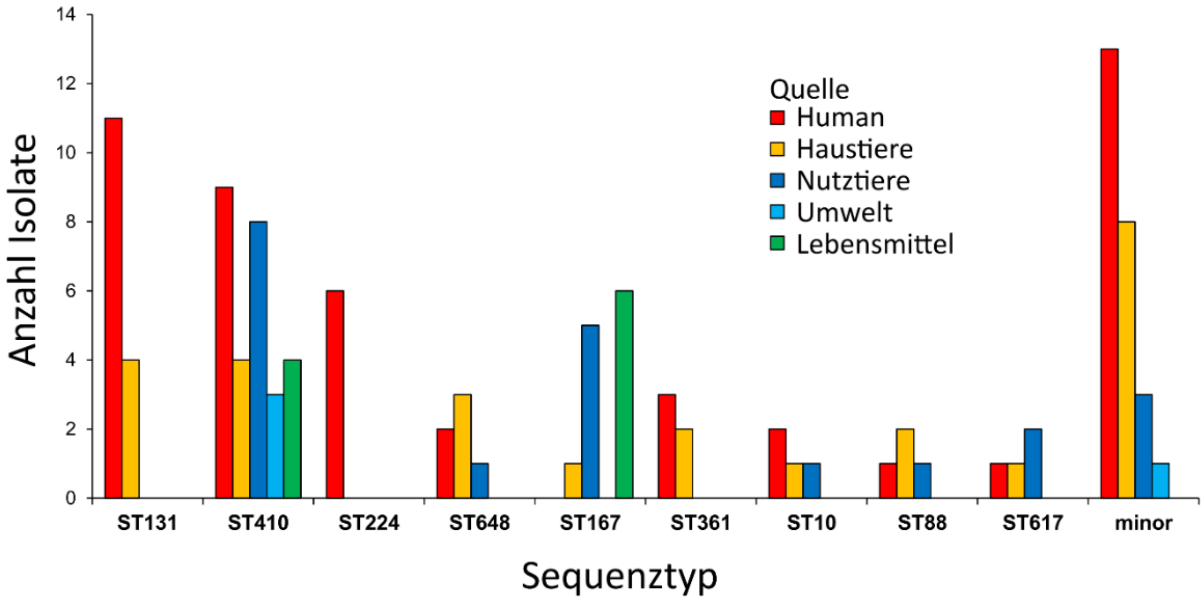


Abbildung 32: Darstellung der in CTX-M-15-Produzenten detektierten Sequenztypen (Daten zusammengefasst aus (34, 42, 60)).

Von neun häufigsten Sequenztypen war nur ein Sequenztyp (ST-410) in allen fünf untersuchten Kompartimenten zu finden – ein Hinweis darauf, dass es sich um eine klonale Übertragung dieses Typs handeln könnte. Die detaillierte Genom-basierte Analyse (Einzelnukleotid-basiert, SNP; Lokalisation des ESBL-Gens) zeigte, dass es innerhalb der ST-410-Isolate fünf differenzierte Cluster gab, die jeweils untereinander eine geringe SNP-Anzahl zeigten (Abbildung 33), also der Definition nach einen Klon darstellten.

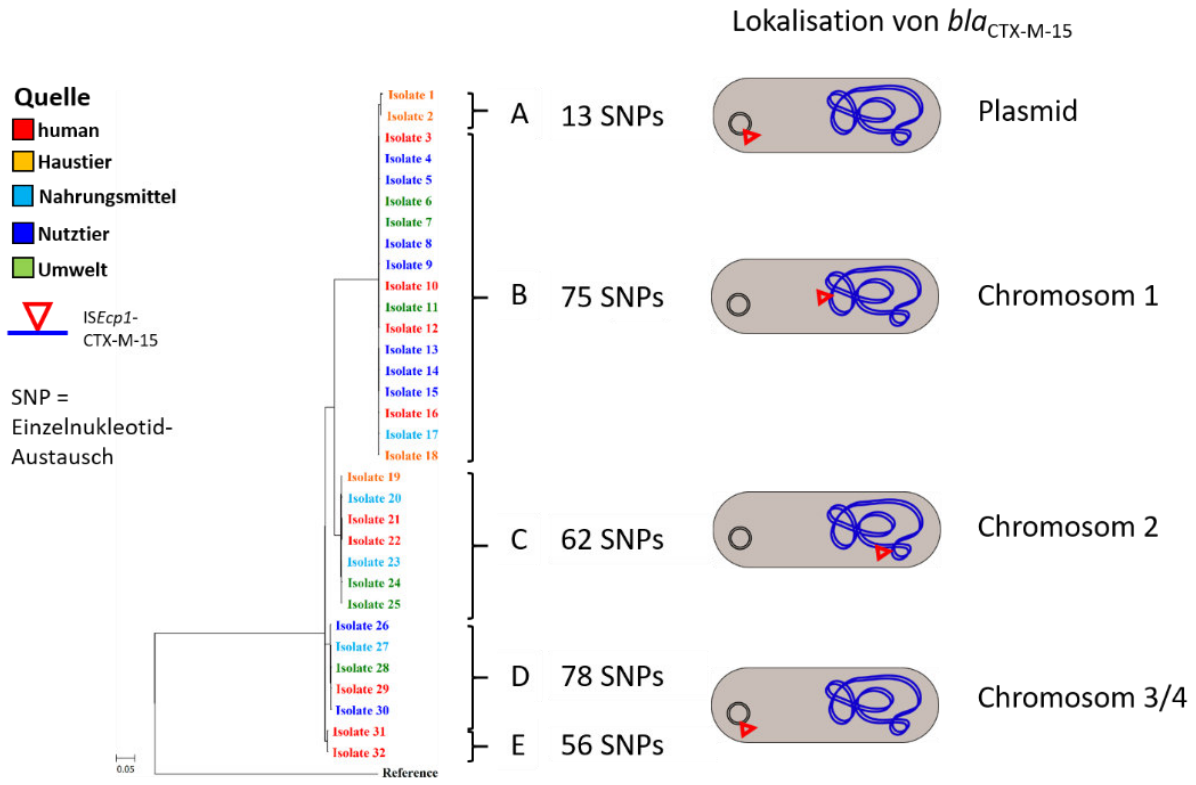


Abbildung 33: Verwandtschaftsanalyse der ST-410-Isolate. Daten zusammengefasst aus (34, 42, 60).

Die Cluster B, C, D und E wiesen eine unabhängige, aber jeweils für den Cluster spezifische, chromosomale Integrationsstelle von CTX-M-15 auf (34, 42, 60). Cluster B wurde in Isolaten aller fünf Kompartimente isoliert. Dieses Ergebnis war von außerordentlicher Bedeutung, da zum ersten Mal identische Isolate in einer so großen Anzahl an Kompartimenten beobachtet worden sind.

Nach Publikation der ST-410-Studie aus Deutschland zeigten andere Studien aus Europa, dass *E. coli* ST-410-Isolate nicht nur in der Lage sind, weitere Antibiotika-Resistenzen, darunter Resistenzen gegen das Reserveantibiotikum Carbapenem, zu akquirieren (85, 86), sondern auch Ausbrüche verursachen können (85). Bei *E. coli* ST-410 handelt es sich somit um einen Klon von krankenhaus- und umwelthygienischer Relevanz.

2.6. Detektion eines international verbreiteten Plasmides (Anhänge H-M)

Ein Vorteil der Verwendung von Ganzgenomsequenzen ist, dass sie immer wieder mit einer neuen Fragestellung analysiert werden können. Wenn z.B. in der Literatur ein neues Antibiotika-Resistenz- oder Virulenzgen charakterisiert wird, können die Ganzgenomsequenzen schnell nach dem Vorhandensein solcher neuen Sequenzen überprüft werden. Ein Beispiel hierfür ist die Detektion des neuartigen Plasmid-lokalisierten Colistin-Resistenz-Gens *mcr-1*, welches eine Resistenz gegen das Reserveantibiotikum Colistin verursacht. Ende 2015 wurde *mcr-1* das erste Mal in China beschrieben (87). Initial in *E. coli* detektiert, wurde *mcr-1* im Laufe der Jahre auch in anderen Spezies wie *Salmonella* spp, *Enterobacter* spp. und sogar in *Pseudomonas aeruginosa* gefunden (88). Da zur Zeit der Erstentdeckung von *mcr-1* bereits zahlreiche bakterielle Genome sequenziert waren, konnte *mcr-1* schnell in anderen Ländern nachgewiesen werden, darunter auch 2016 zum ersten Mal in Deutschland (89). Mittlerweile sind *mcr-1*-kodierende Isolate auf allen Kontinenten gefunden worden (Abbildung 34).

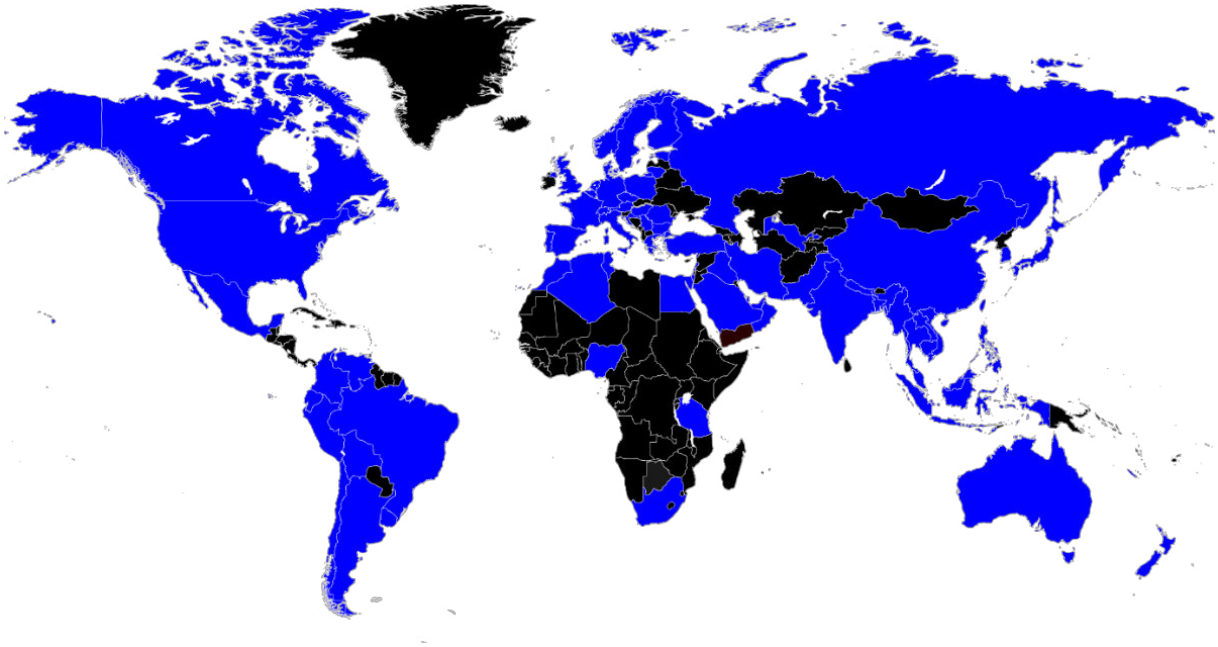
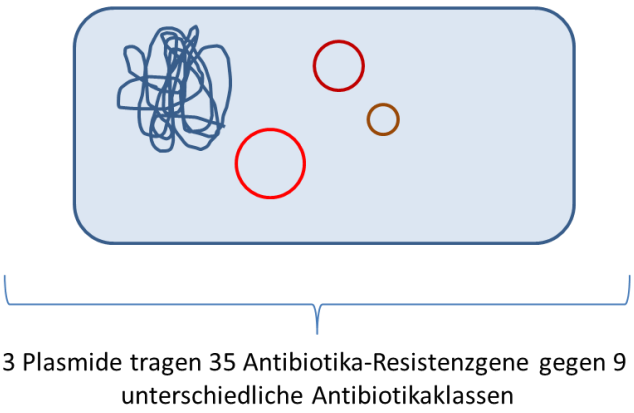


Abbildung 34: Darstellung der Länder, in denen *mcr-1* entdeckt worden ist (blau markiert). Stand: 04.10.2021, Daten zusammengefasst aus (90–103).

mcr-1-positive Isolate wurden überwiegend in Proben aus Nutztieren und in Lebensmitteln gefunden (60, 87, 102, 104, 105), aber auch in humanen Proben (87, 106, 107). Diese Beobachtung führt zu der Hypothese, dass eine Übertragung von

mcr-1-kodierenden Isolaten aus Tieren über Lebensmittel auf Menschen stattgefunden hat. Allerdings sind *mcr-1*-kodierende Isolate auch in Stallfliegen (62) und Wasserproben (66) gefunden worden, sodass eine Nicht-Nahrungsmittelbedingte Übertragung nicht gänzlich auszuschließen ist.

Besonders besorgniserregend an den Isolaten aus Deutschland war, dass sie zusätzliche Antibiotika-Resistenzen trugen, unter anderem Resistenzen gegen Dritt-Generations-Cephalosporine (60, 89), aber auch gegen andere Reserveantibiotika, wie Carbapeneme (106). Eines dieser Isolate trug Resistenzen gegen neun unterschiedliche Antibiotika-Klassen (Abbildung 35).



Antibiogramm:

Antibiotikaklasse	Phenotyp	Bemerkung
Penicilline	R	
Carbapeneme	R	
Fluorchinolone	R	
Monobactame	R	
Colistin	R	
Rifampicin	R	
Sulfonamide	R	
Phenicole	R	
Trimethoprim	R	
Aminoglykoside	R	Sensitiv gegen Amikacin
Tetrazyklin	R	Sensitiv gegen Tigecyclin
Fosfomycin	S	

Abbildung 35: Schematische Darstellung des *E. coli* Isolates NRZ14408, welches zusätzlich zu *mcr-1* auch die Carbapenem-Resistenz *bla*$_{KPC-2}$ trug (106).

Ein solches Isolat ist an der Grenze zur Panresistenz, deren Entstehung und Verbreitung möglichst vermieden werden sollte. Wie in anderen Studien beschrieben (108, 109), wurden in Deutschland *mcr-1*-kodierende *E. coli* mit unterschiedlichen Sequenztypen gefunden (62), ein Hinweis dafür, dass die Übertragung/Weitergabe von *mcr-1* Plasmid-vermittelt und nicht klonal ist. In Deutschland wurden im Gegensatz zu der Situation in China, bei der das meistverbreitete Plasmid der Inkompatibilitätsgruppe IncI2 angehörte (87), überwiegend *mcr-1*-kodierende Plasmide der Inkompatibilitätsgruppe IncX4 detektiert (Abbildung 36).

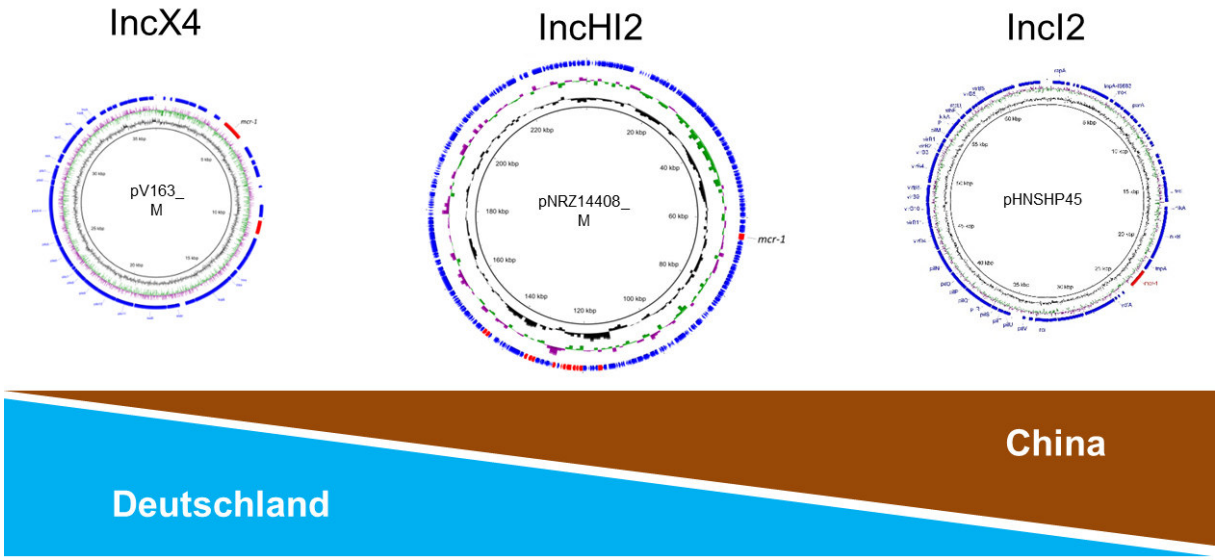


Abbildung 36: Darstellung der drei häufigsten *mcr-1*-kodierenden Plasmidtypen mit ihrer relativen Verteilung in Deutschland und in China.

Bei den *mcr-1*-kodierenden IncX4-Plasmiden handelt es sich um verhältnismäßig kleine Plasmide (ca. 30-40 kbp groß). Den größten Teil der Gene macht die hocheffiziente Konjugationsmaschinerie aus (Abbildung 37). Dies führt zu einer hohen Konjugationseffizienz von bis zu 10^{-1} (89), d.h. die Plasmide (inklusive der Resistenz) können sehr schnell auf andere Bakterienzellen übertragen werden.

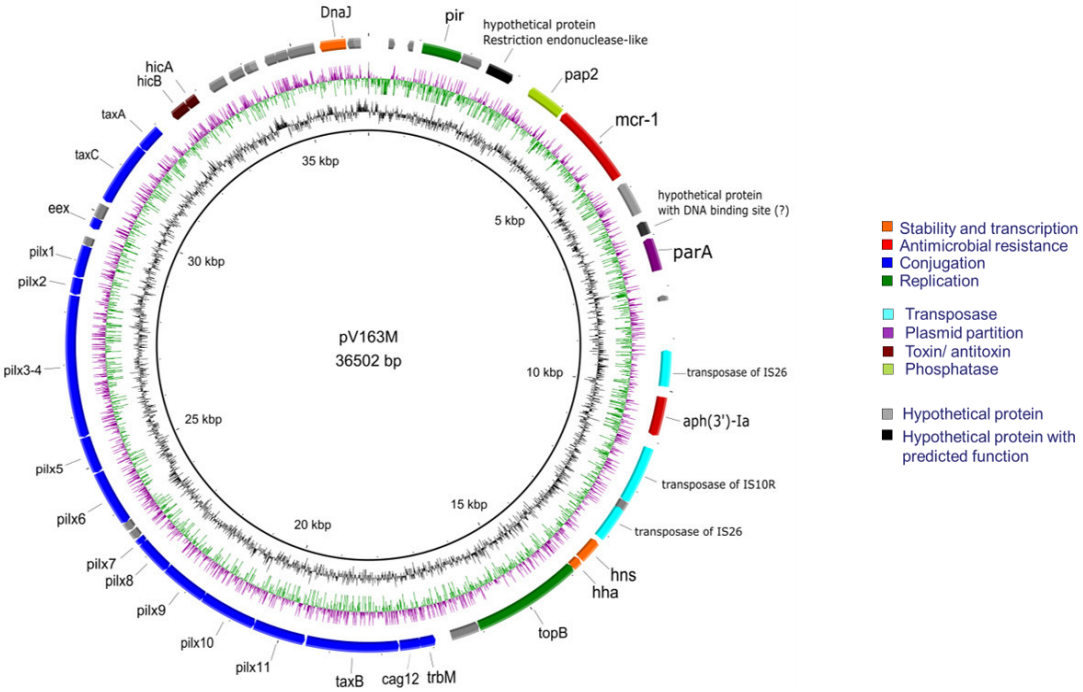


Abbildung 37: Darstellung des weit verbreiteten *mcr-1*-kodierenden IncX4-Plasmides pV163M. Mit Erlaubnis verändert nach: (110).

Die *mcr-1*-kodierenden IncX4-Plasmide wurden in Deutschland in Bakterien mit unterschiedlichen genetischen Hintergründen (= andere STs) detektiert (62, 89, 105). Dies deutet darauf hin, dass es ein sehr erfolgreiches Plasmid darstellt. Auch die weltweite Situation spiegelt diese Hypothese wider: IncX4-Plasmide mit *mcr-1* wurde weltweit auf jedem Kontinent detektiert (111). Folglich kann das *mcr-1*-kodierende IncX4-Plasmid als epidemisches Plasmid bezeichnet werden. Die Gründe für seine weite Verbreitung werden derzeit erforscht.

Eher seltener, aber doch vorhanden, war die Integration von *mcr-1* in das bakterielle Chromosom z. B. bei dem Isolat RL465 (60). Besonders an diesem Isolat war, dass es zusätzlich zu *mcr-1* noch das ESBL-Gen $bla_{\text{CTX-M-15}}$ im Chromosom integriert trug. Es gehörte dem im Abschnitt 2.5 beschriebenen Sequenztyp ST-410 an. Ob diese Konstellation dem Bakterium einen bestimmten Fitness-Vorteil verleiht, konnte noch nicht abschließend geklärt werden.

3. Zusammenfassung und Ausblick

Antibiotika-Resistenz hat sich in den letzten Jahren zu einem immer schwerwiegenderen Problem entwickelt. Mit der Zuspitzung dieses Problems ging aber auch eine Sensibilisierung der Bevölkerung einher mit der Konsequenz, die Forschung auch in diese Richtung auszuweiten. Eine weitere Folge der erhöhten Sensibilisierung in Hinsicht auf die Antibiotika-Resistenz war die Erkenntnis, dass die Lösung des Problems in Händen vieler Akteure liegt, und nicht auf ein Kompartiment, wie z.B. die Humanmedizin, beschränkt bleiben darf. Aus diesem Grund entstanden ab 2010 im Rahmen von Bundes- und Landesinitiativen geförderte Netzwerke, die sich mit dem Thema Antibiotika-Resistenz im One-Health-Kontext beschäftigten und noch beschäftigen, wie z.B. RESET und DZIF. Die in dieser Arbeit dargestellten und diskutierten Publikationen entstanden im Rahmen solcher Netzwerke.

Mit Verwendung modernster Methoden (Ganz-Genom-Sequenzierung, bioinformatorische Analysen) war es möglich, Antibiotika-resistente Bakterien bis auf Nukleotid-Ebene genau zu analysieren. Diese Analyse wurde mit Bakterien aus unterschiedlichen Kompartimenten (Tier, Mensch, Lebensmittel, Haustier, Umwelt) durchgeführt, um zu ermitteln, ob eine Übertragung Antibiotika-resistenter Bakterien oder mobiler genetischer Elemente zwischen den einzelnen Kompartimenten stattfindet.

Die Ergebnisse dieser Analysen waren für alle Beteiligten unerwartet. Neben Klonen, deren Vorhandensein auf ein Kompartiment beschränkt war (z.B. *E. coli* ST206 in Nutztieren) wurde zum ersten Mal in Deutschland ein ESBL-Klon nachgewiesen, der in fünf unterschiedlichen Kompartimenten (Mensch, Nutztier, Haustier, Lebensmittel, Umwelt) zu finden war (*E. coli* ST-410).

Überraschend war auch das Vorhandensein von Klonen, die zwar nur in einem Kompartiment vorkamen, dafür aber national (VREfm ST-117/CT-71) oder sogar international (*E. coli* ST-38) zu finden waren. Aber nicht nur Klone konnten mit den genannten Analysen als wichtige Komponente der Verbreitung von Antibiotika-Resistenzen ermittelt werden. Mit dem *mcr-1*-kodierenden IncX4-Plasmid, welches in nahezu identischer Form in Bakterien aus unterschiedlichen Kompartimenten (Nutztier, Umwelt, Lebensmittel) gefunden wurde, ist ein epidemisches Plasmid detektiert worden, welches die Eigenschaft hat, sich in Bakterien mit verschiedensten

genetischen Hintergründen zu verbreiten. Sein weltweites Vorkommen (111) weist darauf hin, dass dieses Plasmid sehr erfolgreiche Mechanismen zur Übertragung aber auch zur Etablierung in einem bestimmten Stamm haben muss.

3.1. Ausblick

Mit dem Vorhandensein identischer Bakterien/Plasmide in unterschiedlichen Habitaten muss Antibiotika-Resistenz im One-Health-Gedanken mehr als noch bis *dato* bearbeitet werden. Hierzu zählen eine Kompartiment-übergreifende Genom-basierte Surveillance, um zum einen zu ermitteln, ob die Epidemiologie der Antibiotika-resistenten Bakterien identisch ist oder sich im Laufe der Zeit bestimmte Klone durchsetzen können und zum anderen zu ermitteln ob nicht neuartige Antibiotika-Resistenzen gebildet werden (wie am Beispiel von *mcr-1* beschrieben).

Im Rahmen der Studie wurden bakterielle Klone bzw. identische Plasmide national, und sogar über Landesgrenzen hinweg gefunden. Diese Ergebnisse verdeutlichen eindrücklich, dass ein Transfer Antibiotika-resistenter Bakterien und Plasmide stattfindet. Dies ist sicherlich zu einem großen Teil der Globalisierung und dem damit verbundenen internationalen Austausch von Lebensmitteln, aber auch dem starken Reiseverkehr (Abbildung 38) geschuldet.

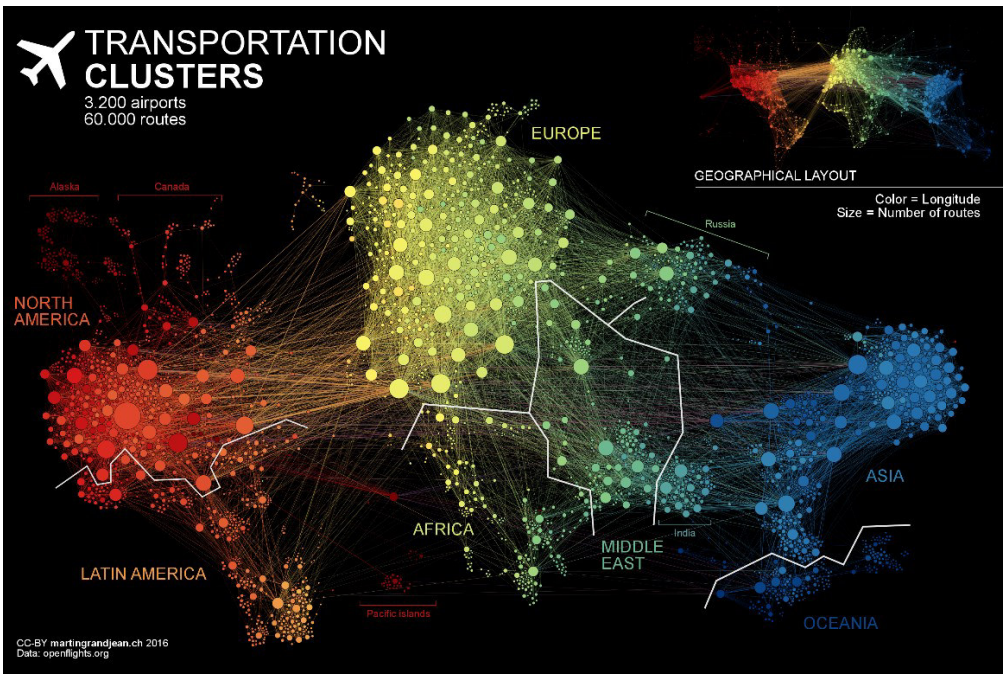


Abbildung 38: Schematische Darstellung der Flugcluster auf der Welt. Mit Erlaubnis von (112) übernommen.

Wie stark der internationale Reiseverkehr an der Verbreitung Antibiotika-resistenter Bakterien beteiligt sein könnte, hat eindrücklich die Verbreitung neuer COVID-19-Varianten während der COVID-19-Pandemie gezeigt. Da nicht davon auszugehen ist, dass sich der Reiseverkehr und der internationale Austausch von Lebensmitteln in Zukunft verringern werden, sondern eher verstärken wird, sollten in Zukunft über solche Wege übertragene Antibiotika-resistente Bakterien detektiert werden, um internationale Ausbrüche nicht zu fördern. Grundvoraussetzung hierfür wäre die kostengünstige und anwenderfreundliche Entwicklung und Bereitstellung von Schnelltests, am besten in Form Ganzgenom-basierter Tests.

Ein weiterer Punkt, der in dieser Arbeit noch nicht angeklungen ist, ist der Einfluss des Klimawandels auf die Verbreitung bestimmter Antibiotika-resistenter Klone und Plasmide. Nach einer Meta-Analyse von Meinen et al. (113) korreliert ein Temperaturanstieg mit einem Anstieg bestimmter Antibiotika-resistenter Bakterien. Dies weist darauf hin, dass der Klimawandel das aktuelle Problem der Antibiotika-Resistenz noch verschärfen könnte. Hitze-resistente Bakterien und solche Antibiotika-Resistenz-Plasmide, die Bakterien eine Hitze-Resistenz verleihen können, könnten sich gegenüber anderen durchsetzen.

Abschließend lässt sich sagen, dass mit der Detektion identischer Klone/Plasmide in mehreren Kompartimenten die eigentliche wissenschaftliche Arbeit erst anfängt. Es muss ermittelt werden, welche Mechanismen diese erfolgreichen Klone/Plasmide haben, die es ihnen erlaubt haben, sich so weit zu verbreiten. Nur mit der Kenntnis dieser Mechanismen wird es möglich sein, neuartige Strategien zur Verhinderung der Ausbreitung Antibiotika-resistenter Bakterien zu entwickeln. Dies soll das Ziel weiterer Projekte sein.

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5. Abbildungs-Lizenzvereinbarungen

In Tabelle 1-3 sind die Lizenzvereinbarungen zu den Abbildungen in der vorliegenden Arbeit zu finden. Die Abbildungen 6-7, 10 - 11, 14 – 16, 18, 21, 29, 32 - 33 und 35 - 36 sind selbst erstellte Abbildungen.

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Gießen, 26.11.2023

Dr. rer. nat. Linda Falgenhauer

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8. Anhänge

Eigene Publikationen, die in dieser kumulativen Habilitationsschrift zusammengefasst und diskutiert wurden (sortiert nach Erstautor, * geteilte Erst-Autorenschaft, $ corresponding author, Tabelle 4)

Tabelle 4: Anhänge

Anhang	Publikation
A	**Falgenhauer L,**$ zur Nieden A, Harpel S, Falgenhauer J, Domann E. 2021. Clonal CTX-M-15-Producing *Escherichia coli* ST-949 Are Present in German Surface Water. Front. Microbiol. 12:857.
B	Ayeni FA, Falgenhauer J, Schmiedel J, Schwengers O, Chakraborty T, **Falgenhauer L**$. 2020. Detection of *bla*$_{CTX-M-27}$-encoding *Escherichia coli* ST206 in Nigerian poultry stocks. J. Antimicrob. Chemother. 75, 3070–3072.
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D	**Falgenhauer L**, Fritzenwanker M, Imirzalioglu C, Steul K, Scherer M, Rhine-Main VREfm study group, Heudorf U, Chakraborty T. 2019. Near-ubiquitous presence of a vancomycin-resistant *Enterococcus faecium* ST117/CT71/*vanB* – clone in the Rhine-Main metropolitan area of Germany. Antimicrob. Resist. Infect. Control. 8, 128**.**
E	**Falgenhauer L**, Nordmann P, Imirzalioglu C, Yao Y, Falgenhauer J, Hauri AM, Heinmüller P, Chakraborty T. 2020. Cross-border emergence of clonal lineages of ST38 *Escherichia coli* producing the OXA-48-like carbapenemase OXA-244 in Germany and Switzerland. Int. J. Antimicrob. Agents 56:106157.
F	**Falgenhauer L**, Imirzalioglu C, Ghosh H, Gwozdzinski K, Schmiedel J, Gentil K, Bauerfeind R, Kämpfer P, Seifert H, Michael GB, Schwarz S, Pfeifer Y, Werner G, Pietsch M, Roesler U, Guerra B, Fischer J, Sharp H, Käsbohrer A, Goesmann A, Hille K, Kreienbrock L, Chakraborty T. 2016. Circulation of clonal populations of fluoroquinolone-resistant CTX-M-15-producing *Escherichia coli* ST410 in humans and animals in Germany. Int J Antimicrob Agents 47:457–465.

Anhang	Publikation
G	Irrgang A, **Falgenhauer L**, Fischer J, Ghosh H, Guiral E, Guerra B, Schmoger S, Imirzalioglu C, Chakraborty T, Hammerl JA, Käsbohrer A. 2017. CTX-M-15-producing *E. coli* isolates from food products in Germany are mainly associated with an IncF-Type plasmid and belong to two predominant clonal *E. coli* lineages. Front Microbiol. 8:2318.
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I	**Falgenhauer L**, Waezsada S-E, Yao Y, Imirzalioglu C, Käsbohrer A, Roesler U, Michael GB, Schwarz S, Werner G, Kreienbrock L, Chakraborty T. 2016. Colistin resistance gene *mcr-1* in extended-spectrum β-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany. Lancet Infect Dis 16:282–283.
J	Fritzenwanker M, Imirzalioglu C, Gentil K, **Falgenhauer L**, Wagenlehner FME, Chakraborty T. 2016. Incidental detection of a urinary *Escherichia coli* isolate harbouring *mcr-1* of a patient with no prior history of colistin treatment. Clin Microbiol Infect 22:954–955.
K	**Falgenhauer L**, Ghosh H, Doijad S, Yao Y, Bunk B, Spröer C, Kaase M, Hilker R, Overmann J, Imirzalioglu C, Chakraborty T. 2017. Genome analysis of the carbapenem- and colistin-resistant *Escherichia coli* isolate NRZ14408 reveal horizontal gene transfer pathways towards pan-resistance and enhanced virulence. Antimicrob Agents Chemother 61:e02359-16.
L	Guenther S, **Falgenhauer L**, Semmler T, Imirzalioglu C, Chakraborty T, Roesler U, Roschanski N. 2017. Environmental emission of multiresistant *Escherichia coli* carrying the colistin resistance gene *mcr-1* from German swine farms. J Antimicrob Chemother 72:1289-1292.
M	Roschanski N, **Falgenhauer L**, Grobbel M, Guenther S, Kreienbrock L, Imirzalioglu C, Roesler U. 2017. Retrospective survey of *mcr-1* and *mcr-2* in German pig-fattening farms, 2011-2012. Int. J. Antimicrob. Agents 50:266-271.

Anhang A

Falgenhauer L, zur Nieden A, Harpel S, Falgenhauer J, Domann E. 2021. Clonal CTX-M-15-Producing *Escherichia coli* ST-949 Are Present in German Surface Water. Front. Microbiol. 12:857.

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Clonal CTX-M-15-Producing *Escherichia coli* ST-949 Are Present in German Surface Water

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Extended-spectrum beta-lactamase (ESBL)-producing bacterial isolates are emerging within the last years. To understand this emergence, a thorough genome-based analysis of ESBL isolates from different sources (One Health approach) is needed. Among these, analysis of surface water is underrepresented. Therefore, we performed a genome-based analysis of ESBL-producing *Escherichia coli* isolates from surface water samples. Water samples were collected from eleven different surface water sites (lakes, river). ESBL-producing *E. coli* were recovered from these samples using filters and chromogenic media. Whole-genome sequencing of ESBL-producing *E. coli* was performed followed by determination of the multilocus sequence type (ST), ESBL-type, and virulence genes. Phylogenetic analysis was done using single nucleotide analysis. From all water samples taken, nineteen ESBL-producing *E. coli* were recovered. All of them harbored an ESBL gene. Nine different multilocus STs were determined, among which ST-949 was the ST detected most frequently. Phylogenetic analysis of ST-949 isolates revealed that all those isolates were closely related. In addition, they harbored an identical chromosomal insertion of $bla_{CTX-M-15}$, indicating a clonal relationship among these isolates. Genetic comparison with isolates from all over the world revealed that these isolates were closely related to human clinical isolates derived from New Zealand and Sweden. An ESBL-producing *E. coli* ST-949 clone was detected in German surface waters. Its close relationship to human clinical isolates suggests its ability to colonize or even infect humans. Our findings reveal that water sources indeed may play a hitherto underreported role in spread of ESBL-producing isolates.

Keywords: CTX-M-15, ST-949, ESBL-*E. coli*, water samples, WGS

INTRODUCTION

Extended-spectrum beta-lactamase (ESBL)-producing bacterial isolates are emerging in the last years (Peirano and Pitout, 2019). The spread of ESBL-producers is a clear One Health issue, as they have been found to be present in different sources, animals, humans, and environment (Hooban et al., 2020). This is true for Germany as well. In Germany, 6.3% of humans are colonized with ESBL-producing *Escherichia coli* isolates (Valenza et al., 2014). In diseased food-producing animals,

the prevalence of ESBL-producing *E. coli* ranges between 0.8 and 11.2% depending on the animal species (Michael et al., 2017).

The commonly accepted opinion is that all different sources play a role in the spread of ESBL-producing bacteria. To be able to track the transfer routes of ESBL-producers among different sources, a thorough understanding of the epidemiology of these bacteria is needed. The method of choice to perform an in-depth epidemiological analysis is to use whole genome sequence-based methods. They have been used a lot in human and veterinary medicine (in particular to track outbreaks), but genome-based data from water sources are still very rare. Few epidemiological studies have been performed to analyze the genomes of ESBL-producing isolates from water samples. These studies showed a high identity between ESBL producers from water samples and clinical samples indicating a spread from either clinical to water sources or vice versa (Fagerström et al., 2019).

In order to gain more insight into this topic, an investigation was performed that included water samples from official and unofficial bathing sites at lakes and a river in Hesse, Germany.

MATERIALS AND METHODS

Sampling Procedure

During the bathing season 2018, samples were taken from swimming lakes in Hesse (n = 10). According to the European Bathing Water Directive (BWD; EG 2006/7) the sites were checked at least monthly for the presence of coliform bacteria. Additionally, samples from unofficial bathing sites of the Hessian river Lahn around Marburg and Giessen were taken (n = 9). Procedures for sampling as well as preparation, filtration, and enumeration were performed conforming with the DIN EN ISO 9308-2 (K6-1) 07-2014, DIN EN ISO 19458 (K19), DIN EN ISO 8199 (K20) 01-2008, and DIN EN ISO 9380-1: 2014 (K12) regulations within 24 h.

Characterization of ESBL-Producing Isolates

For detection of ESBL-producing isolates, water samples were filtered and the filters put onto Brilliance^{TM} ESBL chromogenic medium (OXOID, Wesel, Germany). For isolates growing on the chromogenic agar, species confirmation was performed using MALDI-TOF-MS (Biomérieux, Nürtingen, Germany). Antibiotic susceptibility testing and ESBL phenotype confirmation was performed using the VITEK 2 System (AST-N263 cards, Biomérieux, Nürtingen, Germany). Classification of the antibiotic resistance/susceptibility was performed according to EUCAST criteria[1].

Whole-Genome Sequencing

Short-read whole genome sequencing was performed for all *E. coli* isolates growing on the chromogenic medium (n = 21). DNA from overnight cultures was isolated using the Purelink genomic DNA kit (ThermoFisher, Dreieich, Germany). Short read sequencing was performed on a NextSeq 500 machine (Illumina, Eindhoven, Netherlands) using a Nextera XT sequencing library with an average read length of 115 nt and an average coverage of 33.5 x. Raw reads were processed using the ASA^{3}P pipeline using default parameters (Schwengers et al., 2020).

Long-read sequencing of a representative *E. coli* ST-949 isolate (EDCC5518) was performed using the Nanopore technology. The library was prepared using the native barcoding kit (EXP-NBD103, Oxford Nanopore Technologies Ltd., Oxford, United Kingdom) and 1D chemistry (SQK-LSK108). Sequencing was performed using the SpotON Flow Cell Mk I R9 Version (FLO-MIN106) on a MinION/MinIT machine with an average read length of 4,137 nt. Basecalling was performed directly on the MinIT machine. Demultiplexing was performed using Porechop (v. 0.2.3[2]). Hybrid assembly was performed using Unicycler (v. 0.4.7) (Wick et al., 2017) and the short and long reads with default parameters.

Genome-Based Analyses

In silico multilocus sequence typing of *E. coli* isolates was performed using the scheme presented by Wirth et al. (2006). Antibiotic resistance genes, plasmid incompatibility groups and *fimH* types were determined using the bacterial analysis pipeline of the Center for Genomic Epidemiology[3]. Insertion elements were determined using ISFinder (Siguier et al., 2006). Virulence gene determination was performed using ASA^{3}P (Schwengers et al., 2020). Comparative genome analysis was performed using the HarvestSuite package (Treangen et al., 2014). Publicly available assembled *E. coli* genomes of the multilocus sequence type (ST) ST-949 were downloaded using Enterobase (as of 8th June 2020, **Supplementary Table 1**) (Zhou et al., 2020). Geographical representation of sampling sites was visualized using MicroReact (Argimón et al., 2016).

RESULTS AND DISCUSSION

Detection and Phenotypic Characterization of ESBL-Producing *Escherichia coli* Samples

During the bathing season of 2018 (June–August), fifty-five samples from nineteen sampling sites were collected. Of these samples, forty-four did not show growth of isolates on ESBL chromogenic agar. Notably, the 2018 summer was a comparatively hot summer[4] resulting in low water levels. From the remaining water samples (n = 11, **Figure 1**), nineteen ESBL-producing bacterial isolates were detected (**Table 1**). Only *E. coli* isolates were detected. Environmental data and characterization of the sampling sites are shown in **Table 1**.

For all *E. coli* isolates growing on the chromogenic plates, the ESBL phenotype was confirmed. Phenotypic

[1]https://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_11.0_Breakpoint_Tables.pdf

[2]https://github.com/rrwick/Porechop

[3]http://www.genomicepidemiology.org/

[4]http://www.dwd.de and http://www.wetter.de

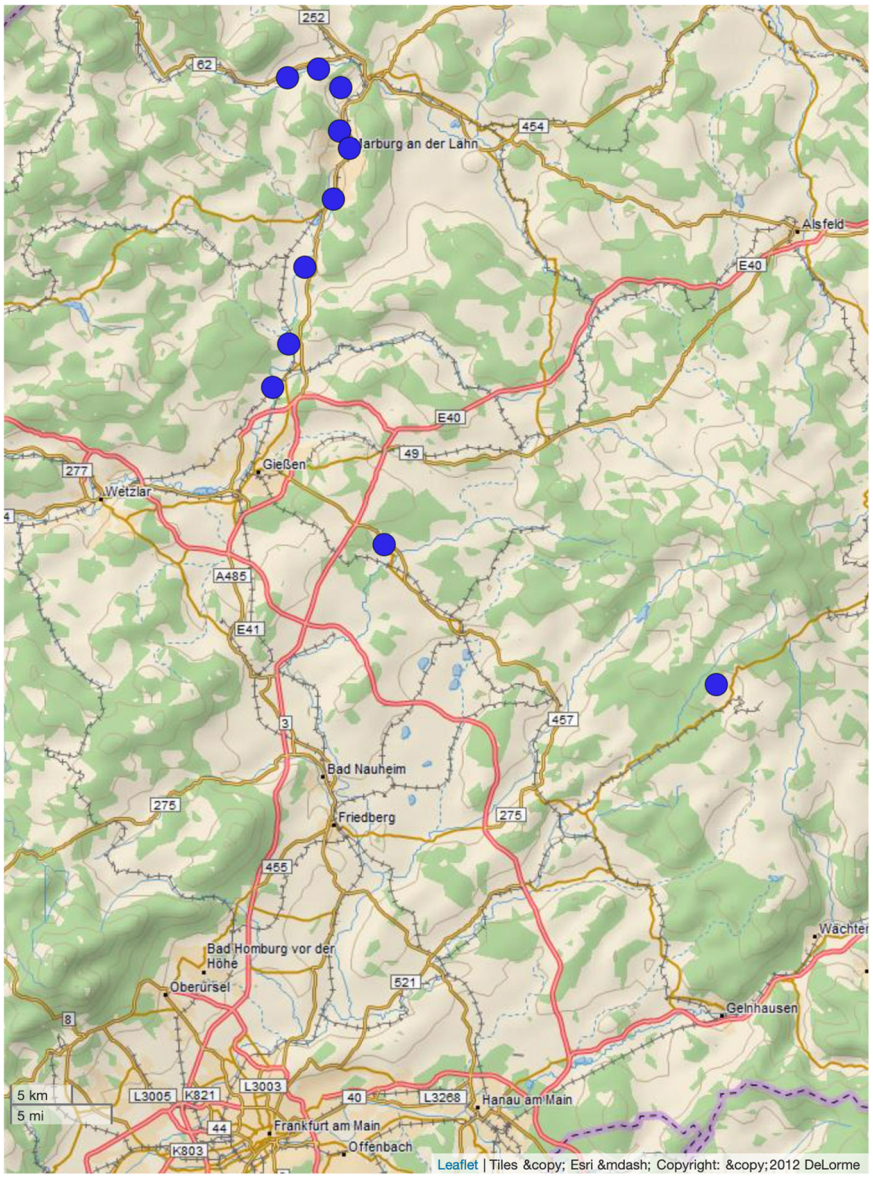

FIGURE 1 | Distribution of sampling sites. The figure was generated using microreact (Argimón et al., 2016).

resistance to antibiotics other than beta-lactams was detected very seldom and included resistance to fluoroquinolones (4/21) and trimethoprim/sulfamethoxazole (5/21) (**Supplementary Table 2**). The isolates were not resistant to carbapenems. According to the classification proposed by Magiorakos et al. (2012), all isolates were multidrug-resistant (resistant to ≥3 different antibiotic classes; **Supplementary Table 2**).

Genome-Based Analysis of *Escherichia coli* Isolates

All *E. coli* isolates harbored an ESBL gene (**Table 2**). The most common ESBL gene detected was $bla_{CTX-M-15}$ (n = 17) followed by $bla_{CTX-M-1}$ (n = 2) and $bla_{CTX-M-27}$ (n = 2). The predominance of $bla_{CTX-M-15}$ in our study is concordant with the results in other studies performed in Europe (Kittinger et al., 2016; Jorgensen et al., 2017). The *E. coli* isolates encoded other antibiotic resistance genes conferring resistance to aminoglycosides (7/21), fluoroquinolones (15/21, *qnrS1*), sulfonamide (5/21), trimethoprim (5/21), and tetracycline (2/21) (**Table 2**). In concordance with previous reports (Rodríguez-Martínez et al., 2011), the presence of *qnrS1* did not lead to high-level fluoroquinolone resistance (MIC > 0.5 mg/L) in our isolates.

Multilocus sequence typing revealed that nine different STs were present (**Table 2** and **Figure 2**). Of these, three were detected

more than once: ST-949 (n = 11), ST-131 (n = 2), and ST-1431 (n = 2). *E. coli* ST-949 and ST-1431 isolates harbored $bla_{CTX-M-15}$, while *E. coli* ST-131 isolates harbored $bla_{CTX-M-15}$ or $bla_{CTX-M-27}$.

To our knowledge, *E. coli* ST-949 have been reported in only five publications worldwide, indicating that this ST is less frequent and might represent an emerging clone (Oh et al., 2014; Potron et al., 2017; Potel et al., 2018; Fagerström et al., 2019; Sedrati et al., 2020). The total number of publicly available *E. coli* ST-949 isolates in the Enterobase database is 41 [as of 11th August 2020 (Zhou et al., 2020)], a very low number compared with frequent multilocus STs as e.g., ST-131 (n = 9202, as of 11th August 2020).

ESBL-producing ST-1431 *E. coli* isolates have been detected more often in animal sources (livestock, pets, wild animals) than in humans (Rocha-Gracia et al., 2015; Bachiri et al., 2017; Seiffert et al., 2017).

In this study, we detected two ST131 isolates. *E. coli* ST-131 are frequently associated with human clinical infections (Nicolas-Chanoine et al., 2014), in particular those depicting the *fim*H type H30 and harboring CTX-M-15 or CTX-M-27 (Nicolas-Chanoine et al., 2014; Stoesser et al., 2016). EDCC5529 depicted the fimH41 *fim*H-type and harbored $bla_{CTX-M-15}$. EDCC5535 depicted a fimH30 *fim*H type and characteristic properties of the ST-131 C1-M27 clade (Matsumura et al., 2016): $bla_{CTX-M-27}$, the GyrA S83L/D87N and ParC S80I/E84V mutations leading to fluoroquinolone resistance and the M27PP1 phage. Therefore, it is a member of the C1-M27 clade usually associated with human isolates (Matsumura et al., 2016; Ghosh et al., 2017). Thus, EDCC5535 might have originated from human sources.

Deeper Analysis of *Escherichia coli* ST-949 Isolates

The most common ST within the ESBL *E. coli* was ST-949 (**Table 2** and **Figure 2**). Therefore, we analyzed these isolates in more detail. *E. coli* ST-949 is known to be associated with carbapenem-resistance (Potron et al., 2017; Potel et al., 2018) or ETEC pathotypes (Oh et al., 2014). They have been isolated from environmental samples (water samples) collected in Sweden, where the authors could show that the water isolates were highly related with isolates derived from a hospital that was adjoining the water source (Fagerström et al., 2019).

Because *E. coli* ST-949 are known to be pathogenic (Oh et al., 2014), we analyzed all available *E. coli* ST-949 isolates for the presence of virulence genes. The ST-949 isolates from this study harbored only ExPEC virulence genes (e.g., iron acquisition genes, Enterobactin, **Supplementary Figure 2**). The isolates detected in New Zealand and Sweden harbored the same sets of virulence genes. Other ST-949 harbored also toxins (Shigatoxin) and hemolysins indicating that ST-949 isolates differ widely in their virulence capabilities.

A whole-genome-based analysis of the *E. coli* ST-949 isolates from this study and those from Enterobase revealed two different findings (**Figure 3** and **Supplementary Table 1**): Firstly, ST-949 isolates are divided into two different clusters. Cluster A (including our isolates) consists of isolates found in water,

TABLE 1 | Environmental data and enumeration results of sampling sites with ESBL-positive samples.

Sampling site #	Isolate #	Species	Site	*E. coli** [CFU/100 ml]	*Enterobacter** [CFU/100 ml]	Temperature air [°C]	Temperature water [°C]	Sampling date	Sampling time
1	EDCC5518	*Escherichia coli*	Bathing lake	77	15	21	20	19.06.18	09:20
	EDCC5519	*Escherichia coli*							
	EDCC5520	*Escherichia coli*							
	EDCC5522	*Escherichia coli*		15	46	23	24	17.07.18	09:00
2	EDCC5521	*Escherichia coli*	Bathing lake	<15	15	23	24	16.07.18	09:30
6	EDCC5523	*Escherichia coli*	Bathing lake	109	161	28	27	07.08.18	10:00
	EDCC5524	*Escherichia coli*							
10	EDCC5525	*Escherichia coli*	River	<15	<15	27	21	08.08.18	9:41
11	EDCC5526	*Escherichia coli*	River	30	<15	28	22	08.08.18	10:21
13	EDCC5527	*Escherichia coli*	River	1,509	144	27	19	08.08.18	11:38
14	EDCC5528	*Escherichia coli*	River	1,749	94	25	19	08.08.18	12:09
	EDCC5529	*Escherichia coli*							
	EDCC5530	*Escherichia coli*							
15	EDCC5531	*Escherichia coli*	River	5,352	640	28	23	08.08.18	12:31
16	EDCC5532	*Escherichia coli*	River	1,931	197	32	23	08.08.18	12:58
	EDCC5533	*Escherichia coli*		1,931	197	32	23	08.08.18	12:58
17	EDCC5534	*Escherichia coli*	River	110	<15	32	24	08.08.18	13:36
	EDCC5535	*Escherichia coli*		110	<15	32	24	08.08.18	13:36
19	EDCC5536	*Escherichia coli*	River	77	<15	32	24	08.08.18	15:53
	EDCC5537	*Escherichia coli*							
	EDCC5538	*Escherichia coli*							

**Enumeration performed based on DIN EN ISO 9308-2 (K6-1) 07-2014 regulation; EDCC, ED culture collection.*

TABLE 2 | Results of the genome-based analysis of the ESBL-producing *E. coli* isolates.

Isolate	ST	Aminoglycoside	Beta-lactam	Macrolide	Phenicol	Quinolone	Sulfonamide	Tetracycline	Trimethoprim	*fimH* type	Plasmid incompatibility groups
EDCC5518	949		$bla_{CTX-M-15}$			*qnrS1*				H121	IncI1 and p0111
EDCC5519	949		$bla_{CTX-M-15}$			*qnrS1*				H121	IncI1
EDCC5520	949		$bla_{CTX-M-15}$			*qnrS1*				H121	IncI1 and p0111
EDCC5521	3,246		$bla_{CTX-M-1}$							H65	IncFIA, IncI1, IncFIB (AP00 1918), IncFII(29), and ColRNAI
EDCC5522	949		$bla_{CTX-M-15}$			*qnrS1*				H121	IncI1 and IncA/C2
EDCC5523	949		$bla_{CTX-M-15}$			*qnrS1*				H121	IncI1
EDCC5524	949		$bla_{CTX-M-15}$			*qnrS1*				H121	IncI1
EDCC5525	949		$bla_{CTX-M-15}$			*qnrS1*				H121	IncI1
EDCC5526	949		$bla_{CTX-M-15}$			*qnrS1*				H121	IncI1
EDCC5527	1,490	*aadA1*	$bla_{CTX-M-15}$			*qnrS1*			*dfrA1*	H25	IncFII, IncFIB (AP00 1918), IncFII (pCoo), and IncB/O/K/Z
EDCC5528	2,797		$bla_{CTX-M-15}$			*qnrS1*				H54	IncFII (pHN7A8) and IncB/O/K/Z
EDCC5529	131	*strA, strB*	$bla_{CTX-M-15}$, bla_{TEM-1B}	*mph(A)*			*sul2*	*tet(A)*		H41	IncFII(29), IncFIB (AP00 1918), and Col156
EDCC5530	155	*aadA5*	$bla_{CTX-M-1}$				*sul2*		*dfrA17*	N.D.	IncI1 and IncFII(pCoo)

(Continued)

TABLE 2 | Continued

Isolate	ST	Aminoglycoside	Beta-lactam	Macrolide	Phenicol	Quinolone	Sulfonamide	Tetracycline	Trimethoprim	*fimH* type	Plasmid incompatibility groups
EDCC5531	226	*aadA1*	$bla_{CTX-M-15}$, bla_{OXA-1}		*catA1*			*tet(B)*		H41	IncFII, ColRNAI, and Col(MG828)
EDCC5532	949		$bla_{CTX-M-15}$			*qnrS1*				H121	IncI1
EDCC5533	949		$bla_{CTX-M-15}$			*qnrS1*				H121	IncI1, Col8282, ColRNAI, Col156, and Col(MG828)
EDCC5534	949		$bla_{CTX-M-15}$			*qnrS1*				H121	IncI1
EDCC5535	131		$bla_{CTX-M-27}$							H30	IncFII (pRSB107), IncFIA, IncFIB(AP001918), Col8282, Col156, and Col(MG828)
EDCC5536	295	*aadA5*	$bla_{CTX-M-27}$, bla_{TEM-1B}	*mph(A)*			*sul1*		*dfrA17*	H54	IncFII (pRSB107), IncFIB (AP001918), IncFII (pCoo), IncY, and ColRNAI
EDCC5537	1,431	*aadA1, aadA2, strA, strB*	$bla_{CTX-M-15}$, bla_{TEM-1B}		*cmlA1*	*qnrS1*	*sul2, sul3*		*dfrA12*	H32	IncI1, IncX1, IncY, and Col156
EDCC5538	1,431	*aadA1,aadA2, strA, strB*	$bla_{CTX-M-15}$, bla_{TEM-1B}		*cmlA1*	*qnrS1*	*sul2, sul3*		*dfrA12*	H32	IncI1, IncX1, IncY, and Col156

EDCC, ED culture collection; N.D., not detected.

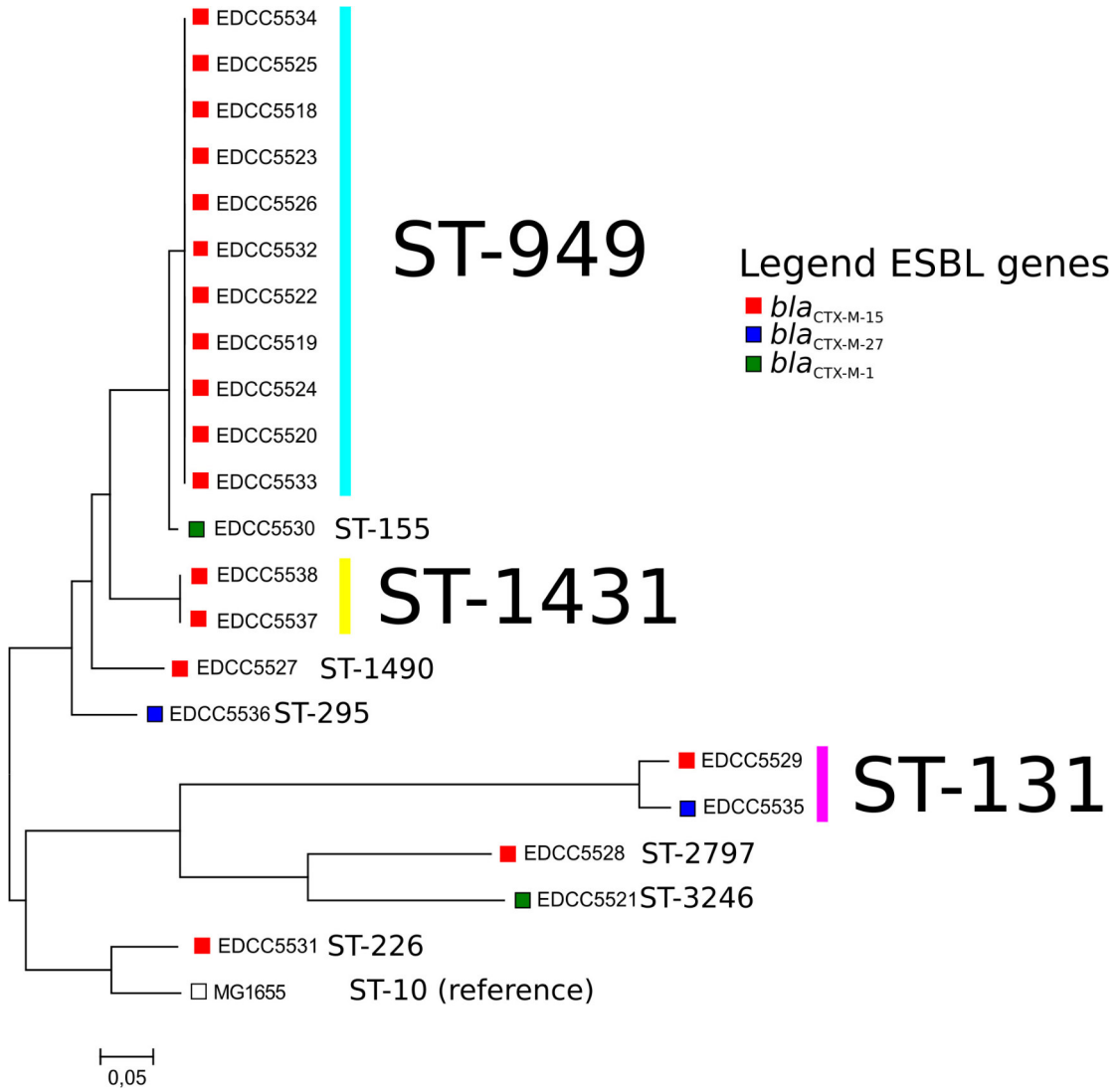

FIGURE 2 | Core-genome-based comparison of the *E. coli* isolates analyzed in this study. Boxes depict the ESBL gene present in these isolates. For a representation of the isolate geographical location and the phylogeny, please see the following link: https://microreact.org/project/bELapwXL1Fi8ZdznzZHF1H.

human, and livestock samples, while cluster B includes also isolates from companion animals. Secondly, only cluster A isolates contain CTX-M-15, while Cluster B isolates harbor either CTX-M alleles other than CTX-M-15, or AmpC beta-lactamases. Cluster B harbored two ST-949 isolates from Germany from livestock and an unknown source.

The *E. coli* ST-949 isolates from this study (Cluster A, n = 11) were highly related to ST-949 isolates found in New Zealand (ESC_FB9670AA_AS, human isolate) and Sweden (n = 4, water and human isolates, **Figure 3**). All these 16 isolates harbored a complex antibiotic resistance gene region including not only the $bla_{CTX-M-15}$ gene, but also the fluoroquinolone resistance gene *qnrS1* and several different insertion sequences (**Supplementary Figure 3**). The antibiotic resistance region was inserted in the chromosome at an identical location in the isolates from this study and the isolates from New Zealand, while the isolates from Sweden harbored the identical region inserted at a different location of the chromosome. This finding indicates that the acquisition of $bla_{CTX-M-15}$ in the two different clones was presumably from a different source and was independent in both clones.

The epidemiological link between Germany and New Zealand is not clear. It may indicate that the ST-949 clone found in Germany is present worldwide, but this is only an assumption as the total number of ST-949 isolates throughout the world is very low. It remains to be clarified whether ST-949 is an emerging ST and whether it is present in other sources.

The epidemiological link between ST-949 isolates from our study is partly explainable. All ST-949 river isolates originate from the same river (sampling sites 10, 11, 16, and 17), indicating a common source of contamination. Possible sources

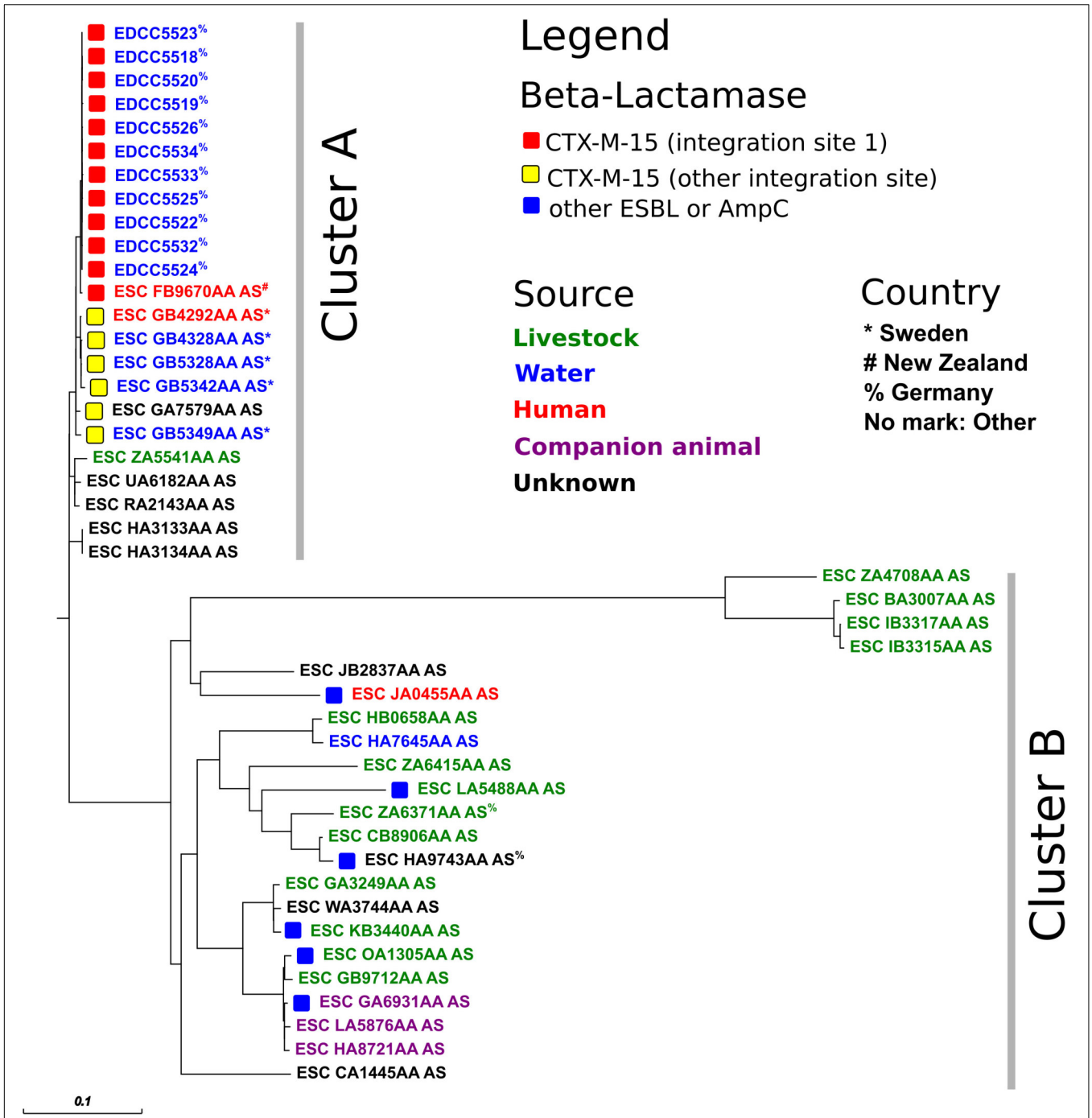

FIGURE 3 | Core-genome-based phylogenetic tree of *E. coli* ST-949 isolates from this study and Enterobase (as of 8th June 2020). For a representation of the isolate sampling site, please see **Supplementary Figure 1**.

of contamination along the river might be either agriculture, two large university hospitals whose cleared wastewater end up in the river itself or human influence through tourism, as the river is frequently used for recreational purposes. Sampling site 6 is located close to the sampled river, indicating a possible contamination through the river by flooding. What is not completely clear, is the epidemiological link between sampling site 1 and the other sites. They are not interconnected by any water flows. A possible connection between those might have been movement of humans or animals (in particular birds).

ST-949 isolates have never been reported in Germany. Therefore, this is the first study detecting ST-949 *E. coli* in Germany. Its predominance in our study indicates that either ST-949 *E. coli* might resemble *E. coli* isolates only present in water

sources or a new emerging multilocus ST in Germany. To prove this hypothesis, more studies are required.

CONCLUSION

In this study, we characterized ESBL-producing *E. coli* isolates from water samples. Our results show that the main MLST type is ST-949, reported in only a few number of very recent publications. In addition, it has been associated with human disease. This indicates that it might be an emerging ST with human pathogenic potential that could spread through water sources.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA656216.

AUTHOR CONTRIBUTIONS

ED designed the study. AN and SH collected samples and data. ED performed antibiotic resistance determination. LF, AN, JF, and ED analyzed the data. LF, AN, and ED wrote the manuscript that was critically reviewed and approved by all authors.

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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.2021.617349/full#supplementary-material

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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.

Anhang B

Ayeni FA, Falgenhauer J, Schmiedel J, Schwengers O, Chakraborty T, **Falgenhauer L**. 2020. Detection of *bla*$_{\text{CTX-M-27}}$-encoding *Escherichia coli* ST206 in Nigerian poultry stocks. J. Antimicrob. Chemother. 75, 3070–3072.

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Detection of *bla*$_{CTX-M-27}$-encoding *Escherichia coli* ST206 in Nigerian poultry stocks

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Sir,

Antimicrobial-resistant Gram-negative bacteria are an emerging problem worldwide. ESBL producers, especially those harbouring *bla*$_{CTX-M}$ genes, have been detected in increasing numbers worldwide, including on the African continent.[1] In Nigeria, there is little information on ESBL-producing *Escherichia coli* in poultry farms with respect to their phylogenetic relationships. Here, we characterized isolates collected from faecal samples of healthy chicken from 11 small commercial poultry farms in five states in Southwestern Nigeria using phenotypic and genotypic methods [see Supplementary Materials and methods and Tables S1 and S2 (available as Supplementary data at *JAC* Online)]. Ethics approval was not needed because faecal samples were collected in a random sampling exercise and not from individual birds.

Samples were collected between September 2015 and February 2016 ($n = 240$). ESBL-producing *E. coli* isolates ($n = 52$) were detected in 21.6% of the samples and in 90% of the farms ($n = 11$). All isolates were susceptible to fosfomycin, glycylcyclines and carbapenems (Figure S1). High resistance rates to aminoglycosides (96.2%) and trimethoprim/sulfamethoxazole (80.8%) were detected. Resistance to quinolones differed for the two compounds tested (ciprofloxacin 65.4%, moxifloxacin 96.2%).

One isolate (fuy0210) did not harbour any ESBL genes and was excluded from further analysis. A total of seven different *E. coli* MLST types were found (Figure S2a; Table S3). The most prevalent ST was ST206 ($n = 35$, 68.6%), followed by ST10 ($n = 5$, 9.8%) and ST3580 ($n = 3$, 5.9%). ST206, ST155 and ST226 were detected in multiple farms (Figure S2b).

Only two different ESBL alleles were detected (Table S3): *bla*$_{CTX-M-27}$ (37/51) and *bla*$_{CTX-M-15}$ (15/51). One isolate harboured two ESBL alleles (fuy0232). CTX-M-15 was present in different STs (Figure S2) and is frequently detected in African isolates from different sources, indicating its circulation through transferable plasmids and/or as bacterial clones.[2–4]

CTX-M-27 was almost exclusively found in ST206 isolates. CTX-M-27-producing *E. coli* have been detected previously in only two other studies performed on the African continent, including one study from Nigeria.[5,6] CTX-M-27 is an CTX-M-14 variant with a broader substrate spectrum and its increased presence, particularly in isolates from Japan and Europe, suggest a recent shift in the epidemiology of CTX-M enzymes.[1,7]

As *bla*$_{CTX-M-27}$-encoding *E. coli* ST206 isolates have not been reported before (to our knowledge), we performed an in-depth analysis of them. All isolates harboured a chromosomal insertion of *bla*$_{CTX-M-27}$ (insertion site pseudogene *yneO*, reference genome *E. coli* MG1655). It was located within a region harbouring a complex antibiotic resistance element (Figure S3) and was flanked by 5 bp DRs (AGTAA).

In addition to *bla*$_{CTX-M-27}$, the Nigerian isolates also harboured a core set of genes conferring resistance to aminoglycosides [*aac(3)-IIa*, *strA*, *strB*], tetracycline [*tet*(A)], sulphonamides [*sul2* (Table S4)], fluoroquinolones (*qnrS1*) and the β-lactamase *bla*$_{TEM-215}$ on an IncN plasmid of the pMLST type ST3 (Figure S4). This plasmid was conserved in all isolates except fuy0057, which carried a deletion of the region including *qnrS1* and *bla*$_{TEM-215}$. TEM-215 has been reported only once previously.[8]

Two subclones were identified (Figure 1; Tables S4, S5; Figure S4). Subclone 1 harboured the above-mentioned antibiotic resistance genes and plasmids while subclone 2 had an additional IncHI2/IncQ1 hybrid plasmid with the pMLST type ST4 and 12 to 14 additional antibiotic resistance genes (e.g. *aadA1*, *dfrA12*, *mphE* and *sul1*).

The *E. coli* ST206/*bla*$_{CTX-M-27}$ isolates were closely related, exhibiting an average difference of 36 SNPs (Table S6). The number of SNPs was independent of the sampling site, date of isolation and the subclone (Table S6) indicating that the subclones were generated by the acquisition of different plasmids, rather than changes within the chromosome. Moreover, both subclones were present in 4/9 farms harbouring *E. coli* ST206/*bla*$_{CTX-M-15}$ (Figure S5). These findings suggest the spread of *E. coli* ST206/*bla*$_{CTX-M-27}$ isolates from a common source (e.g. hatchery) to the different farms and indicate the presence of both subclones in this common source.

To set these results into a global perspective and to estimate possible public health consequences thereof, genomes of 222 publicly available *E. coli* ST206 isolates were analysed for the presence of *bla*$_{CTX-M-27}$ (Table S7).[9] Only 5/222 isolates harboured *bla*$_{CTX-M-27}$ (Table S8). These were isolated in Vietnam but were more distantly related (4292–4293 SNPs, Table S8) and had a different chromosomal location of *bla*$_{CTX-M-27}$. One of these isolates originated from a human sample.

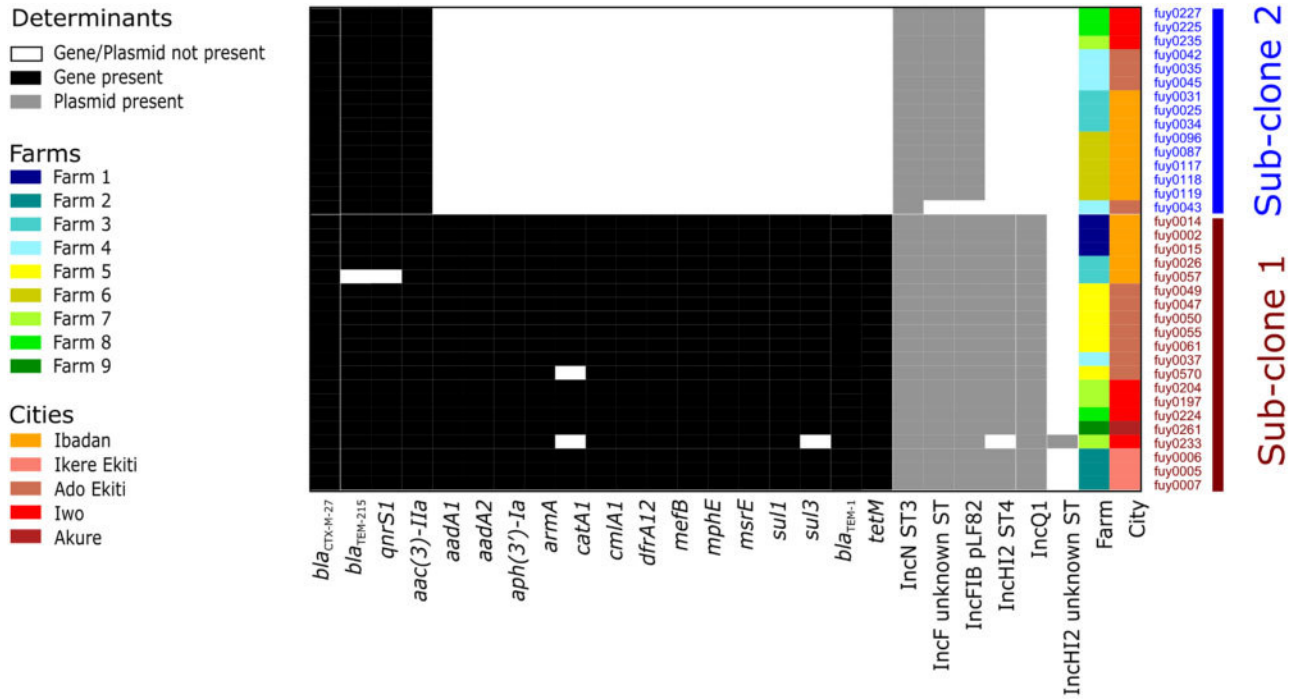

Figure 1. Heat map depicting presence/absence of antibiotic resistance genes and plasmid replicons in Nigerian *E. coli* ST206 isolates. Not depicted are genes present in all isolates [*strA*, *strB*, *sul2* and *tet*(A)]. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

The closest related African *E. coli* ST206 isolate was an avian isolate detected in Kenya (ESC_MA8301AA, 1893 SNPs, Table S7). The closest Nigerian *E. coli* ST206 isolates differed from the clones described here by 3033 and 3057 SNPs, indicating an even more distant relationship (ESC_OA8177AA, human; ESC_OA7828AA, poultry litter, Table S7).

The presence of ST206 *E. coli* isolates in animal and human sources (EnteroBase, Table S7)[9] indicates their potential for dissemination and persistence in different environments. Two human samples were associated with disease, indicating a pathogenic potential of these isolates.

In conclusion, to our knowledge, we report on the first detection of *E. coli* ST206/$bla_{CTX\text{-}M\text{-}27}$-encoding isolates in livestock. Further studies are warranted to determine whether they have already spread to the human population.

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Transparency declarations

None to declare.

Author contributions

F.A.A., T.C. and L.F. conceived the study. F.A.A. performed the formal analysis, supervision and project administration and also performed sampling, isolation and phenotypical characterization of isolates. F.A.A., J.F., O.S. and L.F. performed bioinformatical analyses. J.F., O.S. and L.F. analysed the plasmids. F.A.A., J.S., L.F. and T.C. analysed the data. J.F., J.S. and L.F. created the figures. F.A.A., L.F. and T.C. wrote the manuscript, which all authors approved.

Supplementary data

Supplementary data including Materials and methods, Tables S1 to S8 and Figures S1 to S5 are available as Supplementary data at *JAC* Online.

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Phenotypic and genotypic analysis of KPC-51 and KPC-52, two novel KPC-2 variants conferring resistance to ceftazidime/avibactam in the KPC-producing *Klebsiella pneumoniae* ST11 clone background

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Sir,

Ceftazidime/avibactam has been considered a promising β-lactam/β-lactamase inhibitor combination with activity against serine β-lactamases, including *Klebsiella pneumoniae* carbapenemases (KPCs). Despite limited use of ceftazidime/avibactam on a worldwide scale, ceftazidime/avibactam resistance has been reported in a patient with no history of ceftazidime/avibactam therapy[1] and in patients after short periods of ceftazidime/avibactam exposure.[2] In a recent study, we described our experience with ceftazidime/avibactam at the China-Japan Friendship Hospital in treating nine lung transplant patients with KPC-producing *K. pneumoniae* (KPC-Kp) infections.[3] Although ceftazidime/avibactam treatment for KPC-Kp infection in lung transplant patients is associated with high rates of clinical success, survival and safety, after 13–22 days of treatment, ceftazidime/avibactam resistance was found in the isolates from four out of the nine patients. Demographic and antimicrobial therapeutic details of the

Anhang C

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Changing epidemiology of vancomycin-resistant *Enterococcus faecium*: Results of a genome-based study at a regional neurological acute hospital with intensive care and early rehabilitation treatment

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SUMMARY

Background: Vancomycin-resistant *Enterococcus faecium* (VREfm) are an emerging threat worldwide. In Germany, a VRE-belt with higher VREfm prevalences transversing its central east-west axis and including the state of Hesse was previously described. Recently, we detected a predominant VREfm clone in hospitals throughout the Rhine-Main metropolitan area of Hesse.
Aim: Here we expanded our study on VREfm to a regional neurological acute hospital outside of the metropolitan area with patient referrals from throughout Hesse and the neighboring federal state of Rhineland-Palatinate.
Material/Methods: VREfm isolates obtained between 2016-2018 from a regional neurological acute hospital with intensive care and early rehabilitation units were investigated (n=55). Patient data was collected and analyzed together with whole-genome sequencing data to investigate antibiotic resistance and virulence determinants of the VREfm. The population structure of VREfm was investigated using the Core genome-based multilocus sequence typing (cgMLST).

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Findings: The average age of the patients was 67.1 years. For 96% of the patients, a previous hospital stay was reported. 64% of the patients were treated with antibiotics. All VREfm harbored the *vanB* vancomycin resistance gene. The multilocus sequence types (STs) detected changed abruptly from four different STs in 2016 to a predominant ST in 2017 and 2018 (ST117). Most of the ST117 isolates were members of the cgMLST type CT71.

Conclusion: The results indicate a sudden shift of the VREfm population structure from a semi-heterogeneous population to a pre-dominant clone within an interval of two years. Further investigations are warranted to understand the epidemiology and emergence of this clone.

Introduction

Vancomycin-resistant Enterococci (VRE) are an increasing problem in hospitals in many European countries, as well as in Germany [1—6]. In 2017, WHO has listed vancomycin-resistant *Enterococcus faecium* (VREfm) as a pathogen with high priority in its global priority list of antibiotic-resistant bacteria, emphasizing the paucity of available and effective treatment options. The clinical outcome of patients with VRE infections is significantly poorer [7,8] when compared to infections with non-resistant enterococci, with increased costs for treatment of hospitalized infections [9].

The population structure of VRE-associated infections has changed in recent years in Germany. The German National Reference Center for Staphylococci and Enterococci reported a shift in the predominance of the VRE species from *Enterococcus faecalis* to *E. faecium* and from *vanA* to *vanB*. In addition, the rate of *E. faecium* isolates resistant to vancomycin (VREfm) is increasing [10—14]. This increase has been particularly "dominant" in the central states of Germany, designated the "VRE-belt" [15]. Such an emergence of VREfm could coincide with an expansion of a specific lineage.

This hypothesis has been addressed by studies from some areas within the VRE-belt. In a study at an acute care hospital Weber *et al.* reported recent emergence of the VREfm multilocus sequence type ST117 almost entirely associated with the cgMLST type CT71 [16]. A genome-based analysis of entry screening VREfm specimens collected from 17 hospitals in 2017/2018 and covering a catchment area of 5,000 km^2 and three million inhabitants in the Rhine-Main metropolitan region, also revealed near ubiquitous presence of clonal ST117/CT71 isolates [17]. A predominance of this VREfm type had not been reported until that time.

In the present manuscript, we have extended our studies to include data from a regional referral neurological hospital with units for intensive care and early rehabilitation. Such institutions receive severely debilitated patients with neurological symptoms and provide continued intensive and intermediate care treatment, for in-house patients, and those patients from other acute-care hospitals who no longer require specialized interventions. Clinical specimens were collected from admission-based- and routine-screening from patients admitted from throughout the state of Hesse. Isolates from between 2016 to 2018 were examined to enable a study within a broader geographical range and time.

Materials and methods

Sample inclusion

The regional neurological hospital is located in Weilmünster (50.4310° N, 8.3784° E), in central Hesse, outside of the Rhine-Main metropolitan region. The recruiting area of patient referrals covers the entire State (size: 21.115 km^2) and the neighboring federal state of Rhineland-Palatinate.

All patients, newly admitted to either a neurological intensive care- or early rehabilitation-unit, were screened for VRE on admission ("screening <48h after admission"). Pre-selection of patients for <48h screening based on risk factors was not performed. Additional screening was performed, whenever patients were transferred between wards and when the initial screening had either not been carried out, was not recent, or when a patient had been previously housed in a room with other patients ("screening >48h after admission"). Data on gender, age, major underlying diseases, place of residence, previous hospital stays, and previous antimicrobial therapies were derived from patient's health records.

Isolate characterization

The collection of VRE isolates was performed from patients in the early neurological rehabilitation unit as well as the intensive care unit between January 2016 and December 2018. Colonies were identified using selective media and species determination made using MALDI/TOF. A random selection of these VRE isolates (n=55) was subjected to whole genome sequencing as reported before [17]. Sequencing was performed on an Illumina NextSeq to obtain an average read length of 125 nt and an average coverage of 206x. Quality control and assembly of the raw sequencing data was performed using ASA^{3}P [18]. Resistance gene prediction and multilocus sequence typing was performed applying goseqit (https://www.goseqit.com/). Further differentiation of the ST117 isolates was performed using a core-genome MLST (cgMLST) typing (Ridom SeqSphere+ v. 5.1.0., Ridom GmbH, Münster, Germany; *Enterococcus faecium* scheme developed by de Been *et al.* [19]). Comparative analysis with a representative isolate of the previous study on VREfm was performed using the isolate VRE-11-02-s and the Ridom software [17].

The raw sequencing data is available in the SRA archive, under the project number PRJNA631114.

Statistical analysis

Statistical analysis was performed using the Fisher's exact test.

Results

Patients' characteristics

Within the study period (January 2016—December 2018), patient screening for the presence of VRE on admission (<48h hospital stay) or upon ward transfer (>48h hospital stay) was performed.

The average age of the 55 patients included was 67.1 years with a range of 33—87 years. Fifty-five percent of the patients were male. Sixty-four percent of the participants lived in the region of the MDRO network Rhine-Main (Frankfurt am Main included). The remaining patients lived either outside the Rhine-Main-region of Hesse (n = 17) or in the neighboring federal state of Rhineland-Palatinate (n = 3, Table I, Table A1). Ninety-six percent of the patients had previously been treated at other hospitals including those within the city of Frankfurt/Main (n = 33). Nine of the 55 patients were treated in the Rhine-Main region excluding the city of Frankfurt/Main, while ten patients were from outside the Rhine-Main-region, and one patient from Rhineland-Palatinate. For 4% of the participants, no previous hospital stay was reported. For 64% of the participants, previous antimicrobial therapy had been reported, most often with Piperacillin/Tazobactam (35%), followed by Carbapenems (29%), and Vancomycin (27%) (Table I).

Isolate characteristics

A random selection of the collected VREfm isolates was sequenced (n=55) to enable a genome-based epidemiological analysis of these VRE. Fifty-five *E. faecium* VRE isolates from 2016-2018 were analyzed (2016: n=11; 2017: n=34; 2018: n=10).

All isolates were derived from screening samples (combined nasal/rectal swabs, n=51; tracheal secretion, n=1, urine, n=2, unknown: n=1). Thirty-four isolates derived from screenings performed <48h hours after admission, while 21 isolates derived from screenings >48h after admission. All these isolates encoded the vancomycin resistance determinant *vanB*.

Table I
Participants' and isolate data

		2016—2018	2016	2017	2018
Year of collection	Patients per year	55	11	34	10
Sex	male	30	7	16	7
	female	25	4	18	3
Age (year)	X ± sdev	67.1 ± 12.8	71.3 ± 13.5	65.9 ± 12.9	66.0 ± 10.6
	min - max	33—87	39—86	33—87	46—82
Place of residence	Frankfurt am Main (Region 1)	14	2	10	2
	Rhine-Main-region without Frankfurt (Region 2)	21	5	12	4
	Hessen without Rhine-Main and Frankfurt (Region 3)	17	2	12	3
	Rhineland-Palatinate (Region 4)	3	2	0	1
Place of previous hospital stay	Frankfurt am Main (Region 1)	33	6	20	7
	Rhine-Main-region without Frankfurt (Region 2)	9	4	4	1
	Hessen without Rhine-Main and Frankfurt (Region 3)	10	0	9	1
	Rhineland-Palatinate (Region 4)	1	1		
	no previous hospital stays reported	2	0	1	1
Previous antimicrobial therapy	Vancomycin	15	2	8	5
	Piperacillin/Tazobactam	19	3	10	6
	Carbapenems	16	3	8	5
	other	19	7	11	1
	not reported	20	4	15	1
Sequence type	ST117	51	7	34	10
	NEW	1	1	0	0
	ST18	2	2	0	0
	ST262	1	1	0	0
Sequence type/cgMLST combination	ST NEW, CT NEW	1	1		
	ST117, CT NEW	3	1	1	1
	ST117, CT36	3	3		
	ST117, CT71	45	3	33	9
	ST18, CT NEW	2	2		
	ST262, CT1815	1	1		

Only four MLST sequence types (STs) were detected, with a significant year-wise trend in both screening settings (<48h, >48h) (Figure 1). In 2016, four different STs were detected with a predominance of ST117 (n=7) followed by ST18 isolates (n=2), together with two individual isolates, one exhibiting ST262 and the other a new MLST type (Figures 1 and 2). In 2017 and 2018, only ST117 isolates were detected (2017: n=34; 2018: n=10). Among the ST117 isolates, a significant year-wise trend to the cgMLST type CT71 was detected: In 2016, CT36 and CT71 were found in equal numbers (n=3 each), while in 2017 and 2018 CT36 disappeared and CT71 was detected predominantly (Figure 2).

All of the ST117 isolates (n=51) analyzed harbored a chromosomal insertion of the *vanB* operon. The location of the insertion was dependent on the cgMLST type. In CT71 isolates, the insertion was in an hypothetical gene HMPREF0351_10592, as reported earlier [20], while the CT36 isolates carried an insertion into the *ara*A locus, encoding a putative L-arabinose isomerase (Table II). Forty-nine ST117 isolates (96%) harbored the virulence genes *hylEfm*, *efaAfm* and *acm* (Table II).

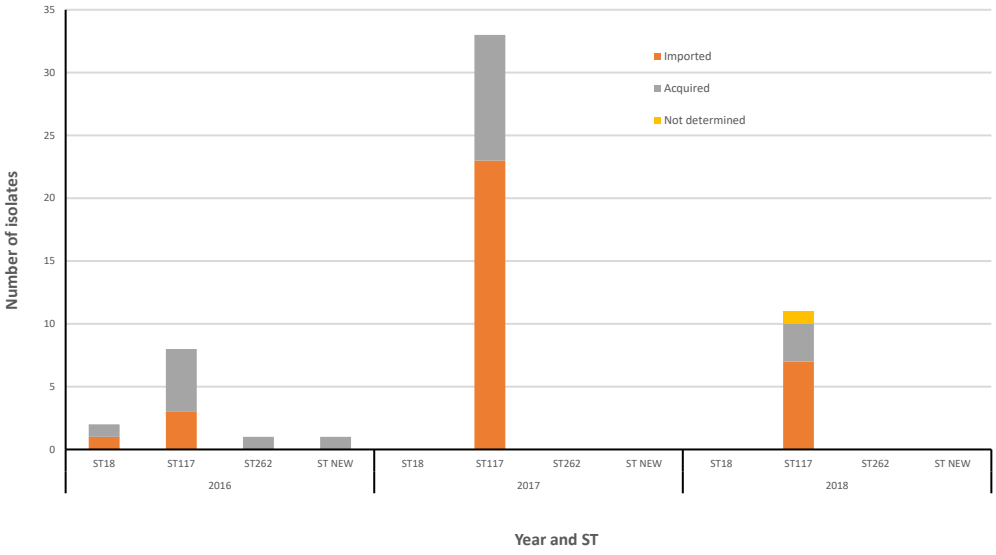

Figure 1. Year-wise depiction of detected STs.

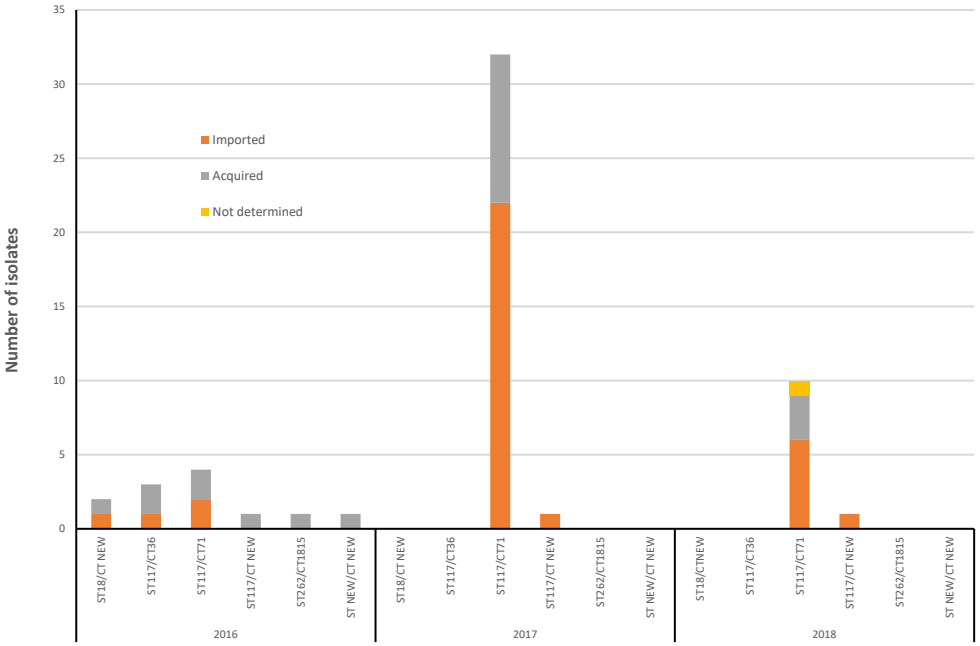

Figure 2. Year-wise depiction of ST/CT combinations detected.

Table II
Isolate characteristics. High relatedness to the Rhine-Main clone indicates a cgMLST allele difference of ≤10 cgMLST alleles

ID	MLST	CT	*van* allele	Acquired/ Imported	Integration site of Van*	virulence genes	Cluster	Highly related to Rhine-Main clone
ING-1	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	5	no
ING-10	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	none	no
ING-11	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	1	yes
ING-12	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	5	no
ING-13	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	1	yes
ING-14	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	3	no
ING-15	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	2	no
ING-16	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	1	yes
ING-17	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	1	yes
ING-18	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	none	no
ING-19	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	1	yes
ING-2	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	1	yes
ING-20	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	none	no
ING-22	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	none	no
ING-23	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	1	yes
ING-24	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	1	yes
ING-25	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	2	no
ING-26	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	1	yes
ING-27	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	1	yes
ING-28	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	1	yes
ING-29	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	1	yes
ING-3	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	1	yes
ING-30	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	2	no
ING-31	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	1	yes
ING-32	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	1	yes
ING-34	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	2	no
ING-35	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	none	no
ING-36	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	none	no
ING-37	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	none	no
ING-38	18	NEW	*vanB*	Imported	ND	*hylEfm, efaAfm, acm*	NA	NA
ING-39	262	1815	*vanB*	Acquired	10592	*efaAfm, acm*	NA	NA
ING-4	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	none	no
ING-40	117	71	*vanB*	Acquired	10592	*efaAfm, acm*	1	yes
ING-41	117	NEW	*vanB*	Acquired	*araA*	*hylEfm, efaAfm, acm*	NA	NA
ING-42	18	NEW	*vanB*	Acquired	ND	*hylEfm, efaAfm, acm*	NA	NA
ING-43	117	36	*vanB*	Acquired	*araA*	*hylEfm, efaAfm, acm*	NA	NA
ING-44	NEW	NEW	*vanB*	Imported	*araA*	*hylEfm, efaAfm, acm*	NA	NA
ING-45	117	36	*vanB*	Imported	*araA*	*efaAfm, acm*	NA	NA
ING-46	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	none	no
ING-47	117	36	*vanB*	Acquired	*araA*	*efaAfm, acm*	NA	NA
ING-48	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	none	no
ING-49	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	4	no
ING-5	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	none	no
ING-50	117	NEW	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	NA	NA
ING-51	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	none	no
ING-52	117	71	*vanB*	Imported	10592	*efaAfm, acm*	none	no
ING-53	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	3	no
ING-54	117	71	*vanB*	Acquired	10592	*hylEfm, efaAfm, acm*	2	no
ING-55	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	none	no
ING-56	117	71	*vanB*	Acquired	10592	*hylEfm, acm, efaAfm*	4	no
ING-57	117	71	*vanB*	not determined	10592	*hylEfm, efaAfm, acm*	2	no
ING-6	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	3	no
ING-7	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	1	yes
ING-8	117	71	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	3	no
ING-9	117	NEW	*vanB*	Imported	10592	*hylEfm, efaAfm, acm*	NA	NA

Statistical analysis on the ST117/CT71 isolates was performed to determine possible correlations of this clone with the place of residence or the year of isolation. It was shown that the presence of ST117/CT71 isolates was dependent on the year of isolation (P-value < 0.001), and not (for example) dependent on the patients' place of residence (P-value $= 0.75$).

Comparative analysis with the common Rhine-Main ST117/CT71 clone

As ST117/CT71/*vanB* isolates were detected in the Rhine-Main area in an earlier study [17], a comparative analysis with a representative isolate from this study was performed. Among the ST117/CT71 isolates, five clusters with <10 cgMLST differences were detected (Figure 3). "Cluster 1" comprises of 15 isolates predominantly isolated in 2017 together with a representative isolate from the earlier Rhine-Main study isolated in 2018 (Figure 3). The cluster harbored isolates from all three regions of Hesse.

Among the lesser-related isolates, four clusters were detected. Cluster 2 (n = 7) and Cluster 3 included isolates from 2018 and 2017, while Cluster 4 included only isolates from 2018. Cluster 5 included two isolates from 2017. These findings indicate that VREfm ST117/CT71/*vanB* from Hesse are constantly evolving.

Discussion

Specialist regional neurological hospitals that have intensive care units and cater for early neurological and neuro-surgical rehabilitations form the bulwark of intermediate and intensive care treatment of patients in Germany. Recent studies have suggested that nearly 25% of all patients entering such facilities are either colonized or infected with multi-drug resistant bacteria [21].

Surveillance studies indicated an increase in VRE from screening- and clinical-samples in the regional neurological specialty hospital [21]. Detailed analysis of isolates using WGS indicated emergence of VREfm MLST type ST117, cgMLST type CT71 with the vancomycin resistance determinant *vanB* (Figure 3). As the neurological hospital with intensive care and early rehabilitation serves the entire federal state of Hesse and neighboring federal state of Rhineland-Palatinate, it was regarded as a representative sentinel point for statewide epidemiology.

Our results reflect the changing VREfm epidemiology in Germany. The national reference center for Staphylococci and

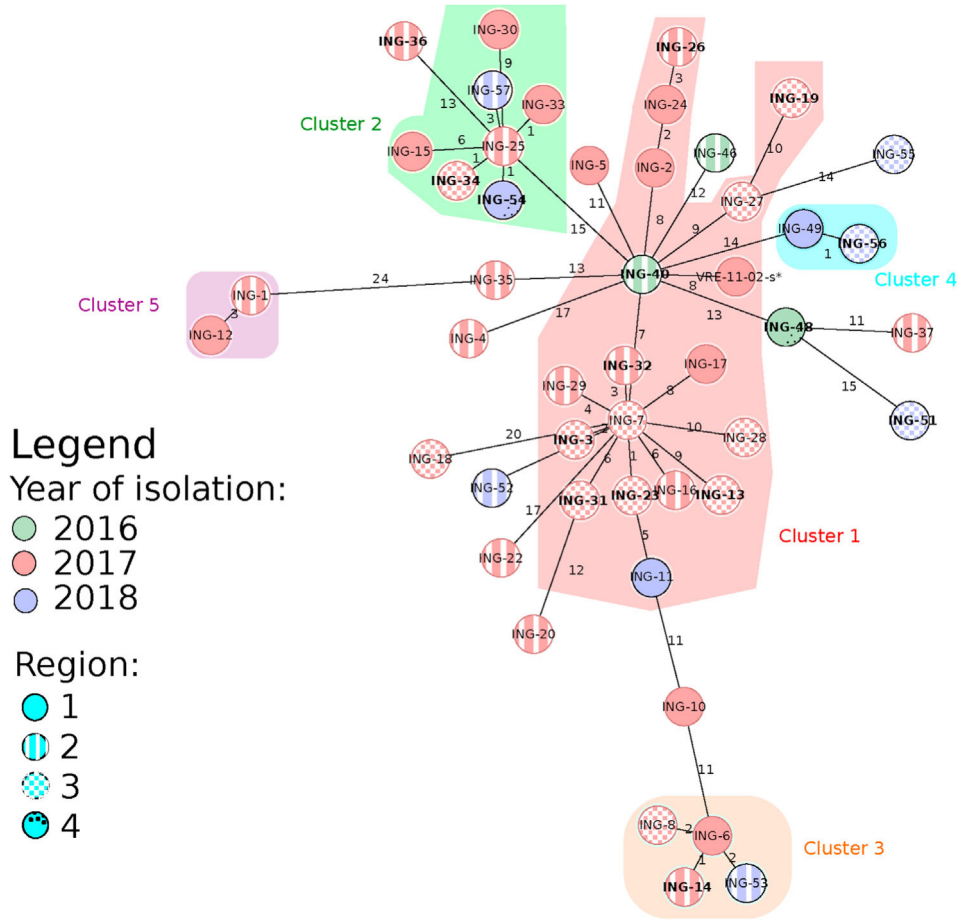

Figure 3. Comparative analysis of VREfm isolates with a representative isolate from the Rhine-Main study (marked with an asterisk). Clusters with a difference of ≤10 cgMLST alleles are marked with shading. Names of those isolates derived from screening performed >48h are marked with bold font.

Enterococci has detected a supra-regional shift in VREfm MLST types, with the emergence of ST117. The total numbers of this ST type have doubled in 2018 as compared to 2016. ST117/CT71 has been detected in ten federal states in Germany [13]. A study performed at a large university hospital in Berlin comprising VREfm isolates from 2008, 2013, 2015 and 2018 [16] has reported a similar shift. From our study here, we document that this shift in the region studied occurred sometime between 2016 and 2017. Unlike the study from Berlin, a shift of *vanA* to *vanB* in the ST117/CT71 isolates was not detected. This could be the result from the shorter period during which the isolates were obtained here. Following our data, VREfm ST117/CT71 replaced other STs and was the predominant clone isolated from the neurological patients of this hospital. This sub-cluster was previously observed in other acute care clinics in the Rhine-Main-region from samples obtained in 2018, where ST117/CT71/*vanB* was detected in patients from intensive care, as well as from hematological and transplantation units [17]. Even though a shift to ST117 could have been expected from previous studies [13,16], the unique predominance of one VREfm sub-cluster type was unexpected.

The predominance of a single sub-cluster type in area with many hospitals is unusual, as VREfm outbreaks are generally oligo- or polyclonal in nature (e.g. Australia [22], UK [23], Germany [24,25]), and clonal outbreaks are only seldom reported (i.e. Turkey [26]). Whenever present, the analysis of the cgMLST types exhibit a broad range of variety of different ST/CT-clones, thus arguing against primary intra-hospital transmission, but rather for the import of new and emerging types [25]. Compared to this diversity, the detection of a single ST117/CT71/*vanB* clone in two independent studies, including in the over 40 isolates in this study which were from patients with geographically distinct places of residence and where previous hospital stays were in institutions from all over Hesse or even in cases without any known previous hospital stay [17], was unexpected and is remarkable. The ubiquitous presence of this clone from widely separated referral institutions clearly suggests inter-hospital spread but could also implicate other hitherto unrecognized vectoral components. It might be hypothesized that this clone has highly adaptive properties for the colonization of the gastrointestinal tract and for effective transmission and persistence stability within the hospital environment. Indeed, ST117/CT71/*vanB* VREfm isolates harbor elements associated with persistence and colonization, such as the collagen-binding gene *acm*, the enterococcal surface protein Esp, required for promoting biofilm formation and the PTS_{clin} phosphotransferase system associated with colonization potential of clinical isolates [27]. Further studies are warranted to understand the impact of these and hitherto undiscovered genes in the distribution and the emergence of the ST117/CT71/*vanB* clone.

Conclusions

The population structure of VREfm at a neurological hospital with intensive care and early rehabilitation changed dramatically within a short time-period. A predominant VREfm type (ST117/CT71/*vanB*) emerged and is currently present throughout Hesse as well as in many regions in Germany. Further studies are needed to understand the epidemiology and emergence of this specific VREfm clone, as well the contribution of other sources (e.g. water, food, animals) to its spread.

Limitations of the study

There are several limitations to our study. Only a representative collection of isolates were sequenced (n=55). The design of the study was retrospective, so that anamnestic data could only be derived from the prior patients' documentation and was not queried individually. While data for sex, age, place of residence and previous hospital stay are correct and complete, data on previous antibiotic therapies may have been less so. A further limitation of the study is that the overall number of isolates examined is relatively small.

Credit author statement

Conceptualization; CB, CI, DM, IP, UH, TC.
Data curation; IP, JF, LF, MF, UH.
Formal analysis; IP, JF, LF, UH, TC.
Funding acquisition; CI, UH, TC.
Investigation; IP, JF, LF, UH.
Methodology; All authors.
Project administration; CB, DM, IP, LF, UH, TC.
Resources; CB, CI, DM, UH, TC.
Software; CI, TC.
Supervision; CB, CI, DM, UH, TC.
Validation; IP, JF, LF, MF, UH.
Visualization; IP, JF, LF, UH.
Roles/Writing — original draft; All authors.
Writing — review & editing: All authors.

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Conflict of interest statement

The authors declare that they have no competing interest.

Ethics approval

The study was approved by the ethics committee of the medical faculty of the Justus Liebig University of Giessen (AZ: 179/16). All samples were taken as part of standard care procedures.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.infpip.2021.100138.

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Anhang D

Falgenhauer L, Fritzenwanker M, Imirzalioglu C, Steul K, Scherer M, Rhine-Main VREfm study group, Heudorf U, Chakraborty T. 2019. Near-ubiquitous presence of a vancomycin-resistant *Enterococcus faecium* ST117/CT71/*vanB* –clone in the Rhine-Main metropolitan area of Germany. Antimicrob. Resist. Infect. Control. 8, 128**.**

Antimicrobial Resistance and Infection Control

SHORT REPORT Open Access

Near-ubiquitous presence of a vancomycin-resistant *Enterococcus faecium* ST117/CT71/*vanB* –clone in the Rhine-Main metropolitan area of Germany

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Abstract

Whole-genome sequencing analysis of Vancomycin-resistant *Enterococcus faecium* isolates from the Frankfurt metropolitan region revealed that 78/94 isolates were MLST type ST117, cgMLST complex type CT71 with a common *vanB* chromosomal insertion site. This indicates circulation of a single VRE clone in a catchment area of 5,000-km^2 with 3 million inhabitants.

Keywords: VRE, ST117, cgMLST, WGS

Background

Vancomycin-resistant *Enterococcus faecium* (VREfm) are an important cause of nosocomial infections worldwide [1]. The WHO ranks VREfm on its high priority list of multidrug-resistant microorganisms because of increasing prevalence and transmission rates in community and healthcare settings [2]. Since 2014 there has been a dramatic increase of VREfm prevalence among clinical samples in Germany. Marked regional differences have been noted, with high VREfm prevalence within an east-west axis in central Germany ("VRE-belt"), that includes the German federal state of Hesse [3, 4].

The presence and impact of epidemic VREfm on individual patients entering the healthcare system particularly within the "VRE-belt" is poorly understood. Here we report on the genome-based analysis and comparison of VREfm isolated from patients with or without a prior history of hospitalization during admission to intensive care units or other wards with patients at risk for VREfm colonization/infection i.e. hemato-oncological and transplantation units.

Sampling area, patient characteristics

Sampling was performed between November 2017 and June 2018 in 17 hospitals within the Frankfurt am Main metropolitan region, all of whom are members of the Network on multidrug-resistant organisms in the Rhine-Main area (MDRO Network Rhine-Main). The size of hospitals varied between 100 and 1488 beds. Among these, 11/17 were tertiary care hospitals, while the remaining six hospitals were either standard care ($n = 3$), general hospitals ($n = 2$) or a specialized clinic ($n = 1$) (Additional file 1: Table S1). Participating hospitals were requested to provide VREfm isolates from samples obtained from patients at admission (within 72 h) to intensive care units or other wards where patients with a high risk for VREfm colonization/infection were treated, i.e. hemato-oncological and transplant units ($n = 85$, anal/rectal swabs, stool specimens). An active admission screening of all patients or of defined risk patients was not performed in this study. Hence, determining the prevalence of VRE carriage at admission was not the purpose of this study. The number of isolates per hospital (Additional file 1: Table S1) was dependent on the

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size of the catchment area of the respective hospital. For hospitals that did not have the requested amount of VREfm-positive screening samples within the study period, VREfm from clinical samples were included ($n = 10$). These comprised of isolates from blood cultures, urine, wound smears, intra-abdominal surgery smears and a central venous catheter isolate. Identification and antibiotic resistance determination of VREfm was performed using standard laboratory methods and technologies (e.g. chromID VRE plates, MALDI MS, VITEK II, BioMérieux, Nürtingen, Germany) in the labs providing regular microbiological service for the participating hospitals.

In total, VREfm isolates from 95 patients were included. Patient meta-data was collected using a questionnaire (Table 1).

Table 1 Depiction of the patient meta-data

Parameter		n	%
Sex	Male	54	56.8
	Female	41	43.2
Age	<60	15	15.8
	60- < 70	21	22.1
	70- < 80	29	30.5
	> 80	29	30.5
	Not reported	1	1.1
Underlying disease	Hemato/oncology	19	20.0
	Cardiology	19	20.0
	Other	36	37.9
	Not reported	21	22.1
Travel abroad during the last 12 months	None	45	47.4
	Yes	8	8.4
	Indeterminate	42	44.2
Previous hospital stays within the last 12 month	No	13	13.6
	Yes	76	80.0
	Not ascertainable	6	6.3
Previous antimicrobial therapy*	Vancomycin	9	9.5
	Teicoplanin	6	6.3
	Piperacillin/Tazobactam	25	26.3
	Carbapenem	19	20.0
	Cephalosporin	20	21.1
	Penicillin	12	12.6
	Metronidazole	12	12.6
	Quinolone	17	17.9
	Treated with antibiotics	72	75.8
	No antibiotic treatment	2	2.1
	Not reported	21	22.1

*multiple answers were possible

The mean age of the patients was 71.2 ± 14.6 years, and ranged from a new-born to 95 years old. Fifty-four patients were male and 41 female. Information regarding a previous hospital stay during the last 12 months was reported in 93.6% (89/95) of the patients. Of these, 85% (76/89) reported a hospital stay. An underlying disease was reported in 77.9%. For 72/95 patients, an antimicrobial therapy during the past 12 months was recorded. Prior treatment with vancomycin was reported in 9.5%. VREfm was a first-time detection in 81 patients. Pre-existing VREfm carriage was known in 14 cases prior to the study.

Characteristics of the VREfm isolates

VREfm were isolated and tested for susceptibility by the participating MDRO Network Rhine-Main centres using standard procedures for clinical diagnostic laboratories. VREfm isolates were generally were ampicillin-, fluoroquinolone- and carbapenem-resistant and linezolid-susceptible.

Whole genome sequencing (WGS) was performed as reported earlier [5, 6]. Resistance gene prediction, and Multilocus sequence typing (MLST) was performed using goseqit tools (https://www.goseqit.com/, Additional file 2: Table S2). Ninety-three VREfm harbored *van*B, and a single isolate harbored *van*A. One isolate did not harbor any *van* gene and was excluded from further analysis.

Analysis of the virulence genes was performed using goseqit tools. The presence of the enterococcal surface protein Esp required for promoting biofilm formation (Additional file 2: Table S2), and the PTS_{clin} phosphotransferase system associated with colonization potential of clinical isolates [7] as well as the uptake and utilization of amino sugars such as β-N-acetylglucosamine commonly found in mucin on the surfaces of epithelial cells and in biofilms were detected using blastn [8]. All *van*-encoding isolates harbored the *efaAfm* gene, suggested to be involved in cell wall adherence, which is concordant with the results from earlier studies [9]. Ninety-two isolates harbored the *hylEfm*, *acm* and PTS_{clin}, while 90/94 isolates carried the *esp* gene.

Almost all isolates (90/94), regardless of source, were ST117 with the remaining four isolates each representing ST80, ST192, ST262 and ST1428. The ST262 isolate harbored a *van*A gene.

The use of MLST-based data to classify VREfm is controversial because of its high recombination rates that masks relatedness of otherwise highly related strains. Therefore, further differentiation of the ST117 isolates using a core-genome MLST (cgMLST) was performed (Ridom SeqSphere+ 5.1.0., Ridom GmbH, Münster, Germany; *Enterococcus faecium* scheme [10]). This analysis revealed that 78/90 (87%) of the ST117 isolates, i.e. from both non-clinical as well as clinical samples, were all members of a single cgMLST complex type (CT71, Fig. 1). Minor CTs detected in ST117 isolates were CT469 ($n = 4$),

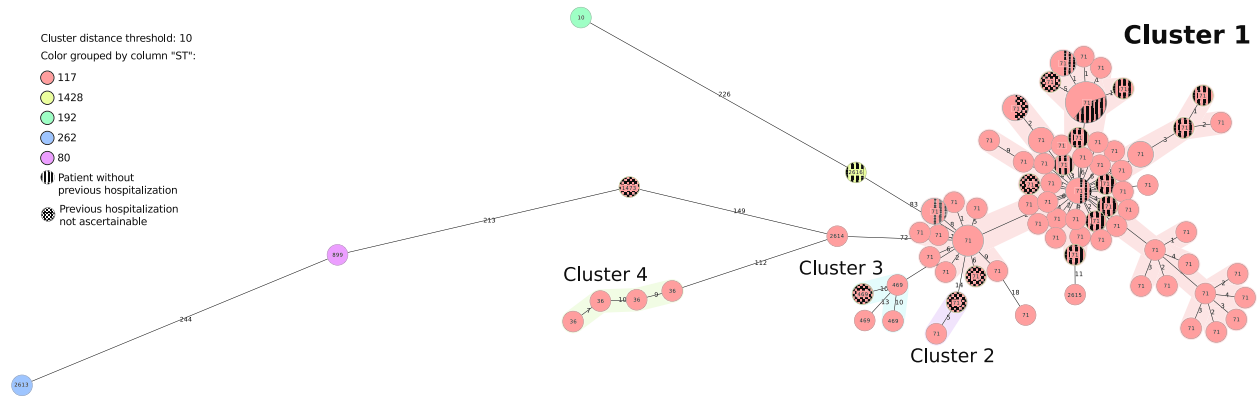

Fig. 1 Minimum spanning tree of the sequenced VREfm isolates based on the cgMLST data. The figure was created according to reference [10]. Cluster distance threshold was set to 10 cgMLST alleles. Size of circles is according to number of isolates. Numbers in the circles indicate the complex type of the respective isolates. Colors of circles indicate multilocus sequence types

CT36 ($n = 4$), CT2614 ($n = 1$), CT2615 ($n = 1$) and CT1473 ($n = 1$).

Of the CT71 isolates, 74/78 clustered into one cgMLST cluster type (Fig. 2, Cluster 1), that exhibited up to 10 cgMLST allele differences. All isolates of Cluster 1 harbored an identical insertion of a *van*B-encoding Tn*1549*-like transposon into a gene of unknown function (HMPREF0351_10592), previously reported for the *van*B-encoding VREfm of the sequence type ST192 [11]. Thus, these isolates constitute a clone, which we designate as the ST117/CT71/*van*B clone.

Statistical analyses using the Mann-Whitney U test [12] did not support an association of the CT71 clone with any of the patient data characteristics detailed in the Table 1 (Additional file 3: Table S3).

Discussion

Surveillance, hygiene/infection control programs and antibiotic stewardship interventions for VREfm have been implemented in healthcare settings throughout Europe [13, 14]. However, the impact of these measures on the VRE influx by individual patients (colonization) entering the healthcare system is poorly understood. Here we characterized VREfm isolated from rectal swabs of patients during admission with and without prior history of hospitalization within the so-called "VRE-belt" in central Germany. Core genome-based phylogenetic analysis classifies all of the VREfm from this study as members of the hospital-associated clade A1 (data not shown) and shows that a single ST117 clonal lineage, with cgMLST complex type CT71 is predominant in the Rhine-Main metropolitan area within a patient population with a high-risk profile for VREfm acquisition. Previous epidemiological data indicated that the emergence of ST117 with its three major CTs, CT36, CT71 and CT469 is recent, and presently accounts for over one-third of all VREfm isolated from bloodstream infections in Germany and the Netherlands [15].

The repeated isolation of a single predominant ST117/CT71/*van*B clone at geographically separated institutions (Additional file 5: Figure S1) throughout the reporting period suggests that it has highly adaptive properties for effective transmission and a capacity for persistence in the hospital environment. Indeed, ST117/CT71/*van*B VREfm isolates harbored elements associated with persistence, such as the collagen-binding *acm* and the enterococcal surface protein Esp, required for promoting biofilm formation (Additional file 2: Table S2). In addition, the PTS_{clin} phosphotransferase system associated with colonization potential of clinical isolates [7] was present in all ST117/CT71/*van*B isolates. Further studies are warranted to understand the impact of these genes in the distribution of the ST117/CT71/*van*B clone.

A limitation of the study is the relatively short collection period and the number of isolates analyzed. Nevertheless, the discovery of a single predominant clone in geographically separated individual institutions within such a large catchment area is unprecedented. There are several possible explanations for this phenomenon: Firstly, our data indicate ongoing inter-hospital spread or even a multihospital outbreak, as near-identical isolates (≤10 cgMLST alleles) of the ST117/CT71/*van*B clone were detected in different hospitals (Fig. 2, Additional file 6: Figure S2). The latter phenomenon would require the movement of patients among the different hospitals sampled. This is true even for the smallest group of patients included in this study, i.e. those who have been reported to have previous stays in other participating hospitals (Additional file 6: Figure S2, Additional file 4: Table S4). Secondly, 14.9% (11/74) of the patients harboring this clone did not report any previous hospital stay within the last 12 months (Table 1). This indicates either acquisition of the VREfm in a

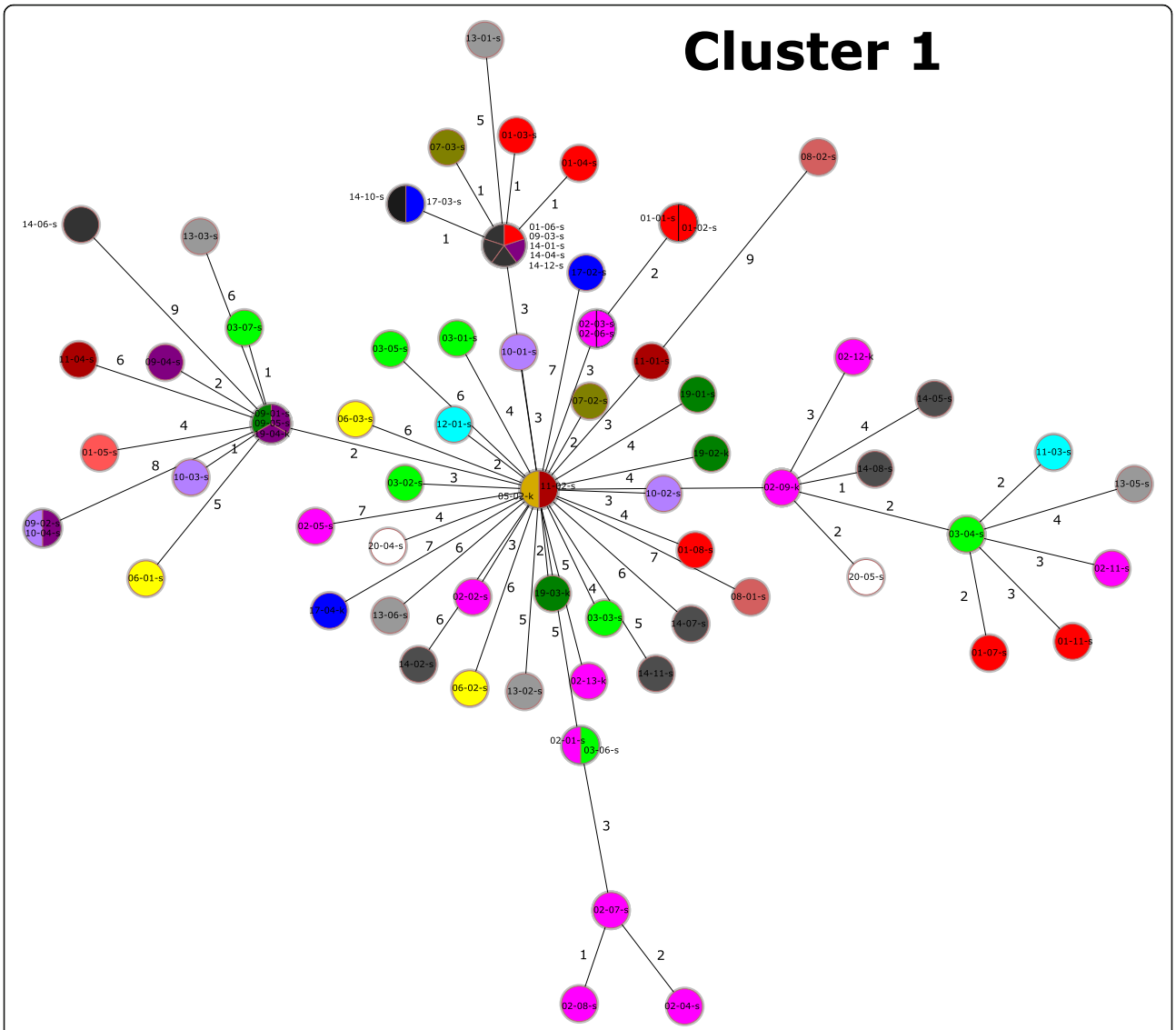

Fig. 2 Minimum spanning tree of Cluster 1 based on cgMLST information. Different colours depict different clinics. Cluster distance threshold was set to 10 cgMLST alleles. Size of circles is representative and reflects the number of isolates

hospital before more than 12 months ago, a nosocomial acquisition during the current hospital stay, or an acquisition through the dissemination of this clone in communal spaces outside of healthcare institutions. Further studies are required to answer the questions raised here, with particular focus on the presence of this clone in the community, healthcare-independent populations and other reservoirs (livestock, food, water).

Conclusion

We report the detection of a near-ubiquitous VREfm clone (ST117/CT71/*van*B) circulating within the metropolitan region in and around Frankfurt am Main/Germany. The presence both of a single clone in such a large catchment area and the detection of a possible multi-hospital VRE transmission in this study has only been revealed as a result of WGS-based analysis. As vancomycin resistance is associated with enhanced mortality among patients in hospital settings, in particular bloodstream infections [16, 17], the prevention of VREfm infections is a major objective. The presence of a VREfm clone within different institutions questions whether infection control and antimicrobial stewardship interventions can be effective without an understanding of the VREfm carriage state and transmission dynamics in human populations within the catchment area studied. The results of our study call for the establishment of a multihospital infection control approach, including rapid detection tools to identify predominant clones and for a genome-based long-term surveillance to be able to

detect newly emerging clones. In addition, the use of clone-based strategies for eradication i.e. based on vaccines or bacteriophages, would be interesting avenues for further pursuit.

Additional files

Additional file 1: Table S1. Characteristics of the sequenced isolates. Depicts the characteristics of each VREfm isolate presented in this study. (DOCX 14 kb)

Additional file 2: Table S2. Characteristics of the participating hospitals. Depicts selected characteristics of the hospitals participating in the study. (DOCX 30 kb)

Additional file 3: Table S3. Statistical analysis of parameters associated with ST117/CT71/*van*B clone carriage. Depicts the statistical analysis of parameters associated with the carriage of the ST117/CT71/*van*B clone. (DOCX 12 kb)

Additional file 4: Table S4. Information on previous hospital stays of the patients. Depicts previous hospital stays of the patients, including information in which hospitals they previously resided. (DOCX 17 kb)

Additional file 5: Figure S1. Regional distribution of the Cluster 1 VREfm ST117/CT71/*van*B isolates. Depicts the regional distribution of Cluster 1 VREfm ST117/CT71/*van*B isolates. Districts may include more than one hospital. The original map was extracted from Googlemaps (https://www.google.de/maps/@50.2354853,8.7072805,11z). (PDF 2079 kb)

Additional file 6: Figure S2. Interaction map between the different participating hospitals. Indicates the patients' previous hospital history, whereever known. Connections between hospitals mark previous hospital stays in another hospital, while circles indicate a previous stay in the same hospital. (PDF 15 kb)

Abbreviations

cgMLST: Core genome multilocus sequence typing; CT: Complex type; MDRO: Multidrug-resistant organisms; MLST: Multilocus sequence typing; ST: Sequence type; VRE: Vancomycin-resistant enterococci; VREfm: Vancomycin-resistant *Enterococcus faecium*; WGS: Whole-genome sequencing; WHO: World Health Organization

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Authors' contributions

UH and TC implemented the study. KS, MS and Rhine-Main VREfm study group provided isolates and gathered information. LF, MF, CI, UH, TC gathered and analyzed the data. TC, UH and LF wrote the manuscript, which all authors approved.

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Availability of data and materials

The raw sequencing data are available in ENA under the accession number PRJEB29744.

Ethics approval and consent to participate

The study was approved by the ethics committee of the medical faculty of the Justus-Liebig-University of Giessen (AZ: 179/16). All samples were taken as part of standard care procedures.

Consent for publication

Not applicable (no individual person's data included).

Competing interests

The authors declare that they have no competing interests.

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Anhang E

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Short Communication

Cross-border emergence of clonal lineages of ST38 *Escherichia coli* producing the OXA-48-like carbapenemase OXA-244 in Germany and Switzerland

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ABSTRACT

Background: Carbapenemase-producing Gram-negative bacteria cause infections that are difficult to treat and represent a rising threat to healthcare systems worldwide. This study analysed isolates of *Escherichia coli* (*E. coli*), a species associated with nosocomial-acquired and community-acquired infections, from hospitals in Germany and Switzerland exhibiting a slight decrease in susceptibility to carbapenems.
Methods: *E. coli* strains from Germany and Switzerland, obtained mainly in 2019, were first screened for carbapenemase genes by PCR and subsequently whole-genome-sequenced and analysed for their clonal relationship using multilocus sequence typing, single nucleotide polymorphisms, virulence and antibiotic-resistance gene content.
Results: The analysis revealed the presence of extended β-lactamase (ESBL)-producing *E. coli* clones producing OXA-244, a point-mutation derivative of OXA-48, with a predominance of isolates exhibiting the sequence type (ST) ST38 in both Germany and Switzerland. These clustered exclusively into two distinct lineages: one encoding CTX-M-27, a recently emerged extended-spectrum β-lactamase, and the other CTX-M-14b. All OXA244/CTX-M-27 ST38 isolates harboured the Dr adhesin operon and a representative isolate exhibited a diffuse adherence (DAEC) phenotype and was invasive for Hela cells.
Conclusion: Clonal lineages of ST38 are members of *E. coli* phylogenetic group D commonly associated with extra-intestinal infections. Their increased isolation in two different European countries indicates ongoing spread of ST38 ESBL-producing and OXA-244-producing *E. coli* clonal lineages. It is possible that members of the multidrug-resistant DEAC ExPEC group have expanded globally, but that this is currently underreported because of the inherent difficulty in detecting isolates expressing the OXA-244 allele.

1. Introduction

Carbapenem-producing Enterobacterales (CPE) are increasingly being identified not only in *Klebsiella pneumoniae* but also in *Escherichia coli* (*E. coli*) [1]. For *E. coli*, NDM- and OXA-48-type enzymes are the main determinants of carbapenem resistance [1]. Their presence in *E. coli*, which is a source of both nosocomial and community-acquired infections, raises the threat of their silent and impossible-to-control spread outside the hospital environment. This study analysed OXA-244-producing *E. coli* isolates collected in two different European countries.

2. Materials and Methods

2.1. Strain collection

OXA-244-producing *E. coli* isolates obtained from two CPE surveillance networks, one in the state of Hesse, Germany

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(ca. 6.2 million inhabitants, SurvCARE http://www.mre-netzwerk-mittelhessen.de/material/SurvCARE), and the other in Switzerland (ca. 8.4 millions inhabitants, National Reference Center for Emerging Antibiotic Resistance, NARA) were analysed.

For SurvCARE, extended-spectrum β-lactam (ESBL)-resistant and carbapenem-resistant isolates were identified using chromogenic media by the laboratories of the participating hospitals. Determination of the MIC towards carbapenems (ertapenem, imipenem and meropenem) was performed using Etest following EUCAST recommendations [2]. Resistance phenotypes of other antibiotics and species were determined with the VITEK 2® system (bioMerieux, Nürtingen, Germany). Enterobacterial strains that displayed a decreased susceptibility to carbapenems were regularly sent to either site, where the molecular mechanisms of resistance were analysed [3].

2.2. Whole genome sequencing and analysis

For whole genome sequencing, DNA was isolated from overnight cultures using the PureLink Genomic DNA kit (Invitrogen, ThermoFischer, Germany). Short read sequencing libraries were prepared for all *E. coli* ST-38/OXA-244 isolates (n = 31) using the Nextera XT kit (Illumina, Netherlands) and sequenced on MiSeq/NextSeq sequencing machines (read length either 2 × 300nt or 2 × 150nt). A long-read sequencing library of the isolate SurvCARE133 was prepared using the native barcoding kit (EXP-NBD103, Oxford Nanopore Technologies) and 1D chemistry (SQK-LSK108, Oxford Nanopore Technologies, UK). Sequencing was performed on a MinION sequencer (Oxford Nanopore Technologies, UK) using a SpotON Mk I R9 Version flow Cell (FLO-MIN106, Oxford Nanopore Technologies, UK). Post-sequencing quality control, assembly and virulence gene determination was performed using ASA^{3}P pipeline [4]. Antibiotic resistance genes, serotypes, plasmid incompatibility groups and pMLST types were determined using the Center for Epidemiology tools (http://www.genomicepidemiology.org/). Phylogenetic analysis was performed using HarvestSuite [5]. Regions exhibiting recombinational hotspots were removed using Gubbins [6]. The raw sequencing data information was stored in the National Center for Biotechnology Information under the project number PRJNA602666 (https://www.ncbi.nlm.nih.gov/).

2.3. Adherence assays

Human HeLa cervical epithelial cells (ATCC CCL2) were grown and assayed at 37°C with 5% CO_2 in Dulbeccos Minimal Essential Medium (DMEM) supplemented with 0.5% (vol/vol) foetal calf serum (FCS). Fresh bacterial cultures grown overnight in LB medium were used to prepare suspensions in PBS adjusted to an OD of 0.1 at 600 nm (~1×10^6 cfu/mL). Subconfluent 10% buffered formalin-fixed or live 48-h of HeLa cells grown on coverslips placed in 3.5-cm petri dishes were washed three times (5 minutes each) with PBS and overlaid with bacterial suspensions. Adhesion was allowed to take place for 3 hours at 37°C in a CO_2 incubator. After incubation, bacterial suspensions were removed, and the monolayers were washed three times in PBS. Live monolayers were fixed with cold methanol for 5 minutes, then both types of monolayers were air-dried and stained with Giemsa diluted 1:20 for 1 hour to visualise bacteria attached to HeLa cells.

2.4. Bacterial internalisation assay

Bacterial suspensions in PBS, prepared as described for the adherence assay, were added to monolayers of HeLa cells (~10 000/well) to give a multiplicity of infection (MOI) of 10–15 in 24 well plates and incubated for 3 hours at 37°C in the CO_2 incubator.

Table 1
Clinical data of the *Escherichia coli* ST38 OXA-244-producing isolates. Origin: CH, Switzerland, GE, Germany.

Isolate	Year of isolation	Clinic	Origin	Sample type	Age (years, if not otherwise stated)	Sex	Infection (I) / Colonisation (C)
N11	2017	CH-04	CH	Liquid drain	78	M	I
N293	2018	CH-03	CH	Urine	67	F	I
N460	2019	CH-04	CH	Urine	77	F	I
N481	2019	CH-05	CH	Urine	75	M	I
N511	2019	CH-02	CH	Rectal swab	64	F	C
N658	2019	CH-05	CH	Urine	48	F	I
N816	2019	CH-04	CH	Urine	41	F	I
N832	2019	CH-05	CH	Urine	28	F	I
N861	2019	CH-01	CH	Rectal swab	44	F	C
N871	2019	CH-06	CH	Urine	24	F	I
N888	2019	CH-07	CH	Urine	62	F	I
N930	2019	CH-08	CH	Urine	14	F	I
sc38-1	2018	GE-08	GE	Rectal swab	37	F	C
SurvCARE133	2018	GE-02	GE	Rectal swab	69	M	C
SurvCARE134	2018	GE-02	GE	Rectal swab	69	M	C
SurvCARE140	2018	GE-07	GE	Punctate lower abdomen	53	F	I
SurvCARE303	2019	GE-06	GE	Rectal swab	80	M	C
SurvCARE318	2019	GE-03	GE	Urine	75	F	C
SurvCARE319	2019	GE-01	GE	Rectal swab	63	F	C
SurvCARE335	2019	GE-10	GE	Gall bladder secretion	84	M	C
SurvCARE377	2019	GE-09	GE	Rectal swab	30	M	C
SurvCARE378	2019	GE-09	GE	Urine	30	F	I
SurvCARE382	2019	GE-05	GE	Vaginal swab	29	F	C
SurvCARE383	2019	GE-05	GE	Nose/throat swab	1 month	F	C
SurvCARE384	2019	GE-05	GE	Nose/throat swab	1 month	F	C
SurvCARE389	2019	GE-05	GE	Rectal swab	1 month	F	C
SurvCARE390	2019	GE-05	GE	Rectal swab	1 month	F	C
SurvCARE392	2019	GE-01	GE	Rectal swab	23	F	C
SurvCARE393	2019	GE-01	GE	Nose/throat swab	1 month	F	C
SurvCARE421	2019	GE-05	GE	Rectal swab	1 month	F	C
SurvCARE438	2019	GE-04	GE	Rectal swab	4	M	C

Origin: CH, Switzerland, GE, Germany.

Cells were subsequently washed thrice with Hanks medium, followed by incubation for 1 hour with DMEM and 0.5% FCS containing 200 µl/mL gentamycin to kill extracellular bacteria. Cells were washed thrice with Hanks buffer and subsequently lysed by the addition of sterile water containing 0.1% Triton X-100 for 30 minutes at room temperature. Appropriate dilutions were plated after the third washing with Hanks buffer and after lysis to determine the number of viable bacteria. Each assay was run in triplicate.

3. Results and Discussion

Carbapenemase-producing *E. coli* isolates obtained between 2017 and 2019 from the two CPE surveillance networks SurvCARE (Federal State of Hesse, Germany; n = 107) and NARA (Switzerland, n = 448) were analysed for the underlying resistance mechanisms. In both networks, the emergence of OXA-244-producing *E. coli* isolates among carbapenemase-producing *E. coli* isolates was noted in 2018 and 2019 (Germany 2018: 22.2%, 2019: 27.5%; Switzerland: 2018: 1%, 2019: 7%) as compared with 2017 (Germany: 0.0%, Switzerland: 0.0%). The enzyme OXA-244 is a point-mutant derivative of OXA-48 (Arg212Gly substitution) with weaker carbapenemase activity [3].

Forty-one *E. coli* isolates harbouring an OXA-244 carbapenemase were detected and whole genome sequenced to determine their epidemiological relatedness. Using multilocus sequence typing, 31 of the 41 OXA-244 producers (75.6%, Supplementary Figure 1) clustered as a single *E. coli* sequence type (ST) 38. Other STs identified included ST69 (n = 3), ST10 (n = 1), ST58 (n = 1), ST361 (n = 1), ST648 (n = 1), ST963 (n = 1), ST131 (n = 1) and ST1722 (n = 1). The predominance of *E. coli* ST38-OXA-244 producers in both countries suggested a possible ongoing outbreak associated with ST38 isolates.

A total of 31 ST38 OXA-244 isolates from both countries were studied (Table 1, Supplementary Table 1). Nineteen *E. coli* isolates were obtained from ten hospitals in Hesse and 12 isolates derived from 12 hospitals/private clinics in Switzerland. Isolates were obtained from urine samples (n = 10), rectal swabs (n = 15), vaginal smear/swab (n = 2) and other sources (n = 4). Isolates originated from different parts of Switzerland (German-, French- and Italian-speaking regions) and in the state of Hesse (Germany) from hospitals distributed within an area half the size of Switzerland. *E. coli* ST38 are representatives of the *E. coli* phylogenetic group D, commonly associated with extra-intestinal infections (ExPEC) [7].

Detailed analysis indicated the presence of two major clonal lineages that segregated almost entirely along the extended-spectrum

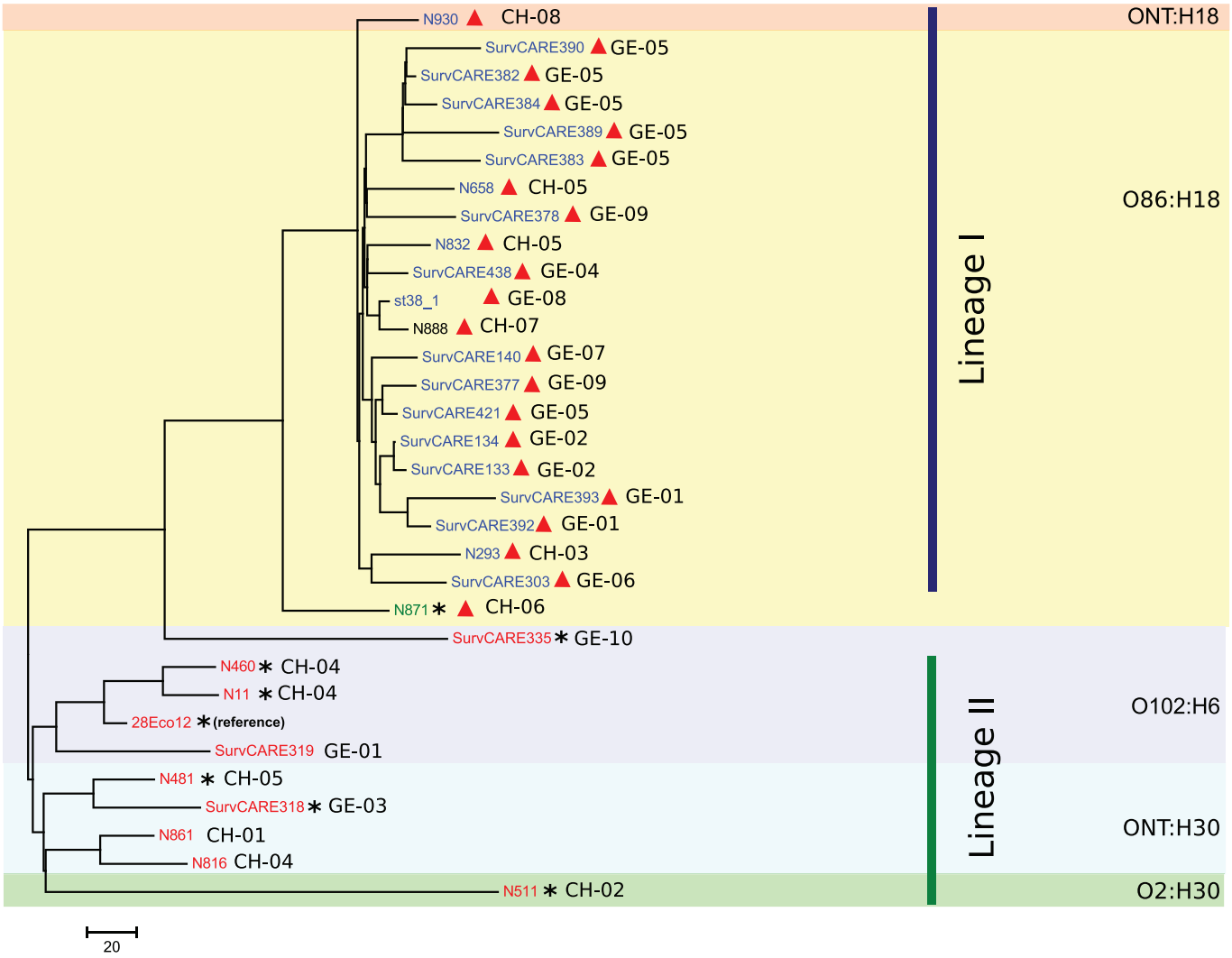

Figure 1. Core-genome-based analysis of OXA-244 producing ST38 isolates. The colour of the isolate names indicates the carriage of additional ESBL/AmpC enzymes; CTX-M-14b, red, CTX-M-27, blue, CMY-2, green; no additional ESBL/AmpC gene, black. A star indicates the presence of a complete Tn*51098* harbouring OXA-244. Red triangles indicate absence of Type I fimbriae genes. As a reference, 28Eco12 was used (marked as reference).

β-lactamase (ESBL) enzyme allele carried (i.e. CTX-M-27 and CTX-M-14b, respectively) (Figure 1, Supplementary Table 2). CTX-M-27 is a point mutant derivative of CTX-M-14.

The ability of OXA-244 to hydrolyse imipenem and temocillin (a specificity of OXA-48 derivatives) is weaker than that of OXA-48. Indeed, many of the isolates analysed here showed variable degrees of susceptibility/resistance to carbapenems and were often labelled as carbapenem-non-susceptible (or as susceptible, increased exposure according EUCAST 2019) when sent to the reference laboratories. Serial testing in the reference laboratories revealed variable results ranging from susceptible to resistant, according to EUCAST criteria in Minimal inhibitory concentration (MIC)-based breakpoint-testing. Using the Etest technique, the MICs for ertapenem, meropenem and imipenem ranged from 0.12–$>$ 32, 0.04–1.5 and 0.04–1 mg/L, respectively. None of the strains was resistant to meropenem or imipenem (Supplementary Table 3, Supplementary Table 4). The low level (if any) of resistance to carbapenems observed for those OXA-244 producers may have been related to the low level of production of the OXA-244 enzyme because the gene was present as a single copy on the chromosome in association with the low-level of carbapenem hydrolytic properties of this enzyme itself.

Co-resistances to other families of antibiotics (fluoroquinolones, tetracyclines, trimethoprim/sulfamethoxazole, aminoglycosides) were also highly similar within the strain collection (Supplementary Table 2, Supplementary Table 3, Supplementary Table 4). The $bla_{\text{OXA-244}}$ gene was chromosomally located in isolates from both lineages.

Clonal lineage I comprises isolates (21 of 31) that harbour the $bla_{\text{CTX-M-27}}$ gene on an IncF plasmid. The $bla_{\text{OXA-244}}$-encoding mobile element of lineage I isolates does not resemble the classical *Tn*51098 or Tn*6327* structure, as reported [8], but are deletion variants thereof (Figure 1, Supplementary Figure 2). Clonal lineage I isolates exhibited differences of $<$ 129 SNPs in their core genomes, regardless of whether the isolates were from Germany or Switzerland (Supplementary Figure 3) and almost exclusively of the serotype 086:H18 (Figure 1, Supplementary Table 11). They carried a Dr adhesin/invasion locus [9], but lacked Type I fimbriae

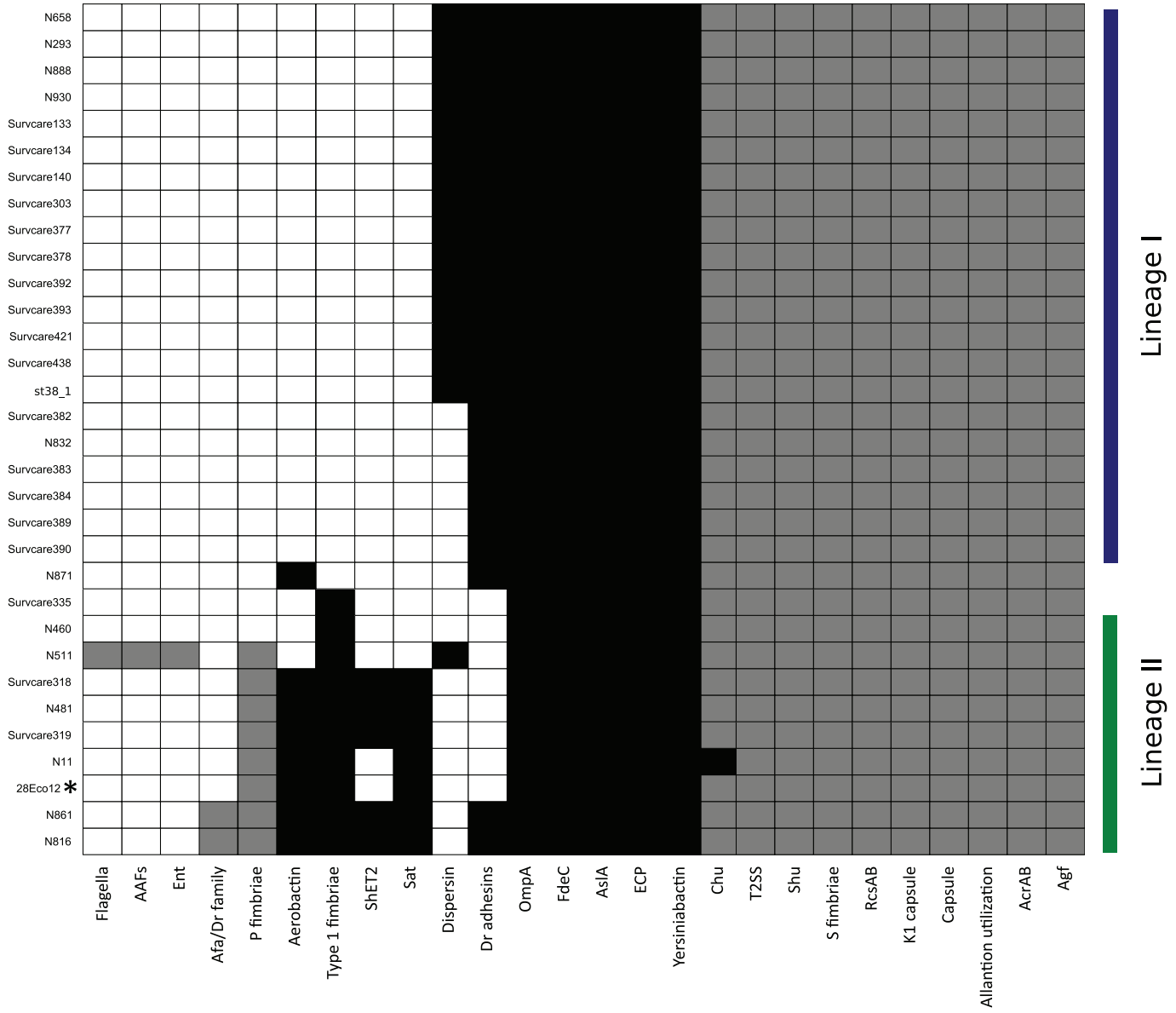

Figure 2. Virulence determinants of *E. coli* ST38 OXA-244 isolates. Complete operons are marked in black, incomplete operons in grey. The functions of the virulence determinants are indicated in Supplementary Table 5. The reference 28Eco12 is marked with an asterisk.

(Figure 2). Isolates expressing the Dr adhesins are often associated with cystitis, pregnancy-associated pyelonephritis and chronic diarrhoea; thus, indicating that isolates of clonal lineage I may have virulence potential [9]. A representative isolate exhibited a diffuse adherent phenotype for adhesion to HeLa cells and was invasive (Supplementary Figure 4).

Clonal lineage II isolates (9 of 31) were slightly more heterogeneous but had a common chromosomal insertion site for the $bla_{CTX\text{-}M\text{-}14b}$ gene. The $bla_{OXA\text{-}244}$-encoding mobile element in lineage II isolates resembled, in five of eight isolates, the previously described *Tn*51098 structure (Figure 1 and Supplementary Figure 2). They presented as several serotypes (O102:H6, ONT:H30 and O2:H30, Figure 1). In contrast to clonal lineage I, they encoded Type I fimbriae. Independent isolates of clonal lineage II from Germany and Switzerland were highly related (Figure 1, Supplementary Figure 3).

All ST38 isolates carried virulence and adaptive genes, including the iron uptake systems aerobactin and yersiniabactin as well as haeme-uptake and degradation systems (Figure 2). In contrast to classical uropathogenic *E. coli* (UPEC), they did not carry haemolysin genes.

It was noted that of the 31 isolates from both countries, 23 isolates were from female patients. This high proportion of female origin of the strains was evidenced among infections (mostly urinary tract infections), as expected, but also from colonisation. Two sets of OXA-244/CTX-M-27 isolates, SurvCARE382/383/384 (383 and 384 were twins) and SurvCARE392/393 represented mother/neonate combinations, while for one combination (SurvCARE 382/383/384/390) the neonates and one mother were present in the same room. The remaining isolates were almost exclusively obtained from elderly male patients (exceptions: SurvCARE377 and SurvCARE438). The fact that these lineages were isolated from patients of different ages and from different clinical specimens indicates an ability to colonise and infect.

A recent meta-analysis of 217 studies on commonly occurring extraintestinal *E. coli* (ExPEC) lineages worldwide in humans did not reveal a single isolate of ST38 prior to 2000 [10]. Nevertheless, a recent report documented an increase in ST38 isolates expressing OXA-244 throughout Germany since 2017 [11]. Also, OXA-244-producing *E. coli* isolates have been identified from a number of countries within the EU, including The Netherlands, Spain, United Kingdom, and France [8]. Previous reports have indicated the presence of OXA-244 producers in the UK, Spain and France in strains belonging to the same ST38 type [12]. The large increase in OXA-244 CTX-M-27/14b producing ST38 isolates in 2019 suggests an ongoing and emerging supranational clonal outbreak over a large area comprising parts of Germany, Switzerland and possibly many other European countries.

The clonal lineage II isolates encoding OXA-244/CTX-M-14b isolates harboured both ESBL and carbapenemase genes on the chromosome. This peculiar genetic situation may stabilise both genes in those *E. coli* strains, leading to a vertical transfer of this resistance trait. Therefore, any β-lactam used for treating *E. coli* infections (penicillin, penicillin/β-lactamase inhibitor, cephalosporin, carbapenem) may select those OXA-244 producers, whereas OXA-244, like other OXA-48-like enzymes, does not hydrolyse cephalosporins. This genetic background may be of the utmost importance for explaining a further spread of community-acquired OXA-244 producers.

It has previously been suggested that DAEC isolates act as 'silent pathogens' that are well-controlled in healthy individuals but express pathogenic potential in susceptible hosts [9]. Thus, the combination of under-detection of multidrug-resistant OXA-244 producing bacteria due to their low level of resistance to carbapenems (if any) and their properties as conditional pathobionts are worrying, and likely to favour their silent spread worldwide.

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Author contributions: LF, PN and TC designed the study. PN, CI, AH, PH and TC provided data and isolates. LF, PN, CI, YY, JF, PH and TC gathered and analysed the data. LF, PN and TC wrote the manuscript, which all authors approved.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijantimicag.2020.106157.

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Anhang F

Falgenhauer L, Imirzalioglu C, Ghosh H, Gwozdzinski K, Schmiedel J, Gentil K, Bauerfeind R, Kämpfer P, Seifert H, Michael GB, Schwarz S, Pfeifer Y, Werner G, Pietsch M, Roesler U, Guerra B, Fischer J, Sharp H, Käsbohrer A, Goesmann A, Hille K, Kreienbrock L, Chakraborty T. 2016. Circulation of clonal populations of fluoroquinolone-resistant CTX-M-15-producing *Escherichia coli* ST410 in humans and animals in Germany. Int J Antimicrob Agents 47:457–465.

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Circulation of clonal populations of fluoroquinolone-resistant CTX-M-15-producing *Escherichia coli* ST410 in humans and animals in Germany

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ABSTRACT

Multidrug-resistant *Escherichia coli* encoding CTX-M-type extended-spectrum β-lactamases (ESBLs) are isolated in increasing numbers from humans, companion animals and livestock, raising concern regarding the exchange and spread of isolates in these populations. In this study, whole-genome sequencing of CTX-M-15-producing *E. coli* isolates recently sampled from humans, companion animals, livestock and farm environments was performed. In total, 26 different sequence types (STs) were detected, of which ST410 was the most frequent and was the only ST present in all populations studied. Five clades (designated A–E) were detected within the ST410 isolates. In particular, isolates of clade B were present in all four populations and had core genomes that differed by less than 70 single nucleotide polymorphisms (SNPs). Isolates of clades B and C were also clonally marked, exhibiting identical chromosomal insertions of $bla_{CTX\text{-}M\text{-}15}$ at distinct loci. These data provide strong evidence for the clonal dissemination of specific clades of CTX-M-15-producing *E. coli* ST410 in human and animal populations.

1. Introduction

Multidrug-resistant Enterobacteriaceae are an emerging problem in human and animal healthcare worldwide. Bacteria-harbouring genes encoding extended-spectrum β-lactamases (ESBLs) are resistant to penicillins, cephalosporins and monobactams. ESBL genes are often located on plasmids and are transferred horizontally [1]. CTX-M-type β-lactamases are the most common ESBL type [2]. In particular, the CTX-M-15 enzyme has a worldwide distribution and is frequently associated in isolates of *Escherichia coli* sequence type 131 (ST131) in humans [3]. However, *E. coli* ST131 isolates have only rarely been detected in companion animals and livestock [3]. Studies

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examining the presence of CTX-M-15-harbouring ESBL-producing *E. coli* in Germany have reported a prevalence of 20–66% among human isolates and a much lower prevalence of 1.3–4% in livestock [4–7].

Clonal transfer of ESBL-producing *E. coli* isolates would result in the presence of related clones in humans and animals that may be propagated via the food chain. To investigate this, several epidemiological studies of ESBL-encoding *E. coli* isolates from humans and animals in the UK, The Netherlands and Germany have been performed, showing that they share identical CTX-M alleles, common STs and have similar resistance plasmids, suggesting a common pool of clones and/or resistance plasmids in these populations [8–10]. These studies were subgenome-based and provided lower resolution than whole-genome sequencing (WGS), which can resolve relationships up to the level of single nucleotide polymorphisms (SNPs). A recent study revisited data from one of these studies performed for ESBL-producing *E. coli* isolates from humans, poultry and pigs [9] to address the comparability of classical genotyping methods with WGS. They showed that although isolates from humans and animals displayed identical STs, the genomes of these isolates were significantly different, exhibiting 4216 SNPs within the core genome even for the most closely related isolates [11]. Nevertheless, they shared highly similar ESBL gene-carrying plasmids, indicating that in this particular scenario, transfer of resistance was plasmid-mediated.

These results raised the question whether the transfer of resistance by highly similar plasmids is the major route for the transfer of ESBL genes between animals and humans and occurs independently from the spread of clonal populations (epidemic *E. coli* clones).

We initiated a study to examine the prevalence of ESBL-producing bacteria in animal and human populations from different locations in Germany [12]. WGS was performed to examine for evidence of clonal exchange between CTX-M-15-producing *E. coli* isolates from livestock, companion animals, farm environments and humans.

2. Materials and methods

2.1. Isolate collection

In various studies conducted between 2009 and 2014 within the national interdisciplinary research consortium RESET (http://www.reset-verbund.de/), ESBL-producing *E. coli* isolates from humans, companion animals and livestock were collected [12]. ESBL production was confirmed using the double disk synergy test, and the presence of a particular ESBL gene (CTX-M-type, TEM-type or SHV-type) was tested by gene- and allele-specific PCR [4]. Of the 429 $bla_{\text{CTX-M-15}}$-encoding isolates obtained, 90 were randomly chosen for sequencing.

In addition, four non-repetitive CTX-M-15-producing isolates from haemato-oncological patients at the German Centre for Infection Research (DZIF, Braunschweig, Germany; http://www.dzif.de/) [13] as well as three isolates obtained from biogas plants [14] were included. The characteristics of all 97 isolates are given in Table 1.

2.2. Whole-genome sequencing

DNA was isolated using a Purelink™ DNeasy Kit (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions. WGS was carried out on an Illumina MiSeq instrument (Illumina, San Diego, CA) using an Illumina Nextera XT library with 2×300 bp paired-end reads. Data were assembled using SPAdes Genome Assembler v.3.0 [15]. Contigs with a size >500 bp were ordered to *E. coli* 789 (GenBank accession no. CP010315.1) using MAUVE [16]. The ordered contigs were then concatenated to a pseudogenome and annotation was performed using *E. coli* MG1655 as a reference.

2.3. In silico analyses

Multilocus sequence typing (MLST) was performed following the scheme of Wirth et al. using the Web-based tool MLST 1.7 [17,18]. Detection of phylogenetic groups was performed in silico according to the literature [19]. Identification of virulence genes was performed using VirulenceFinder [20]. The presence of operons involved in iron acquisition was determined using the NCBI blastn algorithm using the references presented in Supplementary Table S1 [21]. Pathogenicity islands (PAIs) were identified by performing blastn with PAIs from *E. coli*, *Salmonella*, *Klebsiella* and *Shigella* spp. present in the Pathogenicity Islands Database [21,22]. In silico serotyping was performed using SeroTypeFinder [23]. The genetic environment of CTX-M-15 was characterised using ISfinder (https://www-is.biotoul.fr/; identification of insertion sequences and transposons) and Blast search (blastn/tblastn) [21,24]. CTX-M-15-containing contigs that showed high homology to plasmids were considered as being plasmid-derived.

The software package Harvest Suite was used to determine phylogeny, core genome sequences and SNPs [25]. This software identifies the core genome directly without requiring annotation (thereby covering not only core genes but also intergenic core sequences) and performs phylogenetic analysis only with the identified core sequences. For this phylogenetic analysis, *E. coli* 789 was used as reference genome. For SNP analysis, isolate V139, which was the earliest strain isolated in this study, was chosen as a reference. Regions of high recombination (>50 SNPs per 1 kb) and phage sequences (identified by PHAST [26]) were identified by visual inspection and were excluded from the SNP analysis.

2.4. Plasmid characterisation

Plasmids were identified using S1 nuclease digestion followed by pulsed-field gel electrophoresis (S1-PFGE) as reported previously [27]. Plasmid incompatibility (Inc) groups were defined using PlasmidFinder using the assembled contigs [28]. Conjugation experiments were performed using *E. coli* J53 as recipient and sodium azide (200 mg/L) and cefotaxime (2 mg/L) as selective agents as reported previously [27].

2.5. Data availability

All sequences (sequencing reads and the genetic environment of CTX-M-15) are available in the European Nucleotide Archive (ENA) under the project no. PRJEB9568 and the accession numbers LN868272-LN868277.

3. Results

3.1. Sequence typing and phylogenetic analysis of CTX-M-15-producing isolates

Phylogenetic group analysis was performed and the ST of all isolates was determined. Isolates were members of the A ($n = 54$), B1 ($n = 16$), B2 ($n = 15$) and D ($n = 12$) phylogenetic groups [19]. A total of 26 different STs were detected among these CTX-M-15-positive *E. coli*, ranging from 1 to 27 isolates per ST (Fig. 1). The most common STs were ST410 (27 isolates), followed by ST131 (15 isolates), and ST224 and ST648 (6 isolates each). The remaining 43 isolates comprised 22 different known STs (Fig. 1). ST410 was by far the most abundant cluster detected and was present in all population samples (humans, livestock, companion animals and farm environment). Therefore, the relatedness of these isolates was examined in more detail.

Table 1
Properties of the 97 CTX-M-15-producing *Escherichia coli* isolates sequenced in this study.

Name	Date of isolation	Origin	Source	Phylogenetic group	ST
113301	05/01/2011	Livestock	Gut	D	ST648
120768	25/04/2012	Livestock	Milk	A	ST617
123074	02/01/2013	Livestock	Gut	A	ST410
123445	13/03/2013	Livestock	Milk sample	A	ST410
90044	21/01/2009	Livestock	Faeces	A	ST167
94251	19/11/2009	Livestock	Faeces	A	ST167
E001768	13/03/2012	Human	Stool sample	B2	ST131
E003488	28/05/2012	Human	Stool sample	A	ST410
E006910	06/11/2011	Human	Stool sample	A	ST410
E007570	07/12/2011	Human	Stool sample	B2	ST131
ESBL232B15_13_2E	April 2013	Farm environment	Biogas plant input	A	ST410
ESBL370B15_13_2A	July 2013	Farm environment	Biogas plant output	A	ST410
ESBL37B15_13_1E	February 2013	Farm environment	Biogas plant input	A	ST410
H10	09/01/2010	Human	Urine	B1	ST443
H116	01/06/2010	Human	Rectal swab	A	ST361
H119	04/06/2010	Human	Stool sample	A	ST1284
H122	09/06/2010	Human	Cervical swab	A	ST10
H123	10/06/2010	Human	Unknown	D	ST648
H127	20/06/2010	Human	Urine	B2	ST131
H129	22/06/2010	Human	Urine	B2	ST131
H130	23/06/2010	Human	Cervical swab	A	ST88
H134	29/06/2010	Human	Urine	B1	ST58
H141	06/07/2010	Human	Urine	D	ST2141
H143	12/07/2010	Human	Unknown	B1	ST224
H152	16/07/2010	Human	Groin swab	B1	ST224
H153	15/07/2010	Human	Unknown	B1	ST224
H154	15/07/2010	Human	Rectal swab	B1	ST224
H160	20/07/2010	Human	Urine	B1	ST224
H164	22/07/2010	Human	Cervical swab	B1	ST224
H24a	23/01/2010	Human	Urine	A	ST361
H3	29/12/2009	Human	Urine	B2	ST131
H38	04/02/2010	Human	Rectal swab	A	ST44
H45	16/02/2010	Human	Rectal swab	D	ST93
H50	23/02/2010	Human	Urine	D	ST69
H53	02/03/2010	Human	Urine	B2	ST131
H63	12/03/2010	Human	Urine	B2	ST131
H66	16/03/2010	Human	Urine	D	ST405
H71	24/03/2010	Human	Urine	B2	ST131
H75	29/03/2010	Human	Urine	B2	ST131
H89	17/04/2010	Human	Wound swab	D	ST349
H92	16/04/2010	Human	Penis swab	A	ST10
H93	20/04/2010	Human	Urine	B1	ST156
pCT119	09/10/2008	Livestock	Faecal sample (single animal)	A	ST10
R107	29/06/2011	Farm environment	Sock swabs, dairy cattle farm	A	ST410
R208	29/06/2011	Farm environment	Sock swabs, fattener	A	ST410
R261	09/01/2012	Livestock	Pooled faecal samples	A	ST167
R299	15/06/2011	Livestock	Pooled faecal samples	A	ST410
R363	25/04/2012	Livestock	Pooled faecal samples	A	ST617
R37	24/05/2011	Livestock	Pooled faecal samples	A	ST410
R392	08/08/2011	Livestock	Faeces	A	ST410
R423	11/10/2011	Livestock	Faeces	A	ST410
R432	11/10/2011	Livestock	Faeces	A	ST410
R56	14/06/2011	Livestock	Pooled faecal samples	A	ST410
R570	06/02/2012	Livestock	Faeces	A	ST88
R61a	15/06/2011	Farm environment	Dust sample, fed cattle farm	A	ST410
R625	13/03/2012	Farm environment	Sock swabs, cattle farm	B1	ST533
RS099	06/04/2011	Human	Urotube®	B2	ST131
RS119	12/04/2011	Human	Colonised wound	A	ST410
RS149	18/04/2011	Human	Rectal swab	B1	ST443
RS153	23/08/2011	Human	Port catheter infection	A	ST410
RS156	26/08/2011	Human	Groin wound	A	ST361
RS158	19/04/2011	Human	Urine	D	ST648
RS204	01/08/2011	Human	Urine	B2	ST131
RS254	09/12/2011	Human	Urine	A	ST410
RS271	29/01/2012	Human	Urine	A	ST617
RS288	29/02/2012	Human	Urine	A	ST410
RS333	01/02/2011	Human	Stool sample	A	ST410
RS334	01/02/2011	Human	Stool sample	A	ST410
RS371	09/07/2012	Human	Wound swab	A	ST410
V114	19/11/2009	Companion animal	Faeces	B1	ST448
V131	17/12/2009	Livestock	Unknown	A	ST90
V139	16/02/2010	Companion animal	Faeces	A	ST410
V144	24/02/2010	Companion animal	Vagina	B1	ST500

(*continued on next page*)

Table 1 (*continued*)

Name	Date of isolation	Origin	Source	Phylogenetic group	ST
V158	13/04/2010	Companion animal	Faeces	A	ST1480
V161	14/04/2010	Companion animal	Inner organs	A	ST410
V177	12/05/2010	Companion animal	Liver	A	ST410
V182	18/05/2010	Companion animal	Faeces	A	ST10
V195	22/06/2010	Companion animal	Wound	B1	ST533
V201	01/07/2010	Companion animal	Abdominal cavity	B1	ST448
V210	02/08/2010	Companion animal	Unknown	A	ST410
V260	23/09/2010	Companion animal	Urine	B2	ST131
V279	14/10/2010	Companion animal	Urine	A	ST88
V282	14/10/2010	Companion animal	Urine	B2	ST131
V283	15/10/2010	Companion animal	Urine	A	ST88
V291	11/11/2010	Companion animal	Vagina	B2	ST131
V292	16/11/2010	Companion animal	Catheter urine	D	ST405
V295	18/11/2010	Companion animal	Urine	B2	ST131
V70	01/09/2009	Companion animal	Catheter urine	A	ST361
V71	01/09/2009	Companion animal	Skin wound	A	ST1284
V73	16/09/2009	Companion animal	Faeces	D	ST648
V74	21/09/2009	Companion animal	Sperm	B1	ST617
V80	05/10/2009	Companion animal	Faeces	A	ST746
V86	16/10/2009	Companion animal	Faeces	D	ST648
V9	03/06/2009	Companion animal	Faeces	A	ST167
V96	28/10/2009	Companion animal	Faeces	D	ST648
V98	03/11/2009	Companion animal	Faeces	A	ST361
09-0090706-001-01	08/12/2009	Human	Stool sample	A	ST48

ST, sequence type.

3.2. Phylogenetic analysis and in silico serotyping of ST410 isolates

All ST410 isolates obtained were fluoroquinolone (FQ)-resistant and were collected from different regions in Germany between 2010 and 2013 (Table 2). In subsequent studies, three additional FQ-resistant ST410 isolates obtained from biogas plants [14] were included.

Phylogenetic analysis was performed using V139 as the reference. *E. coli* 789 (ST88) was added in the phylogenetic analysis as there is presently no completely sequenced genome of ST410 publicly available. ST88 and ST410 are closely related and differ within their MLST loci only in a single locus (*purA*). Phylogenetic analysis revealed that isolates within the ST410 cluster were subdivided into five clades, designated A–E (Fig. 2). The overall common core sequence was 86%. Clades A and E contained exclusively human and companion animal isolates. Clade D harboured isolates from humans and livestock, whilst clade C comprised of isolates from humans, farm environment and companion animals. Clade B isolates were detected in all four populations studied. In silico serotyping classified isolates of clade A and B as O8:H21, isolates of clade C as O8:H9 and isolates of clades D and E as O-:H9 serotype.

3.3. Single nucleotide polymorphisms within ST410 clades

SNP analysis was performed using V139 as the reference. Members from clade B were separated by between 31 and 70 SNPs in their core sequences. Within clade C the total SNP count was between 51 and 64 SNPs. Isolates of clade A differed from clade B by between 121 and 146 SNPs, and the difference in the core genome between isolates of clades B and C was between 1409 and 1424 SNPs.

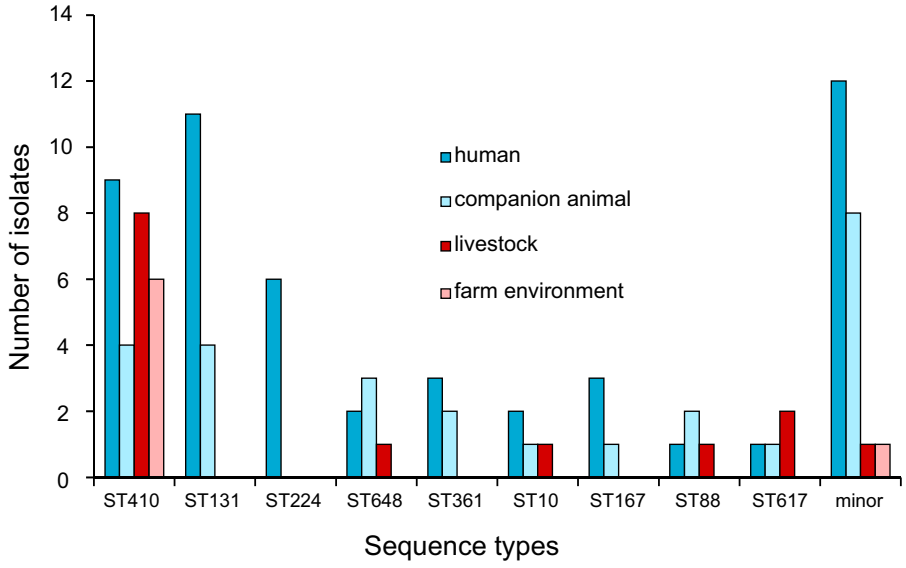

Fig. 1. Distribution of sequence types (STs) by multilocus sequence typing (MLST) analysis of the 97 sequenced *Escherichia coli* isolates encoding CTX-M-15.

Table 2
Characteristics of the investigated *Escherichia coli* sequence type 410 (ST410) isolates.

Strain	Origin	Source	Date of isolation	Site of isolation [a]	Resistance to antibiotics [b]	Clade	Plasmid size (kb) [c]	Inc group [d]	SNPs [e]
V161	Dog	Inner organs	14/04/2010	HE	AMP, FEP, CTX, CAZ, CHL, CIP	A	125, 120, 40	FII, FIA, FIB, Y, Q1	32
V210	Cat	Unknown	02/08/2010	HE	AMP, FEP, CTX, CAZ, CHL, CIP, GEN	A	125, 120, 40	FII, FIA, FIB, Y	1530
123074	Calf	Gut	02/01/2013	BY	AMP, FEP, CTX, CAZ, CHL, CIP, GEN	B	90	FII, FIA, FIB	1424
ESBL232B15_13_2E	Farm environment	Biogas plant input	April 2013	BY	AMP, (FEP), CTX, CAZ, CHL, CIP, GEN	B	85, 60	FII, FIA, FIB	1421
ESBL370B15_13_2A	Farm environment	Biogas plant output	July 2013	BY	AMP, (FEP), CTX, CAZ, CHL, CIP, GEN	B	85	FII, FIA, FIB	43
ESBL37B15_13_1E	Farm environment	Biogas plant input	February 2013	BY	AMP, (FEP), CTX, CAZ, CHL, CIP, GEN	B	90, 70	FII, FIA, FIB	49
R299	Dairy cattle	Pooled faecal samples	15/06/2011	NRW	AMP, FEP, CTX, CAZ, CHL, CIP, GEN	B	90	FII, FIA, FIB, P	49
R392	Dairy cattle	Faeces	08/08/2011	BY	AMP, FEP, CTX, CAZ, CHL, CIP, GEN	B	85	FII, FIA, FIB	1421
R423	Dairy cattle	Faeces	11/10/2011	BY	AMP, FEP, CTX, CAZ, CHL, CIP, GEN	B	85	FII, FIA, FIB	1420
R432	Dairy cattle	Faeces	11/10/2011	BY	AMP, FEP, CTX, CAZ, CHL, CIP, GEN	B	49	FIB	41
R56	Chicken	Pooled faecal samples	14/06/2011	NRW	AMP, (FEP), CTX, CAZ, CHL, CIP, GEN	B	65	FII, FIA, FIB, P	1537
R61a	Farm environment	Dust sample, fed cattle farm	15/06/2011	NRW	AMP, (FEP), CTX, CAZ, CHL, CIP, GEN	B	90	FII, FIA, FIB, P	70
RS119	Human	Colonised wound	12/04/2011	BY	AMP, FEP, CTX, CAZ, CHL, CIP, GEN	B	90, 48.5	FII, FIA, FIB, Col	54
RS153	Human	Port catheter infection	23/08/2011	BW	AMP, FEP, CTX, CAZ, CHL, CIP, GEN	B	90	FII, FIA, FIB	43
RS333	Human	Stool sample	01/02/2011	BY	AMC, AMP, FEP, CTX, CAZ, CHL, CIP	B	90	FII, FIA, FIB	36
RS334	Human	Stool sample	01/02/2011	BY	AMP, FEP, CTX, CAZ, CHL, CIP, (EPM), GEN	B	90	FII, FIA, FIB	58
V139	Cat	Faeces	16/02/2010	HE	AMP, FEP, CTX, (CAZ), CHL, CIP	B	90	FII, FIA, FIB	31
E003488	Human	Stool sample	28/05/2012	NRW	AMP, FEP, CTX, CAZ, (CHL), CIP	C	110, 40	I1, X1	46
E006910	Human	Stool sample	06/11/2011	NRW	AMP, CTX, CAZ, CIP	C	190, 115, 48.5, 6	FII, FIB, I1, X1	1532
R107	Farm environment	Sock swabs, dairy cattle farm	29/06/2011	NRW	AMP, FEP, CTX, CAZ, CIP	C	120	FII, FIB	1536
R208	Farm environment	Sock swabs, fattener	29/06/2011	NRW	AMP, FEP, CTX, CAZ, CIP	C	110, 40	FII, FIB	32
V177	Dog	Liver	12/05/2010	HE	AMP, FEP, CTX, CAZ, CHL, CIP	C	130	FII, FIB	31
123445	Dairy cattle	Milk sample	13/03/2013	BY	AMP, FEP, CTX, CAZ, CHL, CIP, (GEN)	D	110	FII, FIA, FIB	1528
R37	Swine	Pooled faecal samples	24/05/2011	NRW	AMP, FEP, CTX, CAZ, CIP	D	120, 12	FII, FIA, FIB	0
RS288	Human	Urine	29/02/2012	BH	AMP, FEP, CTX, CAZ, CHL, CIP, GEN	D	82, 12	FII, FIA, FIB	121
RS254	Human	Urine	09/12/2011	B	AMP, FEP, CTX, CAZ, CHL, CIP, GEN	E	125	FII, FIA, FIB	1409
RS371	Human	Wound swab	09/07/2012	BY/BW	AMP, FEP, CTX, CAZ, CHL, CIP, GEN	E	125	FII, FIA, FIB, Col	146

[a] Site of isolation: HE, Hesse; BY, Bavaria; NRW, North Rhine-Westphalia; BW, Baden-Württemberg; BH, Bremerhaven; B, Berlin.
[b] Antibiotic resistance tested: AMP, ampicillin; FEP, cefepime; CTX, cefotaxime; CAZ, ceftazidime; CHL, chloramphenicol; CIP, ciprofloxacin; GEN, gentamicin; AMC, amoxicillin/clavulanic acid; EPM, ertapenem; resistance in parenthesis defines isolates with intermediate resistance.
[c] Sizes of the plasmids present in the wild-type isolates were determined by S1 nuclease digestion followed by pulsed-field gel electrophoresis (S1-PFGE).
[d] Incompatibly (Inc) groups were investigated using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/).
[e] Single nucleotide polymorphisms (SNPs) were identified using Harvest Suite [25]. SNPs were calculated using *E. coli* ST410 isolate V139 as a reference.

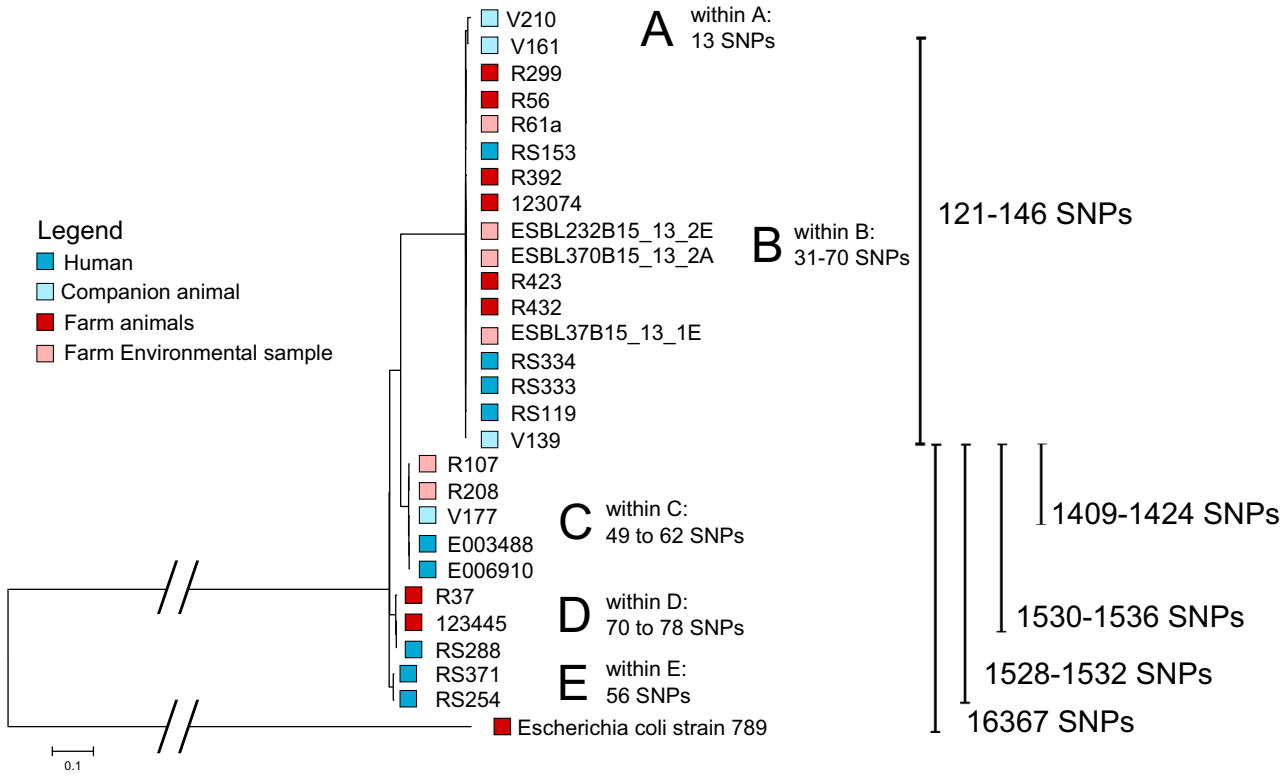

Fig. 2. Phylogenetic analysis of the *Escherichia coli* sequence type 410 (ST410) isolates. The phylogenetic tree was produced using Harvest Suite [25]. Different colours indicate the source of isolation. The letters A, B, C, D and E mark the clades. The overall single nucleotide polymorphism (SNP) numbers were calculated using isolate V139 as a reference. The SNPs within each group were calculated using the oldest isolate in each group.

The highest number of SNPs was detected in isolates of clades D and E that exhibited a total count of between 1528 and 1536 SNPs. In comparison, the closely related ST88 *E. coli* 789 was separated by 16 367 SNPs from the isolate V139. Numbers of SNPs for the individual isolates are given in Table 2.

3.4. Localisation of the $bla_{CTX\text{-}M\text{-}15}$ resistance gene in ST410 clades

Three different chromosomal locations of $bla_{CTX\text{-}M\text{-}15}$ were identified in the ST410 cluster. All isolates of clade B carried a $bla_{CTX\text{-}M\text{-}15}$ transposition unit inserted into a distinct location in the *Rhs*E cassette (Fig. 3a). *Rhs* cassettes are large repetitive elements that can be present in up to seven copies in the genome of *E. coli* (called *Rhs*A–H) and are hotspots for recombination and integration of insertion sequences [29]. The transposition unit carried a remnant of the Tn*3* transposase in between the left direct repeat and *orf477*, which was previously detected in several $bla_{CTX\text{-}M\text{-}15}$-carrying resistance plasmids (e.g. pEC_L8, accession no. NC_014384.1) [30]. All clade B isolates except V139 displayed an additional IS*1* insertion sequence integrated into the IS*Ecp1* element. In isolates of clade C, an intact IS*Ecp1* element in combination with $bla_{CTX\text{-}M\text{-}15}$ and *orf477* was inserted into a defective lambdoid prophage (Fig. 3b) between a putative phage lysin and holin gene. One isolate of clade E (RS254) displayed a third chromosomal location for $bla_{CTX\text{-}M\text{-}15}$ (Fig. 3c). In this case, the $bla_{CTX\text{-}M\text{-}15}$ gene and its adjacent sequences were integrated into yet another defective prophage that was distinct from the one detected in clade C.

The location of the $bla_{CTX\text{-}M\text{-}15}$ antibiotic resistance modules in the remaining isolates of clades A, D and E (123445, V161, V210, RS288, RS371 and R37) were analysed by database searches and were found to be located in three different genetic environments present on various plasmids, depicted in Fig. 3d–f. Plasmids from three of these isolates (123445, R37 and RS371) were transferable by conjugation to *E. coli* J53.

The predominance of chromosomal insertions of the $bla_{CTX\text{-}M\text{-}15}$ allele in ST410 prompted us to examine whether the distribution of chromosomal versus plasmid insertions was also seen with other STs. We observed that in the second largest cluster, which comprised 15 ST131 isolates, plasmid locations predominated over those on the chromosome (Fig. 4). Nevertheless, chromosomally located $bla_{CTX\text{-}M\text{-}15}$ was detected in many STs (Fig. 4).

3.5. Virulence genes and pathogenicity islands

The ST410 isolates were examined for the presence of known virulence factors (Fig. 5). All isolates contained the *lpfA* gene that codes for long polar fimbriae known to be required for intestinal colonisation [31]. Three isolates from clade B (R56, R61a and R299) also harboured fimbriae usually present in uropathogenic *E. coli* (f17 fimbriae), and one isolate (123445) carried the *prfB* gene coding for P-related fimbriae. The serum survival gene (*iss*) was present in members of clade A and C (with the exception of isolate E003488). Isolates of clades C and D, except isolate R37, carried genes encoding microcins or colicins (*mcmA* or *cma*). Toxin genes were present in only two isolates (*astA* in 123445 and *senB* in R37).

All isolates harboured the ferrichrome and ferrous iron-uptake operons (*fhuABCD*, *feoABCD*), the iron(III) dicitrate uptake operon (*fecRI-ABCDE*) as well as the enterobactin siderophore operon (*entABCDEFH*, *entS*, *fepABCDEG*, *fes*, *ybdZ*). The *sit* operon (*sitABCD*, Fe^{2+} transport) was present in all isolates except isolate E003488. The aerobactin operon (*iucABCD*, *iutA*) was present in 22 of 27 isolates (clades A, B, D and E). Clade C isolates (with exception of isolate E003488) harboured the salmochelin operon (*iroBCDEN*). The

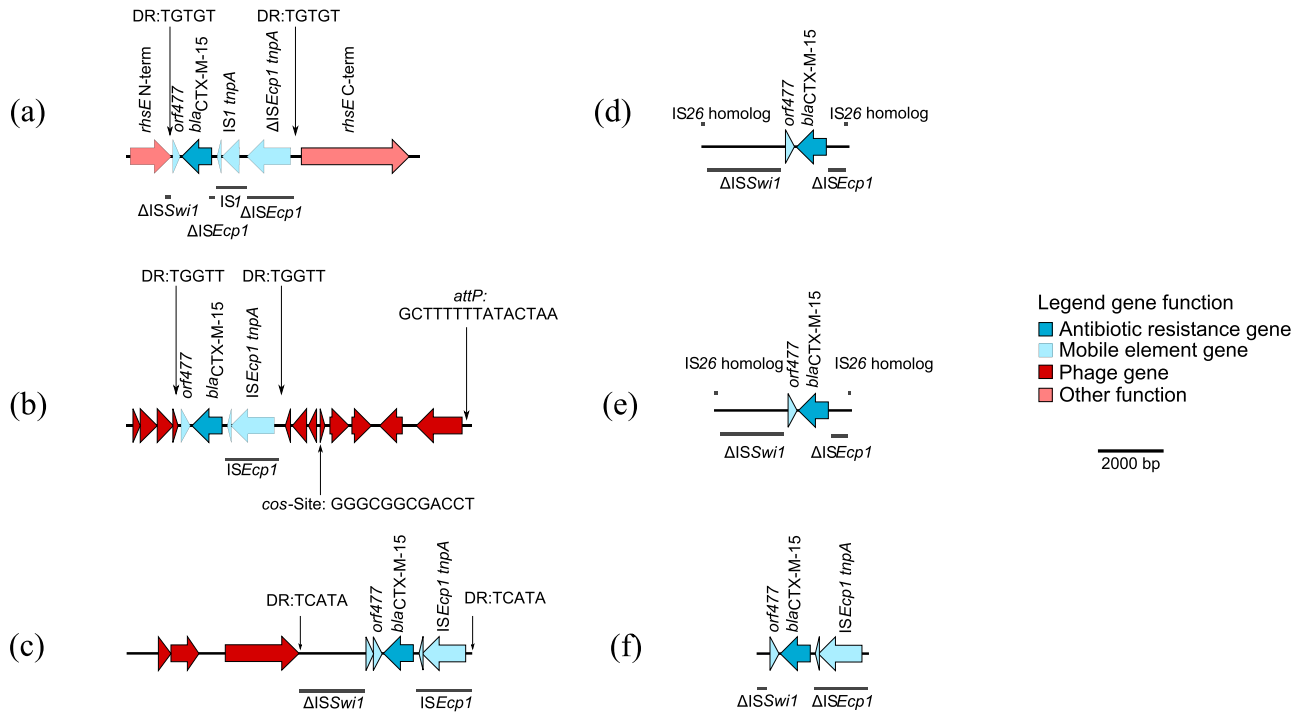

Fig. 3. Location of $bla_{CTX\text{-}M\text{-}15}$ present in the *Escherichia coli* sequence type 410 (ST410) isolates. (a) to (c) = chromosomal insertion; (d) to (f) = plasmid-like structures.

yersiniabactin operon (*fyuA*, *irp1*, *irp2*, *ybtAEPQSTUX*) was present in isolates of clades D and E.

The yersiniabactin operon was part of a PAI similar to a high pathogenicity island (HPI) present in ST131 isolates [3]. A single PAI ($ETT2_{sepsis}$, accession no. DQ077151), originally identified in enterohaemorrhagic *E. coli* O157:H7 isolates, was present in isolates of clades A, B and E. In isolates of clade C and D (with exception of isolate R37), an insertion sequence was integrated into $ETT2_{sepsis}$.

3.6. Plasmid analysis

The number and sizes of plasmids are shown in Table 2. All or nearly all isolates of each clade harboured plasmids that were of similar size. Plasmid sizes ranged from 6 kb to 190 kb. Other than isolate E003488, all isolates harboured IncF incompatibility group (FIA, FIB and/or FII) plasmids. Two isolates of clade C (isolates E006910 and E003488) carried an additional IncI1 plasmid.

4. Discussion

The genetic relationship of recently sampled CTX-M-15-producing *E. coli* isolates from livestock, companion animals, farm environmental sources and humans from different regions in Germany was examined using WGS. The majority of these isolates ($n = 54$) belonged to only one of four STs (ST410, ST131, ST224 and ST648), whilst the remaining 43 isolates represented 22 STs indicating that multiple acquisition of $bla_{CTX\text{-}M\text{-}15}$ gene occurred in many different *E. coli* phylogenetic backgrounds. ST410 presented the largest group of CTX-M-15-producing isolates and was the only ST that was commonly found in companion animals, livestock, farm environmental

Fig. 4. Analysis of the location of the $bla_{CTX\text{-}M\text{-}15}$ gene in the 97 *Escherichia coli* isolates that were sequenced.

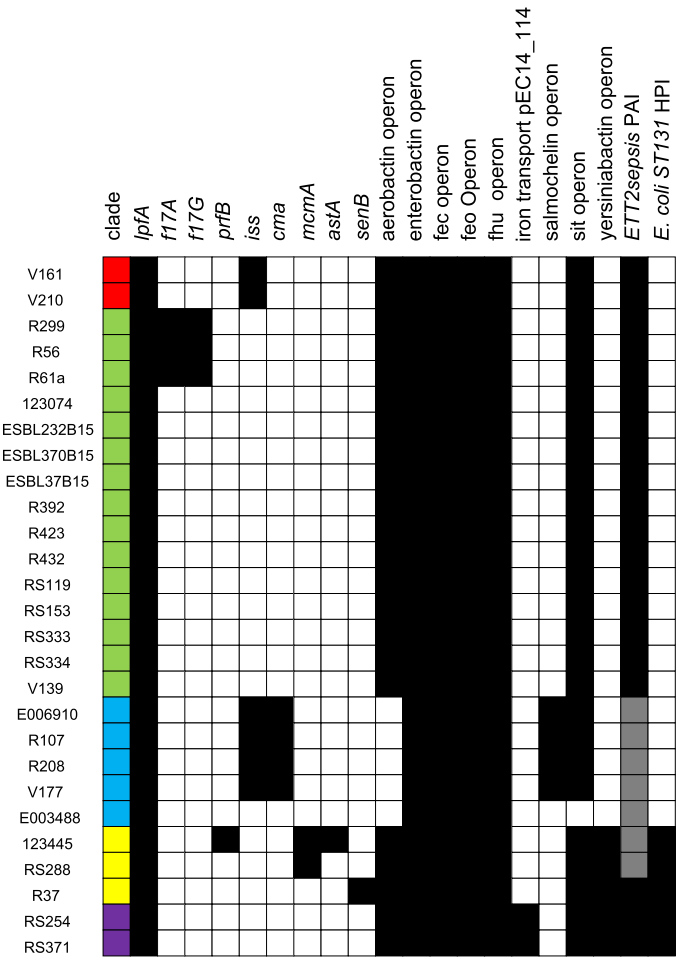

Fig. 5. Virulence genes present in *Escherichia coli* sequence type 410 (ST410) isolates. Colours depict the different clades: red = clade A; green = clade B; blue = clade C; yellow = clade D; and purple = clade E. White/black/grey = absence/presence or insertion within gene/operon.

samples and humans. Whole genome-based epidemiological analysis of this ST provides strong evidence for clonal dissemination and exchange of bacteria within and between these populations.

Isolates of two $bla_{CTX\text{-}M\text{-}15}$-producing *E. coli* clades (B and C) present in humans and animals were found to be virtually identical and separated by <100 SNPs, strongly supporting the hypothesis for interspecies dissemination of *E. coli* isolates and indicative of epidemiological linkage. These clades also harbour identical insertions of $bla_{CTX\text{-}M\text{-}15}$ at distinct chromosomal locations, and clade B in particular is represented in all the host populations and their environment.

It has been previously suggested that chromosomal locations of $bla_{CTX\text{-}M\text{-}15}$ are relatively uncommon [32]. However, the current results demonstrate that chromosomal insertions are, in fact, quite common in the various STs. Insertion of $bla_{CTX\text{-}M\text{-}15}$ into specific regions of the chromosomes can be used as an additional epidemiological marker and, in combination with SNP analysis, is a powerful tool to track clonal dissemination.

These findings differ from data presented by de Been et al. for CTX-M-1 producing *E. coli* of other STs (mainly ST10, ST58 and ST117) [11]. In that study, isolates that were previously deemed to be virtually identical when characterised by conventional genotyping methods (MLST, PFGE, plasmid profile and detection of antibiotic resistance genes) were markedly different when analysed using WGS. Hence, it was postulated that common plasmids and not common clones were responsible for the spread of cephalosporin resistance genes. In the current study, S1-PFGE-derived plasmid profiles indicated that although the various clades harbour commonly occurring FII, FIA and FIB plasmids of similar sizes, resistance to third-generation cephalosporins in clades B and C is not plasmid-encoded but is associated with the dissemination of bacterial clones harbouring distinct chromosomal insertions of $bla_{CTX\text{-}M\text{-}15}$.

ST410 are members of phylogenetic group A, comprising isolates generally considered to be commensals or as being non-pathogenic. A recent phylogenetic classification study carried out by Turrientes et al. suggested that ST410 is in fact a founder member of the clonal complex 23 (CC23) juxtaposed between phylogenetic groups A and B1 [33]. In the isolates in the current study, we detected the presence of genes essential for survival in iron-depleted environments, such as in serum, and the presence of the PAI $ETT2_{sepsis}$, which has been previously shown to contribute to pathogenesis in chickens [34]. In addition, recent publications indicate that isolates of CC23 are associated with infections in animals and humans and may have significant pathogenic potential [35].

The global spread of $bla_{CTX\text{-}M\text{-}15}$ is thought to be mainly associated with spread of the ST131 FQ-resistant H30-Rx clone worldwide [3]. Nevertheless, ST131 isolates are only rarely isolated from animal sources, with the exception of companion animals, as also demonstrated in this study [3]. Here we identified FQ-resistant isolates of a different ST (ST410) that has an even broader distribution than ST131 among different populations (humans, companion animals and livestock) and the farm environment. Unlike ST131, where spread of the $bla_{CTX\text{-}M\text{-}15}$ gene is largely plasmid driven [36], dissemination of FQ-resistant ST410 isolates harbouring this resistance gene is mainly driven by clonal dispersion. Major clades of ST410 may be responsible for the spread of $bla_{CTX\text{-}M\text{-}15}$ into very diverse environments. Indeed, it has recently been reported that highly related ST410 isolates are present both in wild birds and clinical isolates in Germany [37].

Other studies have reported the detection of ST410 isolates resistant to last-line carbapenem antibiotics (e.g. harbouring $bla_{KPC\text{-}2}$, $bla_{NDM\text{-}1}$) [38,39]. In addition, identification of ST410 isolates with multiple ($bla_{KPC\text{-}2}$ with $bla_{CTX\text{-}M\text{-}3}$ or $bla_{CTX\text{-}M\text{-}15}$) antibiotic resistance genes [38] highlights the possibility of the emergence of pan-resistant isolates with epidemic potential within this ST. Currently, most of the data on FQ-resistant ESBL-producing *E. coli* ST410 derive from studies conducted in Germany and may reflect a local situation. Future studies surveying for the presence of ST410 clades will provide information as to whether this is also the case in other countries worldwide.

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Competing interests: None declared.

Ethical approval: This study was approved by the Ethics Committee of the Medical Faculty of the Justus-Liebig University Giessen [AZ: 95/11].

Appendix. Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.ijantimicag.2016.03.019.

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Anhang G

Irrgang A, **Falgenhauer L**, Fischer J, Ghosh H, Guiral E, Guerra B, Schmoger S, Imirzalioglu C, Chakraborty T, Hammerl JA, Käsbohrer A. 2017. CTX-M-15-producing *E. coli* isolates from food products in Germany are mainly associated with an IncF-Type plasmid and belong to two predominant clonal *E. coli* lineages. Front Microbiol. 8:2318.

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CTX-M-15-Producing *E. coli* Isolates from Food Products in Germany Are Mainly Associated with an IncF-Type Plasmid and Belong to Two Predominant Clonal *E. coli* Lineages

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Extended-spectrum beta-lactamases (ESBL) mediating resistance to 3rd generation cephalosporins are a major public health issue. As food may be a vehicle in the spread of ESLB-producing bacteria, a study on the occurrence of cephalosporin-resistantu *Escherichia coli* in food was initiated. A total of 404 ESBL-producing isolates were obtained from animal-derived food samples (e.g., poultry products, pork, beef and raw milk) between 2011 and 2013. As CTX-M-15 is the most abundant enzyme in ESBL-producing *E. coli* causing human infections, this study focusses on *E. coli* isolates from food samples harboring the $bla_{\text{CTX-M-15}}$ gene. The $bla_{\text{CTX-M-15}}$ gene was detected in 5.2% ($n = 21$) of all isolates. Molecular analyses revealed a phylogenetic group A ST167 clone that was repeatedly isolated from raw milk and beef samples over a period of 6 months. The analyses indicate that spread of CTX-M-15-producing *E. coli* in German food samples were associated with a multireplicon IncF (FIA FIB FII) plasmid and additional antimicrobial resistance genes such as *aac(6)-Ib-cr*, $bla_{\text{OXA}-1}$, *catB3*, different *tet*-variants as well as a class 1 integron with an *aadA5/dfrA17* gene cassette. In addition, four phylogenetic group A ST410 isolates were detected. Three of them carried a chromosomal copy of the $bla_{\text{CTX-M-15}}$ gene and a single isolate with the gene on a 90 kb IncF plasmid. The $bla_{\text{CTX-M-15}}$ gene was always associated with the IS*Ecp1* element. In conclusion, CTX-M-15-producing *E. coli* were detected in German food samples. Among isolates of different matrices, two prominent clonal lineages, namely A-ST167 and A-ST410, were identified. These lineages may be important for the foodborne dissemination of CTX-M-15-producing *E. coli* in Germany. Interestingly, these clonal lineages were reported to be widely distributed and especially prevalent in isolates from humans and

livestock. Transmission of CTX-M-15-harboring isolates from food-producing animals to food appears probable, as isolates obtained from livestock and food samples within the same time period exhibit comparable characteristics as compared to isolates detected from human. However, the routes and direction of transmission need further investigation.

Keywords: antimicrobial resistance, CTX-M-15, livestock, genome, plasmid, distribution, ESBL

INTRODUCTION

Resistance to 3rd generation cephalosporins in bacterial pathogens is of great concern in human medicine, since treatment options become increasingly limited in infections caused by multidrug-resistant Enterobacteriaceae. The most common resistance mechanisms in 3rd generation cephalosporin-resistant Enterobacteriaceae is the production of beta-lactamases (ESBL, AmpC and carbapenemases). The emergence and dissemination of ESBL-producing Enterobacteriaceae is mainly driven by horizontal gene transfer, especially conjugation/mobilization, as the enzymes are usually encoded on plasmids (Bonnet, 2004; Carattoli, 2013). Epidemic plasmids, which are detected amongst farm and companion animals, food and humans, belong to the incompatibility groups (Inc.,) F, A/C, N, HI2, I1 and K (EFSA Panel on Biological Hazards, 2011). However, increasing reports of chromosomal localization of antibiotic resistance genes, indicates that spread of the cephalosporin resistance might also be mediated via clonal spread (Hirai et al., 2013; Price et al., 2013; Rodriguez et al., 2014). The ESBL/AmpC genes are sometimes flanked by mobile genetic elements (e.g., transposons, IS elements or class 1 integrons), which are also responsible for successful transmission, and in case of IS*Ecp1* and IS*CR1,* also involved in the expression of the genes (Poirel et al., 2008).

ESBL-producing isolates are frequently reported from samples of livestock origin. Spread and persistence has been demonstrated in different studies (Carattoli, 2009; Liebana et al., 2013). Transmission of ESBL/AmpC-producing *Escherichia coli* from animal to humans is assumed. Contaminated food as a transmission vehicle is often discussed, but direct evidence to support this hypothesis is rare (Leverstein-van Hall et al., 2011). Often, transmission is suggested by indirect evidence through the detection of similar clones, plasmids or sequence types in different populations (EFSA Panel on Biological Hazards, 2011). In Germany, infections with ESBL-producing *E. coli* in humans are most commonly associated with CTX-M-15 enzymes, followed by CTX-M-1, -14 and -27 (Ewers et al., 2012; Valenza et al., 2014; Falgenhauer et al., 2016a; Pietsch et al., 2017). In contrast, the most common ESBL-type in animals is CTX-M-1, whereas CTX-M-15 is underrepresented in samples from animal livestock in European countries (EFSA Panel on Biological Hazards, 2011; Day et al., 2016). Similar observations were also made in food. Studies from Germany on chicken meat revealed that the most detected ESBL enzymes belonged to the CTX-M-1 type or SHV (Kola et al., 2012; Campos et al., 2014). Neither in these studies nor in a comprehensive study on ESBL in food from the UK, CTX-M-15 enzymes could be detected (Randall et al., 2017). Nevertheless, $bla_{\text{CTX-M-15}}$-encoding *E. coli* from animal sources in Europe have been described (Lopez-Cerero et al., 2011; Valentin et al., 2014). The risk of contaminated food for the consumers was clearly shown within the German EHEC-outbreak in 2011 caused by the consumption of fenugreek sprouts contaminated with CTX-M-15-producing *E. coli* O104:H4 clone (Beutin and Martin, 2012; Weiser et al., 2013).

One of the aims of the German national research consortium RESET (2011–2016) was to reveal possible transmission pathways for ESBL/AmpC-producing Enterobacteriaceae. Harmonized protocols were established for the isolation of phenotypically cephalosporin-resistant bacteria from livestock, environment, food, companion animals, and humans to generate a comparable set of data. A previous study on CTX-M-15-producing *E. coli* of livestock origin (Fischer et al., 2014) found a frequent occurrence of isolates belonging to the clonal complex 10 (CC10), as well as clonal spread of ST410 isolates. Supporting this, a phylogenetic analysis based on whole genome data of CTX-M-15-producing isolates obtained from German livestock, companion animals, humans and environment was carried out, revealing interspecies dissemination of ST410 clones (Falgenhauer et al., 2016a). In the present study, CTX-M-15-producing *E. coli* isolated from animal food samples of different matrices were taken in the same period (2011–2013) as the livestock samples and were comprehensively characterized.

MATERIALS AND METHODS

Bacterial Isolates and Cultivation

More than 2,500 food samples of different origins (poultry, cattle, swine, vegetables) and matrices (meat and meat preparations, raw milk, cheese, vegetables) were taken by official food inspectors and investigated by German state laboratories (Saxony, Lower Saxony, Hesse, Bavaria). Food samples from processing plants, retail and raw milk samples were collected at the farm level. From each sample, 25 g were investigated by a non-selective pre-enrichment step for 18–24 h at 37°C in lysogeny broth (LB) following selective cultivation of 10 µl aliquots on MacConkey agar supplemented with 1 mg/L cefotaxime (CTX, Sigma-Aldrich, Munich, Germany) for 18–24 h at 37°C. The identification of *E. coli* was confirmed by MALDI-TOF (Biotyper, Bruker). From each sample one *E. coli* isolate phenotypically resistant to CTX was sent to the German National Institute for Risk Assessment (BfR). Positive samples were obtained from all analyzed matrices, even though only raw milk cheese was burdened and only one vegetable sample was tested positive. The ESBL genotype of 437 isolates was verified by PCR and Sanger sequencing as previously described (Rodriguez et al., 2009). Isolates positive for $bla_{\text{CTX-M-15}}$ were

included in this study and further characterized. Phylogenetic groups were classified as previously described (Doumith et al., 2012). The antimicrobial resistance pattern was determined by microbroth dilution according to CLSI guidelines (CLSI M07-A9) at the National Reference Laboratory for Antimicrobial Resistance (NRL-AR, BfR). The used antimicrobial panel was in concordance to the decision 2013/652/EU of the European Union commission and was carried out with microtiter plates from TREK Diagnostic Systems (Thermo Fisher Scientific, Schwerte, Germany).

Molecular Typing and Characterization

Molecular characterization was performed using pulsed-field gel-electrophoresis (PFGE) and multi-locus sequence typing (MLST). Phylogenetic relationship of the isolates was determined using XbaI-PFGE analysis according to the PulseNet protocol (https://www.cdc.gov/pulsenet/pathogens/protocols.html). MLST was performed using the Achtman scheme (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, *recA*; http://mlst.warwick.ac.uk/mlst/dbs/Ecoli).

The location of the $bla_{\text{CTX-M-15}}$ gene was determined by S1-nuclease PFGE (1–25 s 17 h, 120°, 6 V/cm) with subsequent southern blot hybridization using a $bla_{\text{CTX-M-15}}$ PCR-probe (Rodriguez et al., 2009). A chromosomal location of the $bla_{\text{CTX-M-15}}$ gene was assumed for isolates in which no positive signal had been detected. Plasmids harboring the $bla_{\text{CTX-M-15}}$ gene were isolated by alkaline lysis and transformed into *E. coli* DH10B™ competent cells (Invitrogen™, Thermo Fisher Scientific, Schwerte, Germany) (Birnboim and Doly, 1979; Rodriguez et al., 2009). Selection of transformed cells was carried out on LB agar supplemented with 1 mg/L CTX. Transformation of plasmids was confirmed by PCR. Incompatibility groups of the transferred plasmids were determined by PCR using the PBRT kit (Diatheva, Cartoceto PU, Italy). When transformation experiments were inconclusive, incompatibility group of the $bla_{\text{CTX-M-15}}$ harboring plasmid was determined using PFGE/southern blot hybridization with probes specific for IncF and IncI1. The IS*Ecp*1 element was detected by using modified ALA3/ALA4 Primer (5′-TTTGCGCATACAGCGGCACAC-3′/5′-CTATCCGT**A**CAAGGGAG-3′) (Rodriguez et al., 2014).

Next Generation Sequencing (NGS) and *in silico* Analyses

Additionally, whole genome sequencing of the isolates was performed. Therefore, genomic DNA was isolated from overnight cultures using the PureLink® Genomic DNA Mini Kit (Thermo Fisher Scientific, Schwerte, Germany). A NexteraXT library was generated and sequenced on a MiSeq benchtop sequencer (Illumina, CA, USA) with 2×300 bp paired-end reads. Raw reads were assembled using SPAdes (v 3.5.0) (Bankevich et al., 2012). Whole-genome-based phylogenetic analysis was performed using HarvestSuite (ParSNP) (Treangen et al., 2014).

Resistance genes, virulence genes, serotype and pMLST were predicted using the web-based tools of the Center for Genomic Epidemiology (Zankari et al., 2012; Carattoli et al., 2014; Joensen et al., 2014, 2015).

Accession Numbers

Whole genome sequences of the isolates have been deposited in the European Nucleotide Archive (ENA). Accession numbers of isolates RL16, RL25, RL36, RL40, RL63, RL162, RL195, RL212, RL224, RL230, RL239, RL330, RL331, RL345, RL346, RL364, RL379, RL406-0, RL452,RL464, and RL465 are summarized in the Table S2.

RESULTS AND DISCUSSION

Persistence of the ST167 Clone amongst German CTX-M-15 Food Isolates

From 437 *E. coli* isolates phenotypically resistant to 3rd generation cephalosporins obtained from animal derived food, 404 isolates were confirmed as ESBL/AmpC-producing bacteria. Of these, 21 (5.2%) isolates harbored the $bla_{\text{CTX-M-15}}$ gene. This is in agreement with the observations, that while resistance to 3rd generation cephalosporins in Germany and other European countries is frequently mediated by CTX-M-15 enzymes in isolates from human origin, they are of low prevalence in bacteria from livestock (Pfeiffer et al., 2013; Brolund, 2014; Valentin et al., 2014). A comparable study from the UK even found no CTX-M-15-producing *E. coli* in food samples from animals and non-animal sources while there was an overall prevalence of ESBL-producing *E. coli* of 27.5% of the meat samples (Randall et al., 2017)

The main characteristics of the isolates is given in **Table 1**. There are distinct similarities regarding detected STs, pMLST of IncF plasmids, class 1 integrons or virulence between isolates obtained from food and animal origin. These results suggest a transmission from animal to food (Fischer et al., 2014).

There is a predominance of isolates belonging to clonal complex (CC) 10 ($n = 8$) and CC23 ($n = 4$) of the phylogenetic group A. Although the sequence types (ST) 38 and ST131 are typically observed in humans (Rodriguez et al., 2014),the frequent presence of ST167/ST617 (CC10) and ST410 (CC23) isolates from food samples in this study concurs with previous reports of isolates from animal samples within the same time period (2011–2013) and from human stool samples. In particular, those reported isolates harbored $bla_{\text{CTX-M-15}}$ and were members of the same phylogenetic group (Fischer et al., 2014; Ben Sallem et al., 2015). This underlines a possible transmission from animals to humans via contaminated food.

All CC10 isolates belong to ST167. Five of them showed an almost identical XbaI PFGE pattern (P1; **Figure 1**). These strains have been isolated over a period of 6 months from samples of raw milk ($n = 4$) and beef ($n = 1$) in Saxony (Eastern Germany). Isolates were obtained from four different samples taken at different time points from three different postal code locations. Milk samples were obtained from farms that were nearby, whereas the beef sample was taken about 200 km away. Therefore, a geographical spread of the clone might have occurred. In general, the phylogenetic group A clonal complex 10 (ST10/167/617) represents a successful clonal lineage, which can be found in humans, livestock, as well as in companion animals (Ewers et al., 2012). In this study two additional ST167 isolates

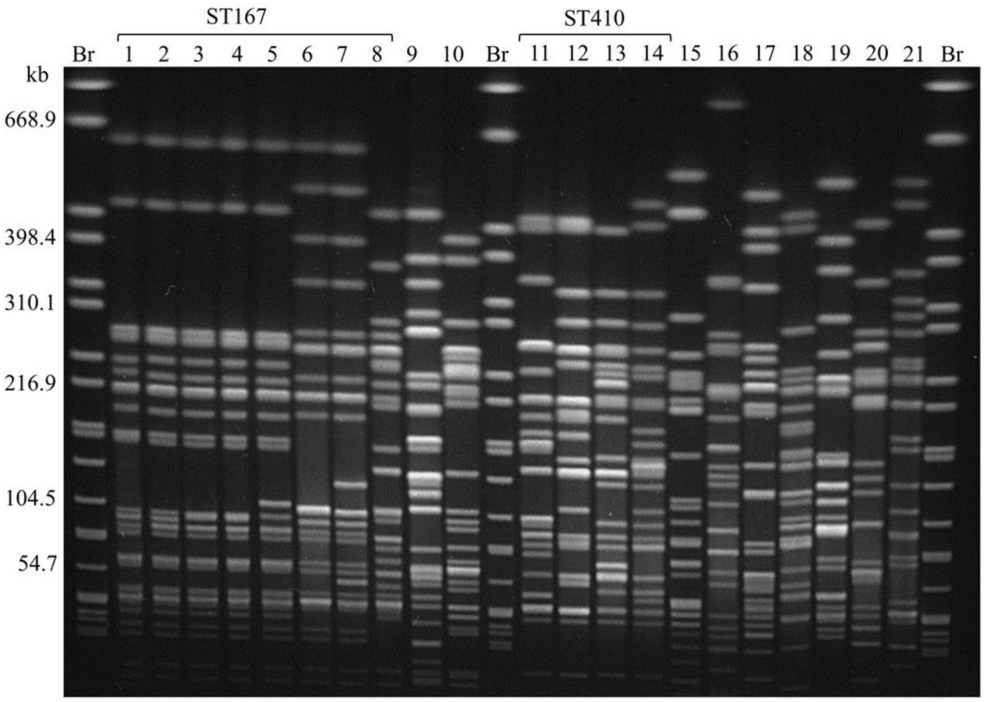

FIGURE 1 | XbaI PFGE of *E. coli* isolates obtained from food harboring the $bla_{CTX-M-15}$ gene. Br = *S.* Braenderup H9812, used as size standard. Lanes:

1: RL16	2: RL40	3: RL330	4: RL331	5: RL364	6: RL345	7: RL346
8: RL36	9: RL162	10: RL63	11: RL25	12: RL212	13: RL406-0	14: RL465
15: RL195	16: RL452	17: RL239	18: RL224	19: RL230	20: RL2379	21: RL464

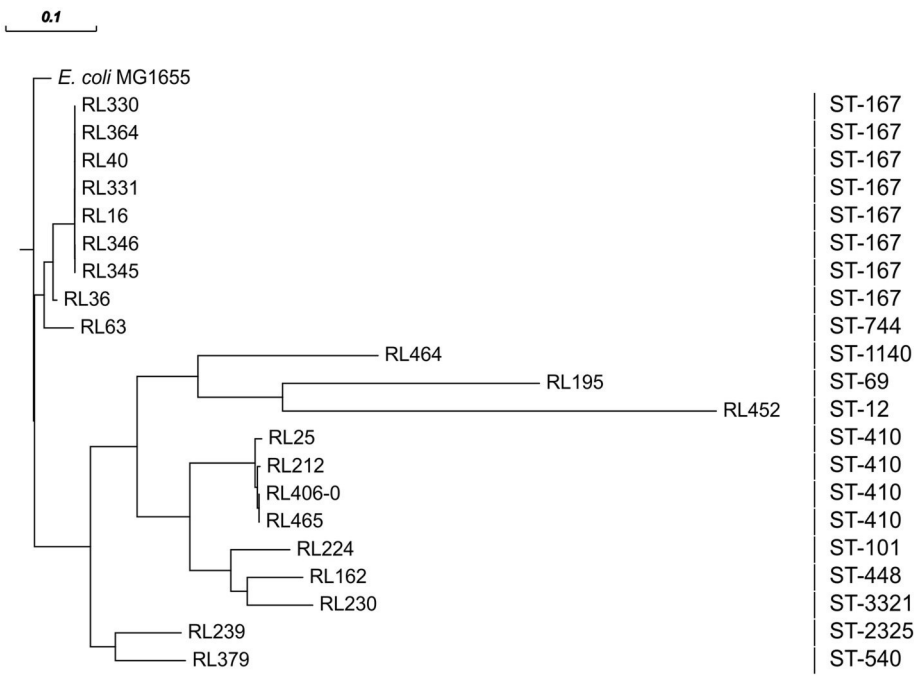

FIGURE 2 | Phylogenetic analysis of all 21 sequenced CTX-M-15-producing isolates obtained from food performed by HarvestSuite (ParSNP), using *E. coli* MG1655 (NC_000913) as a reference. The scale defines the degree of the phylogenetic relationship of the isolates.

TABLE 1 | Overview of the characteristics of CTX-M-15-producing *E. coli* isolates obtained from food samples.

Isolate no.	Source	Isolation date (federal state[a])	Resistance phenotype/acquired resistance genes	Phylogenetic group	PFGE pattern[a]	MLST (Clonal complex)	CTX-M-15 -plasmid size	Inc., Group (pMLST)	Class 1 integron[b]
RL16	raw milk	06/08/2012 (S)	AMP, CIP, FOT, NAL, SMX, TAZ, TET, TMP/*aac(6′)Ib-cr*, *aadA5*, $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, *catB3*-like, *dfrA17*, *mph(A)*, *sul1*, *tet(B)*	A	P1	ST167 (CC10)	160 kb	FII, FIA, FIB (F31:A4:B1)	1,664 kb/ *dfrA17 aadA5*
RL25	turkey meat (steak)	24/08/2012 (H)	AMP, CHL, CIP, FOT, KAN, NAL, SMX, STR, TAZ, TET, TMP / *aadA5*, *aph(3′)-Ia*, $bla_{CTX\text{-}M\text{-}15}$, bla_{TEM-1B}, *catA1*-like, *dfrA17*, *mph(A)*,*strA*, *strB*, *sul1*, *sul2*, *tet(A)*	A		ST410 (CC23)	None[c]	–	1,664 kb/ *dfrA17 aadA5*
RL36	beef (shoulder)	11/05/2012 (S)	AMP, CIP, FOT, SMX, STR, TET, TMP / *aac(3)-IId*, $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, *mph(A),strA*, *strB*, *sul2*,*tet(B)*	A		ST167 (CC10)	None[c]	–	
RL40	beef (chuck)	18/06/2012 (S)	AMP, CIP, FOT, NAL, SMX, TAZ, TET, TMP/*aac(6′)Ib-cr*, *aadA5*, $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, *catB3*-like, *dfrA17*, *mph(A)*, *sul1*, *tet(B)*	A	P1	ST167 (CC10)	160 kb	FII, FIA, FIB (F31:A4:B1)	1,664 kb/ *dfrA17 aadA5*
RL63	raw milk	20/09/2012 (H)	AMP, CHL, CIP, FOT, GEN, KAN, NAL, SMX, STR, TAZ, TET, TMP/*aac(3)-IIa*,*aac(6′)Ib-cr*, *aadA2*, *aadA5*, *aph(3′)-Ia*, $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, $bla_{TEM\text{-}1B}$, *catB3*, *catA1*, *dfrA12*, *dfrA17*, *mph(A)*, *sul1*, *sul2*, *strA*,*strB*, *tet(A)*, *tet(B)*	A		ST744 (none)	165 kb	FII, FIA, FIB (F22:A1: B20)	two: 1,664 kb *dfrA17, aadA5;* ∼1,900kb *dfrA12, aadA2*
RL162	ground beef	31/08/2012 (BAV)	AMP, CIP, FOT, NAL, SMX, STR, TAZ, TET / $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, *catA1*, *strA*, *strB*, *sul2*, *tet(A)*	A		ST448 (CC448)	80 kb	I1 (ST-31)	
RL195	beef	09/10/2012 (BAV)	AMP, FOT, KAN, STR, TAZ, TET/ *aph(3′)-Ic*-like, $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, *strA*-like, *strB*,*tet(B)*-like	D		ST69 (CC69)	78 kb	I1 (ST-31)	
RL212	suckling pig (shoulder)	17/10/2012 (BAV)	AMP, CIP, FOT, NAL, SMX, STR, TAZ, TET, TMP/*aac(6′)Ib-cr, aadA5* ,$bla_{CTX\text{-}M\text{-}15}$, bla_{OXA-1}, *catB3*-like, *dfrA17, mph(A), strA, strB, sul1, sul2, tet(A)*	A		ST410 (CC23)	90 kb	FII, FIA, FIB (F31:A4:B1)	1,664 kb/ *dfrA17 aadA5*
RL224	pork	19/10/2012 (BAV)	AMP, FOT, SMX, STR, TAZ, TET, TMP / $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, *dfrA5*, *strA, strB*, *sul2*, *tet(A)*	A		ST101 (CC101)	48 kb	N (ST-3)	∼700 bp/ *dfrA5*

(Continued)

TABLE 1 | Continued

Isolate no.	Source	Isolation date (federal state[a])	Resistance phenotype/acquired resistance genes	Phylo-genetic group	PFGE pattern[a]	MLST (Clonal complex)	CTX-M-15 -plasmid size	Inc., Group (pMLST)	Class 1 integron[b]
RL230	ground pork	15/11/2012 (H)	AMP, FOT, KAN, SMX, STR, TAZ, TET, TMP / *aph(3′)-Ic*-like, $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, *dfrA5, strA, strB, sul2-like, tet(B)*	B1		ST3321 (none)	90 kb	I1 (ST-31)	~700 bp/ d*frA5*
RL239	raw milk	14/11/2012 (BAV)	AMP, FOT, TAZ/$bla_{CTX\text{-}M\text{-}15}$	A		ST2325 (none)	60 kb	I2 (NA)	
RL330	raw milk	17/12/2012 (S)	AMP, CIP, FOT, NAL, SMX, TAZ, TET, TMP */aac(6′)Ib-cr ,aadA5,* $bla_{CTX-M-15}$, $bla_{OXA\text{-}1}$, *catB3-like, dfrA17, mph(A), sul1, tet(B)*	A	P1	ST167 (CC10)	160 kb	FII, FIA, FIB (F31:A4:B1)	1,664 kb/ *dfrA17 aadA5*
RL331	raw milk	17/12/2012 (S)	AMP, CIP, FOT, KAN, NAL, SMX, TAZ, TET, TMP */aac(6′)Ib-cr*, *aadA5*, $bla_{CTX\text{-}M\text{-}15}$, $bla_{OXA\text{-}1}$, *catB3*-like, *dfrA17*, *mph(A)*, *sul1*, *tet(B)*	A	P1	ST167 (CC10)	160 kb	FII, FIA, FIB (F31:A4:B1)	1,664 kb/*dfrA17 aadA5*
RL345	turkey meat (schnitzel)	21/12/2012 (S)	AMP, CHL, CIP, FOT, KAN, NAL, SMX, STR, TAZ, TET, TMP/*aac(6′)Ib-cr*, *aadA1*, *aadA2*-like, *aadA5*, $bla_{CTX-M-15}$, bla_{OXA-1}, $bla_{TEM\text{-}1C}$, *catB3*-like, *cmlA1*-like, *dfrA17*, *mph(A)*, *strA*-like, *strB*, *sul1*, *sul2*, *sul3*, *tet(A)*	A	P2	ST167 (CC10)	150 kb	FII, FIA, FIB (F31:A4:B1)	1,664 kb/*dfrA17 aadA5*
RL346	turkey meat (schnitzel)	21/12/2012 (S)	AMP, CHL, CIP, FOT, KAN, NAL, SMX, STR, TAZ, TET, TMP/*aac(6′)Ib-cr*, *aadA1*, *aadA2*, *aadA5*, $bla_{CTX-M-15}$, bla_{OXA-1}, $bla_{TEM\text{-}1C}$, *catB3*, *cmlA1*, *dfrA17*, *mph(A)*, *strA*, *strB*, *sul1*, *sul2*, *sul3*, *tet(A)*	A	P2	ST167 (CC10)	190 kb	FII, FIA, FIB (F31:A4:B1)	1,664 kb/*dfrA17 aadA5*
RL364	raw milk	14/02/2013 (S)	AMP, CIP, FOT, NAL, SMX, TAZ, TET, TMP/*aac(6′)Ib-cr*, *aadA5*, $bla_{CTX\text{-}M\text{-}15}$, bla_{OXA-1}, *catB3*-like, *dfrA17*, *mph(A)*, *sul1*, *tet(B)*	A	P1	ST167 (CC10)	160 kb	FII, FIA, FIB (F31:A4:B1)	1,664 kb/ *dfrA17 aadA5*
RL379	pork (rib)	07/03/2013 (H)	AMP, CHL, CIP, FOT, NAL, SMX, STR, TAZ, TET/$bla_{CTX\text{-}M\text{-}15}$, *catA1*-like, *strA*, *strB*, *sul2*, *tet(B)*	A		ST540 (none)	110 kb	FII, FIA, FIB (F1:A1:B49)	
RL406-0	chicken giblets	04/04/2013 (S)	AMP, CIP, FOT, NAL, SMX, STR, TAZ, TET, TMP/*aadA2*, $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B}$, *dfrA12*, *mph(A)*, *strA*-like, *strB*-like, *sul1*, *sul2*, *tet(A)*	A		ST410 (CC23)	None[c]	–	1,913 kb/ *drfA12, aadA2*
RL452	ground beef	06/02/2013 (LS)	AMP, FOT, STR, TAZ, TET / $bla_{CTX\text{-}M\text{-}15}$, $bla_{TEM\text{-}1B-}$, *strA*, *strB*, *tet(B)*	B2		ST12 (CC12)	82 kb	I1 (ST-31)	

(Continued)

TABLE 1 | Continued

Isolate no.	Source	Isolation date (federal state[a])	Resistance phenotype/acquired resistance genes	Phylo-genetic group	PFGE pattern[a]	MLST (Clonal complex)	CTX-M-15 -plasmid size	Inc., Group (pMLST)	Class 1 integron[b]
RL464	turkey meat (breast)	13/11/2013 (LS)	AMP, CHL, CIP, FOT, SMX, STR, TAZ/*aadA1*, *aadA2*-like, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-135}}$, *cmlA1*-like, *qnrS1*, *sul3*	D		ST1140	105 kb	I1 (ST-36)	>4,000 kb/ *sat psp aadA2, cmlA, aadA1*
RL465	turkey meat (breast)	18/11/2013 (LS)	AMP, CIP, COL, FOT, KAN, NAL, SMX, STR, TAZ, TET, TMP/*aadA1*,*aph(3')-Ia*, $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$, *dfrA1*, *mcr-1*, *strA*-like, *strB*-like, *sul2*, *sul3*, *tet(A)*-like	A		ST410 (CC23)	none[c]	-	~1,500 kb/ *dfrA1, aadA1*

Antimicrobials: AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; COL, colistin; FOT, cefotaxim; KAN, kanamycin; NAL, nalidixic acid; SMX, sulfamethoxazole; STR, streptomycin; TAZ, ceftazidim; TET, tetracycline; TMP, trimethoprim; Regions: S, Saxony; H, Hesse; LS, Lower Saxony; BAV, Bavaria.

[a]*Please see* ***Figure 1****.*

[b]*PCR amplicons using CS5'/CS3' Primers (Rodriguez et al., 2009) or identified by analyzing NGS data.*

[c]*Chromosomally located.*

obtained from turkey meat showed similar PFGE restriction patterns (P2; **Figure 1**) which are distinguishable from those of the raw milk isolates. Nevertheless, all eight ST167 isolates cluster when performing phylogenetic analysis based on whole genome sequences (**Figure 2**) and all isolates of this clade belong to the same serotype as shown by NGS data (Table S1).

Detection of a Circulating ST410 Clone and the Impact of the Chromosomal Localization of the Beta-Lactamase Gene

There are several isolates ($n = 4$) belonging to ST410 harboring mainly a chromosomal location of the $bla_{\text{CTX-M-15}}$ gene. Only in one isolate (RL212) the $bla_{\text{CTX-M-15}}$ was located on a 90 kb multireplicon IncF plasmid as described above. However, NGS data revealed a close phylogenetic relationship of all isolates (**Figure 2**). In the Supplementary Material, a comparison based on whole genome sequences with ST410 isolates from German livestock, companion animals, humans and environment is shown (Supplementary Figure 1). The food-related isolates can be found in three of the five clades (B, C, D), which otherwise comprise of isolates from farm or farm environment-related samples as well as samples of human origin. In concordance with the other strains of clade B, $bla_{\text{CTX-M-15}}$ of RL25 integrated at a distinct location in the *rhs*E cassette, which is known as a hotspot for insertion sequences and recombination in *E. coli* (Saier, 2008). The $bla_{\text{CTX-M-15}}$ of RL465 (Falgenhauer et al., 2016b) and RL406-0 also integrated at the same location known for the other members of clade C, at a defective lambdoid prophage region. The results of the current study further extend previous findings of interspecies circulation of ST410 clones to include food and point out the potential risk of contaminated food as transmission vehicles for consumers (Falgenhauer et al., 2016a).

Apart from the three ST410 isolates, a chromosomal localization is also likely for RL63 (ST176). The stable integration of the $bla_{\text{CTX-M-15}}$ genes into the chromosome is reported for different MLST variants (Falgenhauer et al., 2016a). These findings demonstrate that the chromosomal integration of the $bla_{\text{CTX-M-15}}$ gene occurred in several independent events and emphasize that a chromosomal location of this gene might be more common than anticipated (Rodriguez et al., 2014).

$bla_{\text{CTX-M-15}}$ Is Mainly Located on Plasmids of the Incompatibility Group IncF

Apart from strain RL36, where the $bla_{\text{CTX-M-15}}$ seems to be located on the chromosome, the remaining seven ST167 isolates (PFGE pattern P1 and P2) harbor the $bla_{\text{CTX-M-15}}$ gene on 150–190 kb multireplicon IncFIA/FIB/FII plasmids. These large IncF plasmids (>150 kb), as well as the ST410 IncF plasmid, additionally harbored an *aac(6)-1b-cr* gene (plasmid mediated quinolone resistance gene). The further correlation of $bla_{\text{CTX-M-15}}$-encoding IncF plasmids with the detection of $bla_{\text{OXA-1}}$, *catB3* and *tet* genes is also described by Lopez-Cerero et al. (2011). IncFII plasmids carrying $bla_{\text{CTX-M-15}}$ are known to be highly transferable (Carattoli, 2009). Except for plasmids from RL63 and RL379, all multireplicon IncF plasmids of this

study belong to the pMLST F31:A4:B1, indicating plasmid-related spread of *bla*$_{CTX\text{-}M\text{-}15}$ carrying *E. coli* within different food production chains.

The *bla*$_{\text{CTX-M-15}}$ - *aac(6)-1b-cr* - harboring IncF plasmids were also associated with an 1,664 kb large class 1 integron containing a *dfrA17/aadA5* gene cassette (**Table 1**) encoding for trimethoprim and aminoglycoside resistance. Similar class 1 integrons associated with *bla*$_{\text{CTX-M-15}}$ of phylogenetic group A *E. coli* were detected in isolates of livestock and companion origin as well as in samples of healthy humans worldwide (Dureja et al., 2014; Fischer et al., 2014).

Association of *bla*$_{\text{CTX-M-15}}$ with Mobile Genetic Elements

The *bla*$_{\text{CTX-M-15}}$ gene was associated with an upstream located IS*Ecp1* element in all isolates, and has been frequently reported for *bla*$_{\text{CTX-M-15}}$ positive isolates (Lartigue et al., 2004; Smet et al., 2010). The association with IS*Ecp1* was even detected for chromosomally encoded *bla*$_{\text{CTX-M-15}}$ genes. This suggests that the resistance gene can be easily mobilized. Transposition of *bla*$_{\text{CTX–M}}$ genes associated with the IS*Ecp1* element was demonstrated *in vitro* (Lartigue et al., 2004). The PCR for ISEcp1 was positive in all isolates except for one. For the isolate RL25 (ST410, chromosomal *bla*$_{\text{CTX-M-15}}$) insertion event of an IS1-element into the *tnp*A gene (transposase encoding) was detected at identical position to those found in ST410 isolates of different origin in the same clade (Fischer et al., 2014; Falgenhauer et al., 2016a). IS*Ecp1* elements, which are truncated by different IS elements, are occasionally reported and their effects on mobilization and expression of *bla* genes as well as their role in plasmid evolution have been discussed (Smet et al., 2010; Alonso et al., 2017).

Virulence Associated Genes amongst CTX-M-15 Isolates

In addition, the occurrence of virulence genes in the isolates was examined (Table S1). Most of the food isolates contained relatively few virulence genes. These included bacteriocins, glutamate decarboxylase, capsule synthesizing enzymes and serum survival genes. These virulence associated genes were also recognized in CTX-M-15-producing isolates from animals (Fischer et al., 2014). One isolate (RL346) harbored *senB*, which encodes an enterotoxin, that is responsible for enterotoxic activity of enteroinvasive *E. coli* (EIEC) and *Shigella* spp. (Nataro et al., 1995). Another isolate (RL452) carried two toxin genes (*ncf1*–cytotoxic necrotizing factor, involved in urinary tract infection (Mills et al., 2000), and *vat*–vacuolating autotransporter toxin, known to mediate increased fitness of uropathogenic *E. coli* (UPEC) during systemic infections (Nichols et al., 2016). This isolate belonged to phylogenetic group B2. These findings support the general assumption of low pathogenic potential in isolates of phylogenetic group A and B1 (major phylogenetic groups detected in this study) as compared to the higher virulence properties in isolates of the phylogenetic group B2 and D.

CONCLUSION

In conclusion, *bla*$_{\text{CTX-M-15}}$ positive *E. coli* have been detected in ESBL-producing isolates obtained from food, albeit with a low prevalence. There are two major findings regarding the spread of these resistance genes in these isolates: (1) the *bla*$_{\text{CTX-M-15}}$ can either be spread by successful IncF plasmids (pMLST: F31:A4:B1) or (2) it can be transmitted by clonal spread of ST410 isolates harboring a chromosomally encoded gene. This clone was also found in samples of animal and human origin within the same sampling period. Their virtual identity to animal-derived isolates indicates an animal origin of the isolates found in food samples, although cross-contamination cannot be ruled out. Independently, there is a risk for consumers related to exposure to ESBL genes by contaminated food, although a quantification of this issue is not possible. In future, the distribution of CTX-M-types should be closely monitored in a one-health approach, in particular by whole genome analysis of isolates, to detect actual trends and delineate dissemination pathways of the beta-lactamases.

AUTHOR CONTRIBUTIONS

AK, TC, and BG designed the study. AI, JF, EG, and SS performed the experiments. AI, SS, LF, HG, and CI performed WGS-sequencing and bioinformatics. AI, LF, SS, and JAH analyzed the data. AI, JAH, and LF wrote the manuscript and prepared the tables and figures. All authors edited the manuscript.

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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.2017.02318/full#supplementary-material

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Conflict of Interest Statement: 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.

Anhang H

Falgenhauer L, Waezsada S-E, Gwozdzinski K, Ghosh H, Doijad S, Bunk B, Spröer C, Imirzalioglu C, Seifert H, Irrgang A, Fischer J, Guerra B, Käsbohrer A, Overmann J, Goesmann A, Chakraborty T. 2016. Chromosomal locations of *mcr-1* and *bla*$_{\text{CTX-M-15}}$ in fluoroquinolone-resistant *Escherichia coli* ST410. Emerg Infect Dis 22:1689–1691.

Chromosomal Locations of *mcr-1* and $bla_{CTX-M-15}$ in Fluoroquinolone-Resistant *Escherichia coli* ST410

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To the Editor: Recently, Yi-Yun Liu et al. reported on the discovery of *mcr-1*, a plasmidborne resistance gene mediating resistance to colistin, in isolates obtained from humans and animals (*1*). Since the original publication, *mcr-1* with or without the insertion element IS*Apl1* has been detected on plasmids of different incompatibility groups, including IncI2, IncHI2, and IncX4, and in many different countries (*1–3*). Because colistin is a last-resort parenteral antimicrobial drug, the transfer of *mcr-1* by

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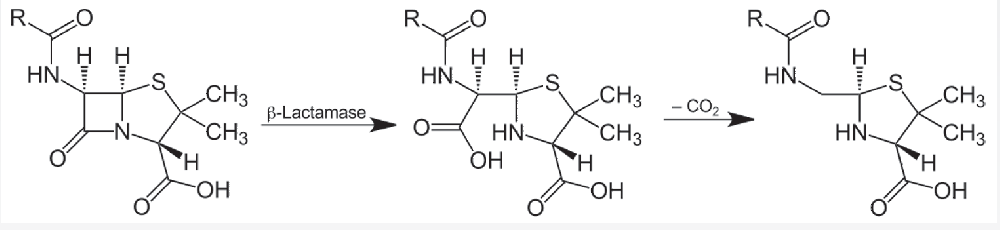
etymologia

β-Lactamase [ba'tə lak'tə-mās]

Enzymes that catalyze the cleavage of β-lactam rings in penicillins, cephalosporins, monobactams, and carbapenems were first described by Abraham and Chain in 1940. These enzymes confer resistance to β-lactam antibiotics on bacteria that produce them. β-lactamases are ancient, theorized to have evolved 1–2 billion years ago, but the emergence and spread of penicillin-resistant staphylococci in hospitals in the 1950s showed how penicillin use could select producers from a population of nonproducers. "Lactam" is a portmanteau of "**lac**tone" (from the Latin *lactis*, "milk," since lactic acid was isolated from soured milk) and "**am**ide." The "β" refers to the nitrogen's position on the second carbon in the ring. The suffix "-ase," indicating an enzyme, is derived from "diastase" (from the Greek *diastasis*, "separation"), the first enzyme discovered in 1833 by Payen and Persoz.



Action of β-lactamase and decarboxylation of the β-lactam ring. Equation by Jü, own work, public domain, https://commons.wikimedia.org/w/index.php?curid=11204303

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conjugation or through mobilizable plasmids raises concern about the emergence of pan-resistant *Enterobacteriaceae*.

We previously described extended-spectrum β-lactamase (ESBL)–producing and carbapenemase-producing isolates obtained from livestock and a human in Germany that harbored the *mcr-1* gene (*2*). Because the transfer of *mcr-1* through the food chain is highly likely, we looked for its presence in 62 whole-genome sequenced ESBL-producing *Escherichia coli* isolates obtained during 2012–2013 from food products sampled in Germany (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/8/16-0692-Techapp1.pdf). We detected 4 isolates harboring the *mcr-1* gene (*E. coli* RL138, RL145, RL158, and RL465) that displayed a colistin MIC of 4 mg/L (online Technical Appendix Table 1). The raw sequencing reads and the assembled contigs of the *mcr-1*–positive isolates were deposited in the European Nucleotide Archive under project accession no. PRJEB13470. We conducted conjugation experiments to analyze the transferability of *mcr-1* (online Technical Appendix). For all isolates except RL465, *mcr-1* was transferable to *E. coli* J53 *Az*^{r}. For isolates RL138, RL145, and RL158, the *mcr-1* gene was present on IncX4 and IncHI2 plasmids (Figure, panel A, http://wwwnc.cdc.gov/EID/article/22/8/16-0692-F1.htm; online Technical Appendix Table 2). The sequence type (ST) 410 *E. coli* isolate RL465 was detected in a turkey hen meat sample from 2013 and harbored $bla_{\text{CTX-M-15}}$ and *mcr-1*, a gene combination hitherto identified only in travelers from the Netherlands and children from China (*4*). Both the $bla_{\text{CTX-M-15}}$ and *mcr-1* genes were not transferable, indicating that neither gene was plasmid-encoded. Examination of the genetic environment of *mcr-1* in the assembled gapped genome showed a chromosomal location for the *mcr-1* transposition unit that included an IS*Apl1* element (Figure 1, panel A; online Technical Appendix Figure 1, panel A) flanked by the inverted repeats (IR-R1, IR-R2, and IR-L1). We verified the chromosomal location for the *mcr-1* gene by sequencing the genome to completion, using long-read single-molecule real-time sequencing (Pacific Biosciences, Menlo Park, CA, USA; online Technical Appendix Figure 2); the resulting contigs of *E. coli* RL465 were deposited in the European Nucleotide Archive under accession no. PRJEB14095. One copy of the IS*Apl1-mcr-1* transposition unit was located in the region between a predicted 4Fe-4S ferredoxin-type protein (*ydhY*) and *ldtE* (L,D-transpeptidase) (bp 2652307–2665241), and flanked on either side by a 2-bp direct repeat (CA). We observed a similar situation for the IS*Ecp1*-$bla_{\text{CTX-M-15}}$–*orf477* transposition unit (online Technical Appendix Figure 1, panel B). However, this insertion mapped to a different chromosomal location in a region encoding a defective lambdoid prophage inserted between the molybdate ABC transporter operon (*modABC*) and the biotin biosynthesis operon (*bioABCDF*) (bp 1662140–1716472). It was flanked by direct repeats (TGGTT).

We reexamined our collection of 424 genome sequenced ESBL- and carbapenemase-encoding *E. coli* isolates, obtained during 2010–2014 (*2*), for isolates that harbored $bla_{\text{CTX-M-15}}$ at a chromosomal location identical to that found in *E. coli* RL465. We detected 3 such isolates from 2010–2011 from companion animals and livestock (R107, sock swab dairy cattle farm, 2011; R208, sock swab pig fattening farm, 2011; V177, sick dog, 2010), and 11 consecutive isolates from a hemato-oncologic patient (*5*), obtained within an 11-month period during 2011–2012 (E006910, E007337, E007651, E007825, E000565, E002592, E002816, E003488, E005417, E006587, E006874) (Figure, panel B). All of these isolates were ST410 and negative for the *mcr-1* gene. Phylogenetic analysis of the core genome of these isolates with *E. coli* RL465 using the program Harvest Suite (*6*) indicated they were highly related and separated from *E. coli* V177 (the oldest isolate) by 66 (E006910, E007651) to 110 (E007337) single-nucleotide polymorphisms (core genome size 94%, representing 4.58 Mbp). Thus, our results suggest that transposition of the IS*Apl*1-*mcr-1* unit to the chromosome in *E. coli* RL465 is a later event and probably occurred after transfer of the $bla_{\text{CTX-M-15}}$ allele to the distinct chromosomal location into this *E. coli* ST410 subclone.

These findings highlight 2 independent points. First, our results extend data on the mobility of IS*Apl*1-*mcr-1* to a chromosomal location and reveal a new dimension in the transmissible nature of *mcr-1* in colistin-resistant *Enterobacteriaceae* isolates and their ecology. Second, clonal isolates of ST410 have been isolated from diverse environments, livestock, companion animals, and humans and, as we demonstrate here, in turkey hen meat (*7*,*8*). Thus, the simultaneous spread of the *mcr-1* and $bla_{\text{CTX-M-15}}$ genes mediated by a single bacterial clone is real and suggests that *mcr-1* is already present in the diverse reservoirs inhabited by these isolates.

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L.F., C.I., and T.C. conceived the study; S.E.W., K.G., H.G., S.D., B.B., C.S., and J.O. performed experiments; A.I., J.F., H.S., B.G., and A.K. contributed isolates and reagents; L.F., B.B., C.I., and T.C. analyzed the data; and T.C. and L.F. wrote the manuscript, which all authors approved.

B.G. is currently employed with the European Food Safety Authority (EFSA) in its BIOCONTAM Unit that provides scientific and administrative support to EFSA's scientific activities in the area of Microbial Risk Assessment. The positions and opinions presented in this article are those of the authors alone and are not intended to represent the views or scientific works of EFSA. The other authors have nothing to proclaim.

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Specificity of Dengue NS1 Antigen in Differential Diagnosis of Dengue and Zika Virus Infection

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To the Editor: Circulation of new arboviruses of the genus *Flavivirus* poses a major problem for differential diagnosis. Zika virus, a mosquitoborne virus of the family *Flaviviridae*, is closely related to other arboviruses circulating in the Americas, including dengue, yellow fever, Saint Louis encephalitis, and West Nile viruses (*1*,*2*). Serologic cross-reactivity between these arboviruses is common; thus, to ensure optimal patient care and accurate epidemiologic surveillance, an effective differential diagnosis is required in regions with active transmission of dengue virus and circulation of Zika virus (*2–4*).

Cross-reactivity between flaviviruses has been reported in antibody assays and in tests for Dengue nonstructural 1 glycoprotein (NS1) antigen. Gyurech et al. (*5*) reported false-positive test results for dengue NS1 antigen in a patient with acute Zika virus infection. Of the 3 NS1 tests used in that study, only the SD Bioline Dengue Duo (Standard Diagnostics, Inc., Gyeonggi-do, South Korea) showed positive results for 3 of 4 sequential serum samples from the patient.

Cross-reactivity in NS1 dengue tests (ELISA and immunochromatographic) using serum samples from patients with acute Zika virus infection would have medically significant consequences. We therefore conducted a retrospective analysis of the differential diagnosis for dengue and Zika virus infections since the beginning of the Zika virus outbreak in French Guiana, a department of France on the northeast coast of South America.

French Guiana is subject to endemoepidemic circulation of dengue and experienced a large outbreak of chikungunya in 2014. We conducted our study from December 17, 2015 (the time of biologic confirmation of the first case of Zika virus disease in French Guiana), through March 2, 2016. During that time, the incidence of dengue virus infection in French Guiana was low, and only 1 sporadic case was confirmed. We studied clinical samples collected during this period from all patients with suspected arbovirus infection.

Anhang I

Falgenhauer L, Waezsada S-E, Yao Y, Imirzalioglu C, Käsbohrer A, Roesler U, Michael GB, Schwarz S, Werner G, Kreienbrock L, Chakraborty T. 2016. Colistin resistance gene *mcr-1* in extended-spectrum β-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany. Lancet Infect Dis 16:282–283.

positive. Notably, the oldest *mcr-1*-positive *E coli* isolate had been collected in 2005. The 106 *mcr-1*-positive *E coli* isolates originated from different individuals located in 94 widely distant farms, and they were clonally unrelated.

Sequencing of the whole *mcr-1* gene in 75 *mcr-1*-positive isolates revealed a 100% identity compared with the original sequence. Co-occurrence of the *mcr-1* and *ESBL* genes was identified in a subset of seven isolates, with *mcr-1* and $bla_{CTX\text{-}M\text{-}1}$ being found on a large and conjugative IncHI2-type plasmid together with genes conferring resistance to sulfonamides and tetracyclines, two antibiotics widely used in veterinary medicine.

See Online for appendix

These findings demonstrate a colocation of the *mcr-1* gene along with an *ESBL* gene on a single plasmid, and additional studies are needed to clarify the diversity of the plasmid backbones spreading these two genes within our collection. Noticeably, the prevalence of the *mcr-1* gene among ESBL producers in veal calves was much higher than that found in ESBL-positive *E coli* isolates in human beings and chicken meat reported in Denmark.[2] This difference may reflect a major spread of the *mcr-1* gene in European live animals. We showed that the dissemination of *mcr-1*, at least in France, had already occurred more than a decade ago, with one *E coli* isolate collected in 2005 identified as *mcr-1* positive.

Altogether, available data reveal the occurrence of *mcr-1* among different animals and human contexts over time.[1–3] Worryingly, we show that selection pressure with broad-spectrum cephalosporins may select for colistin resistance and vice-versa, further highlighting the likelihood of a pandemic spread of *mcr-1*. Of note, the substantial use of tetracyclines and sulfonamides in animals might also substantially contribute to the dissemination of *mcr-1* plasmids.

In a one-health perspective, and considering the renewed importance of colistin in human medicine, our data and those from others underscore the urgent need to limit the spread of *mcr-1*-positive plasmids by reconsidering the massive use of colistin in veterinary medicine worldwide.

We declare no competing interests. This work was supported by the Agency for Food, Environmental and Occupational Health and Safety (ANSES), and by the University of Fribourg, Switzerland, and by a grant of the ANIWHA ERA-NET project (France/Switzerland). We thank all peripheral laboratories of the Resapath network, as well as Charlotte Petitjean for her technical help.

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Colistin resistance gene *mcr-1* in extended-spectrum β-lactamase-producing and carbapenemase-producing Gram-negative bacteria in Germany

A plasmid-encoded gene conferring colistin resistance (*mcr-1*) was recently described by Yi-Yun Liu and colleagues[1] and subsequently reported in isolates from Denmark.[2] Unlike the previously described chromosomally encoded resistance mechanisms to colistin,[3] plasmid-encoded resistance can be transmitted by horizontal transfer from livestock, where colistin is used to treat infected animals, to human beings. Transfer of the resistance to multidrug resistant Enterobacteriaceae would seriously compromise current treatment options.

We searched for the presence of the *mcr-1* gene in our database of 577 whole genome sequences of isolates obtained from different sources (human, animal, and environmental) since 2009 in Germany (appendix). We detected the *mcr-1* gene in four *Escherichia coli* isolates, three originating from swine (R253, V163, 112065) and one from a human wound infection (NRZ14408). R253, V163, and 112065 are extended-spectrum β-lactamase-producing isolates that harbour $bla_{CTX\text{-}M\text{-}1}$, whereas the NRZ14408 human isolate carries a $bla_{KPC\text{-}2}$ carbapenemase gene in addition to *mcr-1* (appendix). The minimal inhibitory concentration of colistin in the isolates ranged from between 2 mg/L to greater than 16 mg/L. The genetic environment of *mcr-1* was variable, and not always associated with ISA*pl1* (figure). Colistin-resistance could be transferred by conjugation at rates of between 10^{-1} and 10^{-7} transconjugants per recipient. The *mcr-1* gene is located on conjugative IncHI2 plasmids in all isolates excepting V163, where it is present on an IncX4 plasmid.

The isolate V163 was obtained in 2010, indicating that the existence of transmissible colistin resistance in animal populations in Germany is not a recent occurrence. The detection of *mcr-1* on different classes of plasmids and their presence in isolates of various sequence types (appendix) suggests that multiple pathways for horizontal transmission of this resistance exist. Our data suggest that the advent of untreatable infections has already

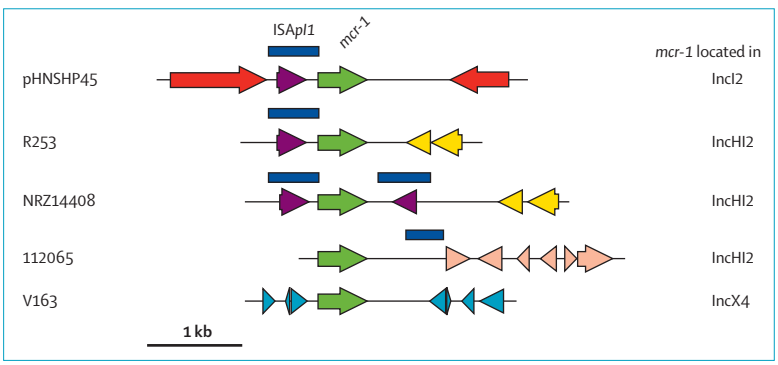

***Figure*: Gene environment surrounding *mcr-1* in 112065, V163, NRZ14408, and R253 compared with plasmid pHNSHP45**
Green arrows depict the *mcr-1* gene, ISA*pl1* is shown by a blue bar with the transposase marked in purple. Other colours refer to unrelated gene segments flanking the *mcr-1* gene.

arrived, as every colistin-resistant isolate described in this study is also resistant to either third-generation cephalosporins or to carbapenems.

This study was supported by grants to the German Center of Infection Research (DZIF), and the Zoonoses Network extended-spectrum β-lactamase and fluoroquinolone resistance in Enterobacteriaceae (RESET) Consortium through the German Federal Ministry of Education and Research (BMBF; grant numbers 8000 701–3 [HZI] to TC [TI06.001] and CI [01KI1313G]). The study was approved by the ethics committee of the medical faculty of the Justus-Liebig-University of Giessen (AZ: 95/11). All samples were taken as part of standard care procedures. We thank Christina Gerstmann for excellent technical assistance and our collaboration partners from DZIF and RESET for providing the isolates. We declare no competing interests.

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Colistin resistance gene *mcr-1* harboured on a multidrug resistant plasmid

In *The Lancet Infectious Diseases*, Yi-Yun Liu and colleagues reported, for the first time, plasmid-mediated colistin resistance in *Escherichia coli* isolated from animals, food, and patients in China.[1] These data bring to the fore an as yet unknown facet of colistin resistance and yet again show the effect of antibiotic use in animal farming on human health.[2,3] We screened a selection of 105 colistin-resistant *E coli* strains (sensititre minimum inhibitory concentration of colistin ≥4 mg/L) isolated during 2011–12 from passive surveillance of *E coli* diarrhoea in 52 calves from Wallonia and 53 piglets from Flanders, both regions of Belgium. All strains were screened for the presence of *mcr-1* using PCR and Sanger sequencing. We detected *mcr-1* in 13 (12·4%) of 105 *E coli* (macrobroth dilution minimum inhibitory concentration of colistin 4 and 8 mg/L), of which six (11·5%) of the 52 strains were isolated from calves and seven (13·2%) of 53 were isolated from piglets. The *mcr-1* allele showed 100% sequence similarity to the Chinese allele.[1] Plasmid sequencing (MiSeq, Illumina) from one bovine strain isolated pKH-457-3-BE that showed an IncP backbone and a size of 79 798 bp (figure). Blast comparison with pHNSHP45 showed 100% similarity only in a short, 2604 bp region that included *mcr-1* (1626 bp) and a truncated ISA*pl1* mobile element that did not include the transposase-encoding *tnpA* gene. pKH-457-3-BE showed 99% similarity (73% query coverage) to plasmid pHXY0908 (GenBank access number KM877269) identified in *Salmonella enterica* serotype Typhimurium isolated from chicken stool in China. By contrast with pHNSHP45, pKH-457-3-BE harboured several resistance-encoding genes to trimethoprim (*dfrA1*), tetracycline (*tetA*), aminoglycoside (*aadA1, aph(6)-Id* or *strA,* and *aph(3″)-Ib/strB*), and sulphonamide (*sul1*) antibiotics. Phenotypic testing showed absence of extended-spectrum β-lactamase and carbapenemase production in all *mcr-1* positive strains.

We show a marked presence of *mcr-1* in animal pathogenic bacteria in Europe, an indication that this is already a truly global phenomenon. That *mcr-1* was present in *E coli* circulating in Belgian farm animals during 2011–12 and was harboured on a different plasmid backbone than the one isolated from pigs in China (IncI2) or from imported chicken meat in Denmark (IncX4),[1,4] indicates a high promiscuity of this gene guided by the adjoining mobile element. Of note, most of the *mcr-1* positive *E coli* strains we isolated were enterotoxigenic and verocytotoxic strains that affect animals but do not cause pathology in humans. Assessment of the transfer

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†Members of the RESET consortium listed in appendix

Anhang J

Fritzenwanker M, Imirzalioglu C, Gentil K, **Falgenhauer L**, Wagenlehner FME, Chakraborty T. 2016. Incidental detection of a urinary *Escherichia coli* isolate harbouring *mcr-1* of a patient with no prior history of colistin treatment. Clin Microbiol Infect 22:954–955.

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Letter to the Editor

Incidental detection of a urinary *Escherichia coli* isolate harbouring *mcr-1* of a patient with no history of colistin treatment

The recent emergence of the transmissible colistin resistance genes *mcr-1* and *mcr-2* has raised concern among public health specialists, as colistin is a last-line antibiotic used in the treatment of multidrug-resistant bacteria, especially carbapenem-resistant *Enterobacteriaceae* (CRE) [1–3]. These genes have been identified mostly in livestock receiving antibiotics as growth promotors and only rarely in humans. Writing in this journal, Nordmann *et al.* [3] have recommended that detection of colistin-resistant bacteria should be encouraged, particularly in isolates also exhibiting resistance to carbapenems. Here we report detection of *mcr-1* in a urine isolate with an unremarkable antibiotic resistance profile from a patient without a history of colistin treatment.

In an ongoing study initiated at the beginning of 2016, samples obtained from urologic wards at a university hospital were analysed. We examined 162 consecutive *Escherichia coli* isolates, irrespective of their antibiotic resistance profiles, obtained from urine specimens of hospitalized patients and outpatients. Nextera XT libraries of the isolates were sequenced on a NextSeq with a NextSeq500 High Output Kit with 300 cycles (Illumina, the Netherlands) and contigs generated using a local assembly pipeline. Genomewide screening for resistance genes with Resfinder (https://cge.cbs.dtu.dk/services/ResFinder) detected a single isolate containing *mcr-1* in addition to other antibiotic resistance genes ($bla_{\text{TEM-1B}}$, *aadA1, sul1, dfrA1, tet*(*A*) and *mph*(*B*)). The isolate was assigned to multilocus sequence type ST-1851 (https://cge.cbs.dtu.dk/services/MLST/) using *E. coli* scheme 1. Antibiotic resistance genes were present on IncX4 and IncFII plasmids as determined by Plasmidfinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/). The *mcr-1* gene was located in a genetic environment that was similar to previously published IncX4 plasmids from *E. coli* (pAF48, accession no. KX032520; pOW3E1, accession no. KX129783) and *Klebsiella pneumoniae* (pMCR1.2-IT, accession no. KX236309; pmcr1_IncX4, accession no. KU761327). The conjugative IncX4 plasmids are promiscuous and are associated with the spread of *mcr-1* between different *E. coli* multilocus sequence types and in other Gram-negative species worldwide [1,4]. Sequence data for the isolate is available at the European Molecular Biology Laboratory ENA archive (accession no. ERR1562562).

The isolate was obtained from a man in his 60s hospitalized for chronic obstructive pulmonary disease to investigate ongoing unintended weight loss. Urine was sampled as a routine measure during consultation. The urine culture harboured *E. coli* (>100 000 CFU/mL), and susceptibility testing indicated that the isolate was resistant to ampicillin, ampicillin/sulbactam, cefuroxime, piperacillin, tetracycline and trimethoprim/sulfamethoxazole. The patient was discharged without antibiotic treatment. No prior colistin treatment in the patient's medical history was reported. Because the isolates resistance phenotype was not remarkable, it was not tested for colistin resistance during routine microbiologic testing. Phenotypic resistance testing, however, confirmed colistin resistance (minimum inhibitory concentration (MIC) 4 μg/mL). Because there were no other *mcr-1* isolates from the collection time period, it is not known whether the brief stay resulted in transmission to other patients in the same ward, as there is no screening implemented for *mcr-1*.

Unlike standardized screening media and automated susceptibility testing panels for other antibiotics, a commercial screening medium for colistin resistance is presently not available. In addition, MIC testing of colistin resistance is fraught with pitfalls, and joint Clinical and Laboratory Standards Institute/European Committee on Antimicrobial Susceptibility Testing subcommittee has recently issued warnings related to the overall poor quality of colistin antimicrobial susceptibility testing (http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/General_documents/Recommendations_for_MIC_determination_of_colistin_March_2016.pdf). Recommended testing now requires performing the cumbersome reference broth microdilution test—at least for all CRE and other enterobacterial isolates from nosocomial outbreaks [3]. Nevertheless, on the basis of these criteria, the isolate described here would have evaded detection; its incidental detection was only possible as a result of an unbiased approach that used whole-genome sequencing. Thus, in the absence of adequate screening, the true prevalence of *mcr-1* in human populations will remain undetermined. Given the transmissible nature of *mcr-1,* the emergence of outbreaks of CRE-resistant bacteria harbouring colistin resistance genes is an epidemiologic disaster in waiting. We suggest investigating the prevalence of *mcr-1* in representative healthcare settings, preferably with simple genetic point-of-care assays, to assess the risk for susceptible patient populations.

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Transparency Declaration

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Anhang K

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Anhang L

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Environmental emission of multiresistant *Escherichia coli* carrying the colistin resistance gene *mcr-1* from German swine farms

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Objectives: Pigs have been the focus of the worldwide spread of colistin resistance. However, there is little information on the transmission of *mcr-1*-containing bacteria into the environment of pig farms. We therefore rescreened environmental *Escherichia coli* isolates from the surrounding farm areas of three previously *mcr-1*-positive swine herds in Germany.

Methods: Thirty-five mixed bacterial cultures obtained from boot swabs, flies, dog faeces and manure from three pig farms in Germany in 2011–12 were non-selectively recultivated and the presence of the *mcr-1* gene was checked by real-time PCR. After separation, single *E. coli* colonies were subsequently isolated and the presence of *mcr-1* was confirmed by PCR and sequencing. In addition, phenotypic antimicrobial resistance screening and WGS followed by phylogenetic analysis and resistance genotyping as well as plasmid typing were performed.

Results: Seven *mcr-1*-positive *E. coli* strains originating from environmental boot swabs, dog faeces, stable flies and manure were found. The isolates belonged to five different STs (ST10, ST1011, ST1140, ST5281 and ST342) and harboured extensive additional resistance genes. Comparative plasmid analysis predominantly located *mcr-1* on IncX4 plasmids, which are strongly related to a recently described plasmid of human clinical origin (pICBEC72Hmcr).

Conclusions: WGS-based analysis of the environmental *E. coli* isolates of farm surroundings showed clear links to *mcr-1*-harbouring *E. coli* recovered from pig production in Europe as well as from human clinical isolates worldwide, presenting another piece of the puzzle, which further complicates the rapidly evolving epidemiology of plasmid-mediated colistin-resistant *E. coli* strains.

Introduction

A plasmid-encoded colistin resistance gene named *mcr-1* was described in Enterobacteriaceae isolated from humans, livestock and raw meat in China in 2015.[1] Since then, plasmid-mediated colistin resistance by *mcr-1* has been reported worldwide in humans, livestock, companion animals, food and wildlife.[2] Colistin is approved for use in veterinary medicine and widely used to treat gastrointestinal infections in pigs and poultry.[3] Livestock was therefore the focus of *mcr-1* spread right from the outset.

We rescreened *Escherichia coli* isolates originally obtained in 2011–12, recovered from environmental samples of three previously *mcr-1*-positive swine farms in Germany. In these farms *E. coli* strains with colistin resistance encoded on incX4 and incHI2 plasmids were detected in pooled pig faeces and boot swabs from the stables. This study was undertaken to investigate whether environmental samples from areas surrounding the farms and possible vectors such as flies and barn dogs harboured *mcr-1*-positive strains back in 2011–12.

Materials and methods

During the national research project RESET (www.reset-verbund.de) seven pig farms were investigated between 2011 and 2012.[4] The resulting mixed bacterial cultures derived from farm samples, such as pooled faeces and indoor boot swabs, were recently screened for the presence of *mcr-1*. The results obtained showed that three out of seven investigated farms (farm 1, farm 2[4] and farm 3[4]) contained *mcr-1*-positive *E. coli* isolates within pooled faeces samples. In an attempt to reveal possible emission sources, 35 mixed bacterial cultures previously obtained from the environment of the three pig farms[4] were rescreened for the presence of *mcr-1*.

The cultures were originally derived from boot swabs ($n = 25$) of the surrounding areas (up to 500 m downwind and 100 m upwind of the barn), barn flies ($n = 3$), a barn dog ($n = 1$), manure ($n = 5$) and mice faeces ($n = 1$). Details on sampling and bacterial cultivation can be found in the original publication.[4,5] In brief, samples were selectively cultivated on MacConkey agar plates containing 1 mg/L cefotaxime, Endo-Enro (2 mg/L) or Gassner agar plates. Mixed sets of bacteria, able to grow on these plates, were stored at −80°C for further investigation.

Previously obtained bacteria were recultured in non-selective LB-broth and DNA was prepared from the enriched cultures and screened for the presence of the *mcr-1* genes by real-time PCR.[6] Out of the 35 investigated cultures, 7 turned out to be *mcr-1* positive, originating from two boot swabs taken 50/150 m from the downwind side of the barn, manure ($n = 3$), one dog faecal sample and a stable fly (*Musca domestica*). The overnight cultures of the seven samples were spread on MacConkey agar plates containing 2 mg/L colistin and single *E. coli* colonies were picked. The presence of the *mcr-1* gene was confirmed by conventional PCR and subsequent sequencing. Antimicrobial resistance screening using a VITEK-2® compact system (AST-card N248, bioMérieux, Germany) was performed, confirming phenotypic colistin resistance (MIC of 8 or ≥16 mg/L) for the seven *E. coli* isolates (Table 1).

All phenotypically colistin-resistant *E. coli* isolates were used for WGS using Illumina NextSeq 300 bp paired-end sequencing (Illumina, Eindhoven, The Netherlands) and *de novo* assembled (CLC Genomics workbench v. 9.0, http://www.clcbio.com/). Draft genomes of the isolates were annotated using RAST.[7] In addition, data were used for *in silico* MLST (MLSTFinder[8]), resistance genotyping (ResFinder 2.1[9]) and plasmid typing (PlasmidFinder 1.3[10]). Large contigs containing putative plasmid sequences were verified using the Blast option of Geneious 7.1.2 and visualized using BRIG.[11] The number of single nucleotide polymorphisms (SNPs) in the core genome (defined as orthologous sequence conserved in all seven aligned genomes) was calculated for isolates with the same ST, using Harvest suite 1.0 (parsnp)[12] and MEGA 6.0 (http://www.megasoftware.net). In addition, XbaI digestion and S1-PFGE were performed.

Results and discussion

Among the environmental samples we detected seven *mcr-1*-positive *E. coli* strains originating from boot swabs and emission sources such as dog faeces, stable flies and manure. One *E. coli* isolate per culture was further investigated and showed that five different STs were present (ST10, ST1011, ST1140, ST5281, ST342). Most of the isolates also harboured extensive additional resistance genes (Table 1). Some of those STs have been recently described as carriers of the *mcr-1* gene. ST1011 was reported from livestock and meat samples in the Netherlands.[13] Furthermore, strains of ST10 have been found in porcine and bovine samples in Belgium[14] and in travellers returning from India,[15] and have been found to be carriers of the new colistin resistance gene *mcr-2*.[16]

The number of SNPs in the core genome indicated that the two isolates of ST10 [boot swab 150 m (farm 1) and dog faeces (farm 2)] were highly related, differing by 7 SNPs (1.3 SNPs/Mbp) in the core genome only, while the two isolates of ST1011 [manure (farm 2) and stable fly (farm 3)] displayed 109 SNPs (21 SNPs/Mbp).

In addition, we comparatively analysed the plasmid content of the isolates; *mcr-1* was predominantly located on an IncX4-type plasmid (Table 1), previously reported as one of the major types in the plasmid-driven *mcr-1* dissemination. Using Geneious 7.1.2, we screened the WGS data for the recently described IncX4 plasmid pICBEC72Hmcr (ENA accession number CP015977.1, originating from a human patient,[17] 33 kb). Using the BLAST option of Geneious 7.1.2 we were able to detect almost the complete

Table 1. Information on seven colistin-resistant *E. coli* isolates from environmental samples including ST, plasmid content and phenotypic and genotypic resistances

Pig farm, location	Environmental isolate no.	Type of environmental sample (downwind distance)	Phenotypic resistance	ST	Resistance genes	Plasmid(s)	Contig information on IncX4 plasmids [number, size (bp)]
Farm 1, OPR	S-477-1	boot swab (150 m)	PIP, CST	10	*blaTEM-1B-like*, ***mcr-1***, *strA-like*, *strB-like*, *tet(A)*	**IncX4**, IncN	1 (Node_46, 35107)
Farm 2, UM	S-488-1	barn dog faeces	CTX, CST*, SXT	10	*blaTEM-1B-like*, *dfrA1-like*, ***mcr-1***, *strA-like*, *strB-like*, *tet(A)*	**IncX4**, IncN	1 (Node_42, 35107)
	S-492-1	boot swab (50 m)	PIP, CIP, CST*, SXT	1140	*aadA1*, *aadA2*, *aph(3')-Ia-like*, *blaTEM-1B*, *cmlA1-like*, *dfrA1*, ***mcr-1***, *sul3*	**IncX4**, IncFII, IncX1, IncY	2 (Node_52, 29857; Node_140, 1249)
	S-30-1	manure	CTX, CST*, SXT	5281	*aadA5*, *aph(3')-Ic-like*, *blaCTX-M-1*, *dfrA17*, ***mcr-1***, *strA*, *strB*, *sul2-like*, *tet(B)*	IncI1	
	S-275-1	manure	PIP, CIP, CST*, SXT	1011	*aadA1*, *blaTEM-1B*, *dfrA1*, ***mcr-1***, *strA*, *strB*, *sul1*, *sul2*, *tet(A)*	IncFII, IncFIB, IncX1, IncQ1	
Farm 3, SPN	S-504-1	stable fly	CTX, CIP, CST, SXT	1011	*aadA1*, *blaTEM-1B*, *dfrA1*, *dfrA14-like*, ***mcr-1***, *strA*, *strB*, *sul1*, *sul2*, *tet(A)*	**IncX4,** IncFII, IncFIB, IncN, IncQ1	1 (Node_42, 34173)
	S-38-1	manure	CTX, CST*	342	*aadA1*, *aadA2*, *blaCTX-M-1*, *cmlA1-like*, ***mcr-1***, *mph(A)*, *sul3*, *tet(A)-like*	**IncX4**, IncFII, IncFIB, IncX1	2 (Node_49, 20956; Node_59, 9361)

OPR, Ostprignitz County; UM, Uckermark County; SPN, Spree-Neisse County.
CTX, cefotaxime; CIP, ciprofloxacin; CST, colistin (MIC 8 mg/L); CST*, colistin (MIC ≥16 mg/L); PIP, piperacillin; SXT, trimethoprim/sulfamethoxazole.

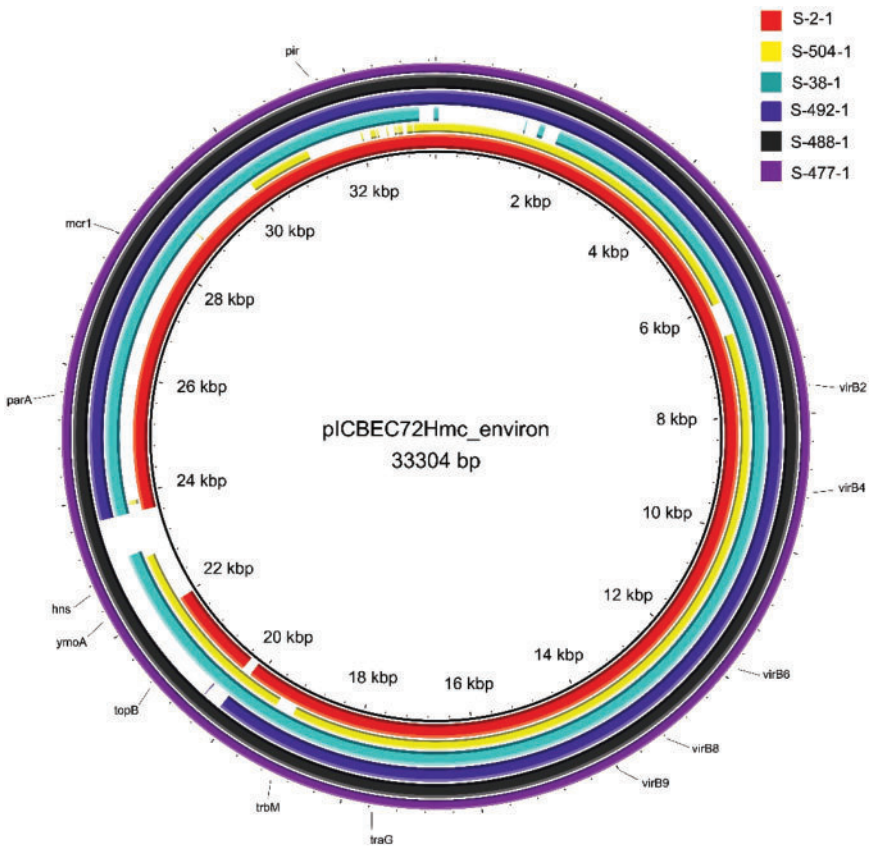

Figure 1. Circular visualization of the six *mcr-1*-containing IncX4 plasmid sequences as compared with *E. coli* IncX4 plasmid pICBEC72Hmcr using BRIG.[11] S-2-1, pooled animal faeces/farm 1; S-504-1, stable fly/farm 3; S-38-1, manure/farm 3; S-492-1, boot swab/farm 2; S-488-1, barn dog faeces/farm 2; S-477-1, boot swab/farm 1.

plasmid sequence of the plasmid pICBEC72Hmcr for four IncX4 isolates (Figure 1, Table 1). For S-477-1 and S-488-1 a single large contig was present comprising the full pICBEC72Hmcr sequence. For S-492-1 and S-38-1 two contigs (30 and 1.5 kb, and 20 and 10 kb, respectively) with 95% coverage and 99% identity compared with pICBEC72Hmcr could be found (Figure 1). In contrast, the IncX4 plasmid of S-504-1 was different from the other plasmids and showed 99% identity to a plasmid recently described in Chinese pigs (NCBI number KM580533).[18]

As the pICBEC72Hmcr-like plasmid was found in environmental samples from all three farms tested, its transmission potential seems to be high. In addition, we also detected an almost identical plasmid in an isolate (S-2-1, ST540) derived from pooled faeces of the animals in farm 1 (1 contig, 30 kb; Figure 1) and its plasmid showed similar S1 nuclease digestion profiles to the plasmid of S-477-1 (data not shown), pointing towards the original source of this plasmid being in the surrounding farm area.

Additional information regarding the antibiotic treatment of the pigs provided further support for the assumption that the original source of the *mcr-1*-containing IncX4 plasmid might have been located inside the pig farm. In two out of the three farms investigated (farms 1 and 3) colistin was given to the animals during the pre-fattening period,[4] suggesting a possible origin for the emission of colistin-resistant bacteria/plasmids into the environment. To the best of our knowledge, this is the first report of *mcr-1* isolated from environmental surface samples close to swine farms. The additional finding in manure is rather unsurprising; however, the positive results for stable flies and barn dog faeces indicate possible additional transmission routes. Our data suggest both the plasmidal spread of the *mcr-1* gene in the surroundings of pig farms in north-east Germany as well as to a certain extent a clonal spread, e.g. for two isolates of ST10 differing by only 1.3 SNPs/Mbp. This very low number of SNPs per Mbp is even smaller than the one described for clonal enterohaemorrhagic *E. coli* (EHEC) strains during the German outbreak[19,20] (1.8 SNPs/Mbp). WGS-based analysis of the environmental isolates at the core genome and plasmid level showed clear links to *mcr-1*-harbouring *E. coli* recovered from pig production in Europe as well as from human clinical isolates worldwide. The presence of the *mcr-1* gene in natural environments therefore presents another piece of the puzzle which further complicates the rapidly evolving epidemiology of plasmid-mediated colistin-resistant *E. coli* strains and simultaneously underlines the importance of the One Health approach.

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Transparency declarations

None to declare.

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Anhang M

Roschanski N, **Falgenhauer L**, Grobbel M, Guenther S, Kreienbrock L, Imirzalioglu C, Roesler U. 2017. Retrospective survey of *mcr-1* and *mcr-2* in German pig-fattening farms, 2011-2012. Int. J. Antimicrob. Agents 50:266-271.

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Short Communication

Retrospective survey of *mcr-1* and *mcr-2* in German pig-fattening farms, 2011–2012

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Multidrug resistance

ABSTRACT

In November 2015, the first plasmid-encoded colistin resistance gene, *mcr-1*, was described in animals and in humans in China. Subsequently, a multitude of further studies was performed and quite recently the global spread of *mcr-1* as well as the occurrence of a new gene variant, *mcr-2*, was reported. To obtain an overview of the occurrence of the colistin resistance genes *mcr-1* and *mcr-2* in German pig farms, a retrospective study, including 436 boot swab and pooled faecal samples collected from 58 pig-fattening farms throughout Germany, was performed. Whilst *mcr-2* was not detected, the presence of *mcr-1* was confirmed in 43 *Escherichia coli* isolates from 15 farms, indicating that the *mcr-1* gene was present in 9.9% of the analysed samples and 25.9% of the investigated pig farms. Subsequent characterisation of the isolates showed colistin minimum inhibitory concentrations (MICs) of 4–8 μg/mL, with most isolates being resistant to several antibiotics including cephalosporins and/or fluoroquinolones. Pulsed-field gel electrophoresis (PFGE) showed great heterogeneity among the tested *mcr-1*-positive isolates. However, further analyses of 15 selected *E. coli* isolates (one per *mcr-1*-positive farm) indicated that the colistin resistance genes were predominantly located on IncX4 plasmids, highly similar to a plasmid initially isolated from an *E. coli* derived from a human patient in Brazil. The results described herein support the already expressed concern for public health and further underline the need for monitoring programmes in veterinary practice as well as in human medicine.

1. Introduction

Increasing number of multidrug-resistant (MDR) bacteria as well as the lack of new antimicrobials has caused a resurgence of old antimicrobial substances such as colistin (polymyxin E) in human medicine. For example, the consumption of polymyxins increased significantly in Denmark, Hungary and Italy during 2010–2014 [1]. Importantly, colistin is currently a last-line treatment option for infections caused by carbapenemase-producing Gram-negative Enterobacteriaceae (*Escherichia coli*, *Klebsiella* spp.) as well as MDR *Pseudomonas* and *Acinetobacter* spp. [2,3]. On the other hand, colistin has been widely used for many years in veterinary medicine and is of therapeutic importance for the treatment of enterobacterial infections in food-producing animals such as pigs, broilers, turkeys, cattle, sheep, goats and meat rabbits [3]. With the first description of a plasmid-mediated colistin resistance gene (*mcr-1*), observed in November 2015 in China [4], a major concern for public health was voiced. Since then, further investigations performed throughout the world have exposed the global spread of the *mcr-1* gene. Until now, *mcr-1* has been found in various bacterial species isolated from a wide range of different sources, including the environment, wild birds, various food-producing animals, companion animals, different kinds of food (meat and vegetables), infected human patients and asymptomatic carriers [5]. Quite recently, a novel plasmid-mediated colistin resistance gene, named *mcr-2*, was detected in porcine *E. coli* from Belgium [6].

Moreover, the presence of the first *mcr-1* genes in clinical carbapenem-resistant isolates has been described, thus representing an alarming situation [7–9]. Taken together, these findings indicate that the occurrence of plasmid-mediated colistin resistance is an important topic that needs to be investigated carefully with regard to

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the One Health aspect in human and veterinary medicine. To gain more detailed information about the occurrence of the colistin resistance genes *mcr-1* and *mcr-2* in German pig-fattening farms, a retrospective study including 436 bacterial cultures derived from 58 German pig-fattening farms sampled within the years 2011–2012 was performed.

2. Materials and methods

2.1. Investigated bacteria and positive control strains

A total of 436 mixed bacterial cultures primarily isolated from pooled faeces and boot swab samples, taken on 58 pig-fattening farms throughout Germany, were investigated. The primary samples were taken between January 2011 and October 2012 [10,11]. This study focused on estimation of the prevalence of extended-spectrum β-lactamase (ESBL-) and AmpC-producing as well as fluoroquinolone-resistant Enterobacteriaceae in German livestock farms. Therefore, the major portion of initially taken samples (e.g. faeces and boot swabs) were selectively cultivated on either MacConkey agar plates containing 1 μg/mL cefotaxime (232 cultures; 53.2%) or Endo agar containing 2 μg/mL enrofloxacin (200 cultures; 45.9%). The remaining four cultures (0.9% of the total investigated samples) were initially cultivated on non-selective Gassner agar plates. Mixed sets of bacteria that were able to grow on the respective plates (primary mixed bacterial cultures) were stored at –80 °C for further investigation.

Isolates R253 (*mcr-1*) [7] and KP37 (*mcr-2*) [6] served as positive controls for screening of the colistin resistance genes *mcr-1* and *mcr-2*.

2.2. Screening for mcr-1- and mcr-2 positive bacteria

Previously stored bacteria were taken from the stock and were re-cultured non-selectively in LB broth (Luria/Miller) (Carl Roth, Karlsruhe, Germany) overnight at 37 °C. DNA preparation for the following PCR was performed as previously described [12].

As the recently published screening protocol only covers the detection of *mcr-1* [13], the assay was extended to include the following primer/probe combination for the detection of *mcr-2*: mcr-2_fwd, TTGTCGTGCTGTTATCCTATCG; mcr-2_rev, CCGTGCCA TAAGTATCGGTAAA; and mcr-2_Probe, ROX-ACTGATTATGGG TGCGGTGACGAG-BHQ-2. PCR amplification was performed in 25 μL reactions [12.5 μL of ABsolute qPCR Mix (Thermo Scientific, Darmstadt, Germany), 1 μL of each forward and reverse primer (10 pmol) and 0.2 μL of each *Taq*Man probe (10 pmol)]. When mixed bacterial cultures were investigated, 2 μL of the DNA preparation was added; when pure cultures or the positive controls were used, 1 μL of DNA was sufficient. To achieve maximum activity of the polymerase, a preliminary step at 95 °C for 15 min was necessary. Subsequently, 30 cycles of 95 °C for 15 s and 60 °C for 1 min followed. Fluorescence signals were detected in two different channels: orange (533–610 nm)/ROX and red (618–660 nm)/Cy5. Samples showing a fluorescence signal above the cycle threshold (C_t) were assessed as positive.

In the case of positive PCR results, stored LB overnight cultures were spread on MacConkey agar plates containing 2 μg/mL colistin sulphate (Carl Roth) and single colonies were picked. Presence of the *mcr-1* gene was confirmed by conventional PCR [7] and subsequent sequencing of the PCR product.

2.3. E. coli phylotyping and pulsed-field gel electrophoresis (PFGE)

PCR-based determination of *E. coli* phylogenetic groups was performed according to Clermont et al [14]. In addition, *Xba*I-PFGE was performed for all of the *mcr-1*-positive bacterial isolates. Gels were analysed using BioNumerics 6.6 (Applied Maths, Sint-Martens-Latem, Belgium). All IncX4 plasmids were analysed by S1-nuclease PFGE using the following running conditions: 1 s to 25 s; 17 h; 6 V/cm; and 120 V.

2.4. Minimum inhibitory concentration (MIC) determination

MICs for 14 different antimicrobials were determined by broth microdilution following the Clinical and Laboratory Standards Institute (CLSI) protocol [15] and the results were evaluated in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [16].

2.5. Detection of extended-spectrum β-lactamase, AmpC and fluoroquinolone resistance genes

Presence of the most common ESBL genes was tested by real-time PCR [12]. The results were confirmed using conventional PCR and subsequent sequencing of the PCR products. Fluoroquinolone-resistant isolates were tested for the presence of the plasmid-encoded fluoroquinolone resistance genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *aac(6′)-Ib*-cr and *qepA*.

2.6. Further characterisation of selected mcr-1-containing isolates

From each farm, one *mcr-1*-positive isolate was selected for further characterisation (if available, derived from pooled faeces). Plasmids were isolated and were transferred into the electrocompetent *E. coli* strains NEB-5-α or NEB-10-β (New England Biolabs, Frankfurt, Germany). The incompatibility (Inc) group of the transferred plasmids was determined by PCR-based replicon typing (PBRT Kit; Diatheva, Fano, Italy). Transferability of the *mcr-1*-containing plasmids was tested by conjugation [7]. Whole-genome DNA was isolated from overnight cultures of the wild-type isolates. Nextera XT sequencing libraries were sequenced using a NextSeq 500 machine with 2 × 150 cycles (Illumina, Eindhoven, The Netherlands). Average read length and coverage were 121 nt and 55×, respectively. The raw data were assembled using SPAdes [17]. Resistance genes, virulence genes and plasmid Inc groups were identified and multilocus sequence typing (MLST) was performed using the Web tools ResFinder [18], PlasmidFinder [19] and MLST 1.8 [20]. The contigs of the *mcr-1*-encoding IncX4 plasmid sequences were extracted from the whole-genome data using the software Geneious 7.1.2 (http://www.geneious.com) and a subsequent sequence comparison was performed against the reference plasmid pICBEC72Hmcr (accession no. **CP015977.1**) [21]. Contigs containing plasmid sequences were plotted against the reference plasmid using the BLAST Ring Image Generator (BRIG) [22].

3. Results

3.1. Occurrence of mcr-1 and mcr-2 in German pig-fattening farms

Of the 436 investigated primary cultures in 2011–2012, 43 (9.9%) were *mcr-1*-positive. Of the *mcr-1*-positive cultures, 2 were initially cultured on non-selective Gassner agar plates, 23 were selected on MacConkey agar + cefotaxime and the remaining 18 cultures were initially derived from Endo agar containing enrofloxacin. In contrast, none of the tested samples was positive for *mcr-2*. On farm level, these data showed that at least one *mcr-1*-positive sample was detected in 15 (25.9%) of the 58 investigated pig-fattening farms. The distribution of *mcr-1*-positive pig farms occurred in all four agricultural regions of Germany (north/west, south, middle and east) with almost similar quantities (25%, 25%, 21% and 36%, respectively). A single *E. coli* colony was picked from each of the 43 positive-tested stock cultures and the presence of the *mcr-1* gene was confirmed by PCR and subsequent sequencing of the PCR product.

Table 1
Minimum inhibitory concentration (MIC; in μg/mL) determination in accordance with the Clinical and Laboratory Standards Institute (CLSI) broth microdilution protocol [15].

Region	Farm	Isolate	Origin	Phylo-group	PFGE-Pattern	AMP	AZI	CHL	CIP	COL	FOT	GEN	MERO	NAL	SMX	TAZ	TET	TGC	TMP	Enzyme
North-West	104	S-222-1	bs1, MC+	A	Ec26	>64	4	≤8	≤0.015	4 (8)	>4	≤0.5	≤0.03	≤4	16	8	≤2	0.5	0.5	Mcr-1, CMY-2
	105	S-228-1	bs1, MC+	E	Ec16	>64	4	64	0.5	4 (8)	≤0.25	32	≤0.03	>128	>1024	≤0.5	>64	0.5	≤0.25	Mcr-1, TEM-1
		S-454-1	bs1, EE+	E	Ec14	>64	4	≤8	8	8	≤0.25	>32	≤0.03	>128	>1024	≤0.5	64	≤0.25	>32	Mcr-1, TEM-1
Middle	4	S-296-1	bs1, EE+	B1	Ec11	>64	8	64	8	4	≤0.25	≤0.5	≤0.03	>128	>1024	≤0.5	>64	≤0.25	>32	Mcr-1, TEM
		S-297-1	pf1, EE+	B1	Ec22	>64	4	≤8	>8	4 (8)	≤0.25	≤0.5	≤0.03	>128	>1024	≤0.5	>64	≤0.25	>32	Mcr-1, TEM-1
		S-299-1	pf2, EE+	B1	Ec22	>64	4	≤8	>8	4	≤0.25	≤0.5	≤0.03	>128	>1024	≤0.5	>64	0.5	>32	Mcr-1, TEM
	7	S-60-1	bs, MC+	B1	Ec5	>64	4	64	>8	4 (2[a])	2	>32	≤0.03	>128	>1024	4	>64	≤0.25	>32	Mcr-1,TEM-1, CMY-2
	14	S-318-1	bs1, EE+	A	Ec20	>64	16	64	>8	4	≤0.25	≤0.5	≤0.03	>128	>1024	≤0.5	>64	0.5	>32	Mcr-1, TEM
		S-319-1	pf1, EE+	A	Ec20	>64	16	64	>8	4	≤0.25	1	≤0.03	>128	>1024	≤0.5	>64	≤0.25	>32	Mcr-1, TEM
		S-320-1	bs2, EE+	A	Ec20a	>64	16	32	>8	4	≤0.25	≤0.5	≤0.03	>128	>1024	≤0.5	>64	≤0.25	>32	Mcr-1, TEM
		S-321-1	pf2, EE+	A	Ec20	>64	16	64	>8	4 (8)	≤0.25	≤0.5	≤0.03	>128	>1024	≤0.5	>64	≤0.25	>32	Mcr-1, TEM-1
	16	S-322-1	bs, EE+	A	Ec20	>64	16	64	>8	4 (4)	≤0.25	≤0.5	≤0.03	>128	>1024	≤0.5	>64	≤0.25	>32	Mcr-1, TEM-1
	20	S-330-1	bs, EE+	E	n.t.	>64	4	32	8	8 (8)	≤0.25	1	≤0.03	>128	>1024	≤0.5	≤2	≤0.25	>32	Mcr-1, TEM-1
East	1	S-2-1	pf, Ga	A	Ec24	>64	≤2	≤8	≤0.015	4 (8)	≤0.25	≤0.5	≤0.03	≤4	>1024	≤0.5	>64	≤0.25	>32	Mcr-1, TEM-1
	3	S-508-1	pf, Ga	D	Ec23	>64	8	>128	>8	8 (8)	≤0.25	>32	≤0.03	>128	>1024	≤0.5	>64	0.5	>32	Mcr-1, TEM-1
	45	S-141-1	bs1, MC+	A	Ec8	>64	8	≤8	≤0.015	8	>4	≤0.5	≤0.03	≤4	>1024	2	>64	0.5	>32	Mcr-1, CTX-M, TEM
		S-366-1	bs1, EE+	B1	Ec6	>64	4	≤8	8	8	>4	8	≤0.03	>128	>1024	2	64	≤0.25	>32	Mcr-1, CTX-M-1, TEM-1
		S-142-2	pf1, MC+	B1	Ec10	>64	≤2	≤8	8	4 (4)	>4	16	≤0.03	>128	>1024	≤0.5	64	≤0.25	>32	Mcr-1, CTX-M-1, TEM-1
		S-144-1	bs2, MC+	B1	Ec4	>64	4	≤8	≤0.015	4	>4	≤0.5	≤0.03	≤4	>1024	1	≤2	≤0.25	>32	Mcr-1, CTX-M
	52	S-93-1	bs1, MC+	B1	Ec18	>64	4	≤8	8	8	>4	16	≤0.03	>128	>1024	1	64	≤0.25	>32	Mcr-1, CTX-M, TEM
		S-103-1	pf1, MC+	B1	Ec3	>64	4	≤8	≤0.015	4	>4	≤0.5	≤0.03	≤4	>1024	4	64	≤0.25	>32	Mcr-1, CTX-M, TEM
		S-337-1	bs1, EE+	B1	Ec12	>64	4	≤8	8	4	>4	>32	≤0.03	>128	>1024	1	64	≤0.25	>32	Mcr-1, CTX-M-1, TEM
		S-94-1	bs2, MC+	B1	Ec3a	>64	4	≤8	0.03	4	>4	≤0.5	≤0.03	≤4	>1024	2	>64	0.5	>32	Mcr-1, CTX-M, TEM
		S-338-1	bs2, EE+	B1	Ec17	>64	4	≤8	8	8	>4	16	≤0.03	>128	>1024	1	64	≤0.25	>32	Mcr-1, CTX-M-1, TEM-1
		S-105-1	pf2, MC+	B1	Ec1	>64	4	≤8	≤0.015	4 (2[a])	>4	≤0.5	≤0.03	≤4	>1024	1	64	0.5	>32	Mcr-1, CTX-M-1, TEM-1
		S-95-1	bs3, MC+	B1	Ec7a	>64	4	≤8	≤0.015	4	>4	≤0.5	≤0.03	≤4	>1024	1	>64	≤0.25	>32	Mcr-1, CTX-M, TEM
		S-96-1	bs4, MC+	D	Ec9	>64	4	≤8	≤0.015	4	>4	≤0.5	≤0.03	≤4	>1024	2	>64	0.5	>32	Mcr-1, CTX-M, TEM
		S-108-1	pf5, MC+	E	Ec13	>64	8	>128	≤0.015	8	>4	1	≤0.03	≤4	>1024	4	>64	0.5	>32	Mcr-1, CTX-M, TEM
		S-98-1	bs6, MC+	E	Ec19a	>64	4	>128	≤0.015	4	>4	≤0.5	≤0.03	≤4	>1024	2	>64	≤0.25	>32	Mcr-1, CTX-M, TEM
		S-99-1	bs7, MC+	B1	Ec21	>64	4	≤8	≤0.015	4	>4	≤0.5	≤0.03	≤4	>1024	1	64	0.5	>32	Mcr-1, CTX-M
		S-112-1	pf9, MC+	B1	Ec7	>64	4	≤8	0.03	4	>4	≤0.5	0.06	≤4	>1024	1	64	≤0.25	>32	Mcr-1, CTX-M, TEM
		S-102-1	bs10, MC+	E	Ec19	>64	4	>128	≤0.015	8	>4	≤0.5	≤0.03	≤4	>1024	2	>64	≤0.25	>32	Mcr-1, CTX-M, TEM
	115	S-462-1	bs, EE+	D	Ec2	>64	4	≤8	>8	4	≤0.25	1	≤0.03	>128	>1024	≤0.5	>64	0.5	>32	Mcr-1, TEM-1
South	55	S-176-1	pf1, MC+	A	Ec25g	>64	64	128	>8	4	>4	>32	≤0.03	>128	>1024	1	>64	≤0.25	>32	Mcr-1, CTX-M-1
		S-177-1	pf2, MC+	A	Ec25b	>64	64	>128	>8	4	>4	>32	≤0.03	>128	>1024	2	>64	≤0.25	>32	Mcr-1, CTX-M-1
		S-391-1	pf2, EE+	A	Ec25a	>64	4	>128	>8	4 (8)	>4	>32	≤0.03	>128	>1024	2	≤2	≤0.25	>32	Mcr-1, CTX-M-1
		S-179-1	bs2, MC+	A	n.t.	>64	4	≤8	>8	4	>4	≤0.5	≤0.03	>128	>1024	2	≤2	≤0.25	>32	Mcr-1, CTX-M-1
	56	S-159-1	pf1, MC+	A	Ec25	>64	64	128	>8	4	>4	>32	≤0.03	>128	>1024	≤0.5	>64	≤0.25	>32	Mcr-1, CTX-M-1
		S-393-1	bs1, EE+	A	Ec25c	>64	64	64	>8	4	>4	>32	≤0.03	>128	>1024	1	>64	≤0.25	>32	Mcr-1, CTX-M
		S-394-1	bs2, EE+	A	Ec25f	>64	4	128	>8	4	>4	>32	≤0.03	>128	>1024	1	>64	≤0.25	>32	Mcr-1, CTX-M
		S-160-1	pf2, MC+	A	Ec25e	>64	64	>128	>8	4	>4	>32	≤0.03	>128	>1024	2	>64	≤0.25	>32	Mcr-1, CTX-M
		S-395-1	pf2, EE+	A	Ec25d	>64	>64	128	>8	4 (4)	>4	>32	≤0.03	>128	>1024	2	>64	≤0.25	>32	Mcr-1, CTX-M-1
	59	S-189-1	bs2, MC+	A	Ec15	>64	8	≤8	0.03	8 (8)	>4	≤0.5	0.06	≤4	16	8	≤2	≤0.25	≤0.25	Mcr-1, CTX-M-1

PFGE, pulsed-field gel electrophoresis; AMP, ampicillin; AZI, azithromycin; CHL, chloramphenicol; CIP, ciprofloxacin; COL, colistin; FOT, cefotaxime; GEN, gentamicin; MERO, meropenem; NAL, nalidixic acid; SMX, sulfamethoxazole; TAZ, ceftazidime; TET, tetracycline; TGC, tigecycline; TMP, trimethoprim; bs, boot swab; pf, pooled faeces (when different stables within one farm were *mcr-1*-positive, the index indicates the respective stable number); MC+, MacConkey agar + 1 μg/mL cefotaxime; EE+, Endo agar + 2 μg/mL enrofloxacin; Ga, Gassner agar (non-selective); n.t., non-typeable; EUCAST, European Committee on Antimicrobial Susceptibility Testing.
Isolates highlighted in dark grey represent the 15 *Escherichia coli* isolates in which whole-genome sequencing was performed.
MICs highlighted in light grey represent resistance according to EUCAST.
In the 'COL' column, respective *mcr-1*-containing transformants were investigated for their COL MICs (shown in parentheses).
According to EUCAST guidelines [16], a colistin MIC of 2 μg/mL cannot be interpreted as resistant. However, the recipient strains NEB-5-α and NEB-10-β (New England Biolabs, Frankfurt, Germany) were tested in the same way and both of them possessed complete sensitivity (MIC ≤ 0.5 μg/mL).

Further characterisation of the isolates by PFGE depicted a quite heterogeneous pattern of *mcr-1*-positive isolates (Table 1). All of the isolates were resistant to colistin (MIC ≥4 μg/mL) [16]. In addition, most of the isolates also possessed resistance to several other antimicrobial agents (Table 1). Whilst the detected cephalosporin resistances were explainable by the presence of either an ESBL or AmpC gene, screening for plasmid-encoded fluoroquinolone resistance genes was negative. In this case, the existence of chromosomally encoded point mutations within the topoisomerase II and IV genes (*gyrA* and *parC*) was most likely. However, none of the isolates showed resistance to meropenem.

3.2. Detailed characterisation of selected mcr-1-containing isolates

Fifteen *mcr-1*-containing *E. coli* isolates (one per positive tested farm) were chosen for detailed characterisation. An overview of the whole-genome data is given in Table 2. These data confirmed the large heterogeneity among the *mcr-1*-carrying isolates as determined by PFGE. Among the 15 analysed isolates, 13 different *E. coli* MLST sequence types (STs) were detected. Only two STs were found twice: isolates derived from farms 14 (S-321-1) and 16 (S-322-1) displayed ST410, whilst two *E. coli* isolates from farms 55 (S-391-1) and 56 (S-395-1) belonged to ST2509. In the case of S-321-1 and S-322-1, even the same resistance genes and almost identical plasmid replicon types were detected. S-391-1 and S-395-1 both belonged to the same ST but the resistance gene pattern as well as the plasmid replicon type composition were different among these isolates (Table 2). Beside the colistin resistance gene *mcr-1*, the investigated isolates harboured several additional antibiotic resistance genes conferring phenotypic resistance to different classes of antimicrobials (Tables 1 and 2). Plasmid isolation and transformation experiments showed that the *mcr-1* gene, as well as the related colistin resistance, was transferable in 14 of 15 cases (Table 1). Plasmid replicon typing showed that in 9 of the 14 isolates the *mcr-1* gene was located on an IncX4 plasmid, in 3 cases the *mcr-1* gene was located on an IncHI2-plasmid and in 2 isolates the *mcr-1*-containing plasmids displayed multiple replicon types (IncX4/N or IncHI2/FIB/FII/X3). For S462-1, neither electroporation nor conjugative transfer of an *mcr-1*-containing plasmid was possible, indicating that the gene might have been integrated into the bacterial chromosome. In addition, conjugative transfer of the *mcr-1*-containing plasmid was not possible for isolates S-105-1 and S-395-1, indicating that these plasmids were not self-transmissive. However, a more detailed analysis of the ten isolates containing the *mcr-1* gene located on an IncX4 plasmid indicated that a highly conserved IncX4 plasmid is present in all farm isolates, highly similar to the already described plasmid pICBEC72Hmcr [21] (Fig. 1). This plasmid has been detected in an *E. coli* isolate derived from a urinary tract infection in a Brazilian hospital. S1-nuclease PFGE confirmed a plasmid size for all IncX4-carrying isolates in the same range, described for pICBEC72Hmcr (33.67 kb, data not shown).

4. Discussion

The results described here showed that during the years 2011 and 2012, 9.9% of the investigated bacterial cultures and 25.9% of the investigated pig-fattening farms were positive for the presence of the *mcr-1* gene. This value was almost the same across the different agricultural regions of Germany. However, one has to consider the data derived from mostly pre-selected bacterial cultures, which might have influenced the number of *mcr-1*-positive isolates in total. Nevertheless, the numbers appear to be comprehensive, as an earlier study on antibiotic usage in German pig farms reported that among 495 farms, colistin was by far the most used antibiotic for the treatment of intestinal diseases in piglets and weaners in 2011 [23]. However, one has to keep in mind that most of the isolates analysed here contained several different antibiotic resistance genes causing phenotypic resistance against a multitude of antibiotics. Therefore, even in the absence of colistin usage, co-selection can lead to further spread of *mcr-1*-containing isolates and/or plasmids.

Table 2
In-depth investigation of 15 selected *mcr-1*-containing *Escherichia coli* isolates derived from 15 different pig farms.

Isolate (accession number)[a]	ST	Resistance genes	Plasmids[b]
S-508-1 (**ERS1507261**)	n.c.	*aac(3)-IIa*, *aadA1*-like, *aph(3′)-Ia*-like, *strA*, *strB*, *mcr-1*, *sul1*-like, *sul2*, *sul3*, *dfrA1*, *tet*(A), *tet*(D)-like, *catA1*-like, $bla_{TEM-1B-like}$	**IncHI2**, IncFIC(FII), IncHI2A, **IncFII**, IncI1, **IncFIB**(AP001918), IncX1, **IncX3**, IncQ1, IncX4, ColRNAI
S-462-1 (**ERS1507260**)	ST1011	*aadA1*, *strA*, *strB*, *mcr-1*, *sul1*, *sul2*, *dfrA1*, *tet*(A), bla_{TEM-1B}	IncFII, IncFIB(AP001918), IncI2, IncX1, p0111, IncQ1, IncX4
S-395-1 (**ERS1507259**)	ST2509	*aac(3)-IId*-like, *aadA1*, *aadA2*, *aadA5*, *mcr-1*, *sul2*, *sul3*, *dfrA12*, *dfrA17*, *tet*(A)-like, *cmlA1*-like, *floR*-like, $bla_{CTX-M-1}$, *mph(A)*	IncI1, IncFIC(FII), IncY, IncR, Col(MG828), **IncX4**, ColRNAI
S-330-1 (**ERS1507257**)	ST57	*aadA2*, *mcr-1*, *sul1*, *sul3*, *dfrA1*, *tet*(B), *catA1*-like, *cmlA1*-like, bla_{TEM-1A}	IncFIB(pLF82), IncI1, IncFIC(FII), IncHI1B(R27), IncFIA(HI1), IncFIB(AP001918), IncHI1A, IncI2, p0111, **IncX4**
S-322-1 (**ERS1507256**)	ST410	*aadA1*, *aadA2*, *mcr-1*, *sul3*, *dfrA12*, *tet*(B)-like, *tet*(M)-like, *cmlA1*-like, bla_{TEM-1B}, *mef*(B)	IncFII, IncR, Col(MG828), Col(BS512), ColRNAI, Col156, ColE10, **IncX4**
S-321-1 (**ERS1507255**)	ST410	*aadA1*, *aadA2*, *mcr-1*, *sul3*, *dfrA12*, *tet*(B)-like, *tet*(M)-like, *cmlA1*-like, bla_{TEM-1B}, *mef*(B)	IncFII, IncR, Col(MG828), Col(BS512), ColRNAI, Col156, IncX1, ColE10, **IncX4**
S-297-1 (**ERS1507254**)	ST448	*aadA1*, *aph(3′)-Ia*, *strA*, *strB*, *mcr-1*, *sul2*, *dfrA1*, *dfrA5*, *tet*(A)-like, bla_{TEM-1B}	IncFIB(pLF82), IncFII, IncI1, IncFIB(AP001918), IncQ1, **IncX4**
S-228-1 (**ERS1507253**)	ST1842	*aac(3)-VIa*-like, *aadA1*, *aadA2*, *aph(3′)-Ia*, *mcr-1*, *sul3*, *tet*(A), *cmlA1*-like, $bla_{TEM-1B-like}$	IncFIB(pLF82), IncI1, p0111, Col(MG828), **IncX4**, ColRNAI
S-222-1 (**ERS1507252**)	ST2040	*mcr-1*, bla_{CMY-2}	IncFIC(FII), IncI1, IncFIB(AP001918), IncI2, Col(MG828), Col156, **IncX4**, ColRNAI
S-189-1 (**ERS1507251**)	ST34	*mcr-1*, $bla_{CTX-M-1}$, *mph*(A)	IncI1, **IncN**, Col8282, ColRNAI, **IncX4**, Col(MG828)
S-142-2 (**ERS1507250**)	ST2067	*aac(3)-IVa*-like, *aadA1*, *aph(4)-Ia*, *mcr-1*, *sul1*-like, *sul2*, *dfrA1*, *tet*(A), $bla_{CTX-M-1}$, bla_{TEM-1B}	IncHI2A, **IncHI2**, IncB/O/K/Z, ColRNAI, Col(MG828)
S-105-1 (**ERS1507249**)	ST4398	*aadA1*, *aadA2*, *aadA5*, *mcr-1*, *sul2*, *sul3*, *dfrA17*, *tet*(A), *cmlA1*-like, $bla_{CTX-M-1}$, $bla_{TEM-1B-like}$	**IncHI2**, IncFIC(FII), IncI1, IncFIB(AP001918), IncHI2A, IncI2, IncX4, Col(MG828)
S-60-1 (**ERS1507248**)	ST1196	*aac(3)-IIa*, *aadA1*-like, *aadA2*, *aph(3′)-Ic*-like, *strA*, *strB*, *mcr-1*, *sul1*, *sul2*, *sul3*, *dfrA1*, *dfrA12*, *tet*(A)-like, *catA1*-like, *cmlA1*-like, bla_{CMY-2}, bla_{TEM-1B}	IncHI2A, IncFII, **IncHI2**, IncFIB(AP001918), IncR, IncQ1, ColRNAI
S-2-1 (**ERS1507247**)	ST540	*aadA1*, *strA*, *strB*, *mcr-1*, *sul1*, *sul3*, *dfrA1*, *tet*(A), *tet*(B), bla_{TEM-1B}	IncI1, IncX1, Col8282, **IncX4**, Col(MG828)
S-391-1 (**ERS1507258**)	ST2509	*aad1*, *aadA5*, *aac(3)-IId*, *aadA2*, *mcr-1*, *sul2*, *dfrA12*, *dfrA17*, *tet*(M), *floR*, *cmlA1*, $bla_{CTX-M-1}$	IncY, IncR, Col (MG828), **IncX4**, ColRNAI

ST, sequence type; n.c., not completely characterised.
[a] Whole-genome sequence data were deposited in the database of the European Nucleotide Archive (ENA); accession numbers are included within the table.
[b] Bold replicon types indicate localisation of the *mcr-1* gene.

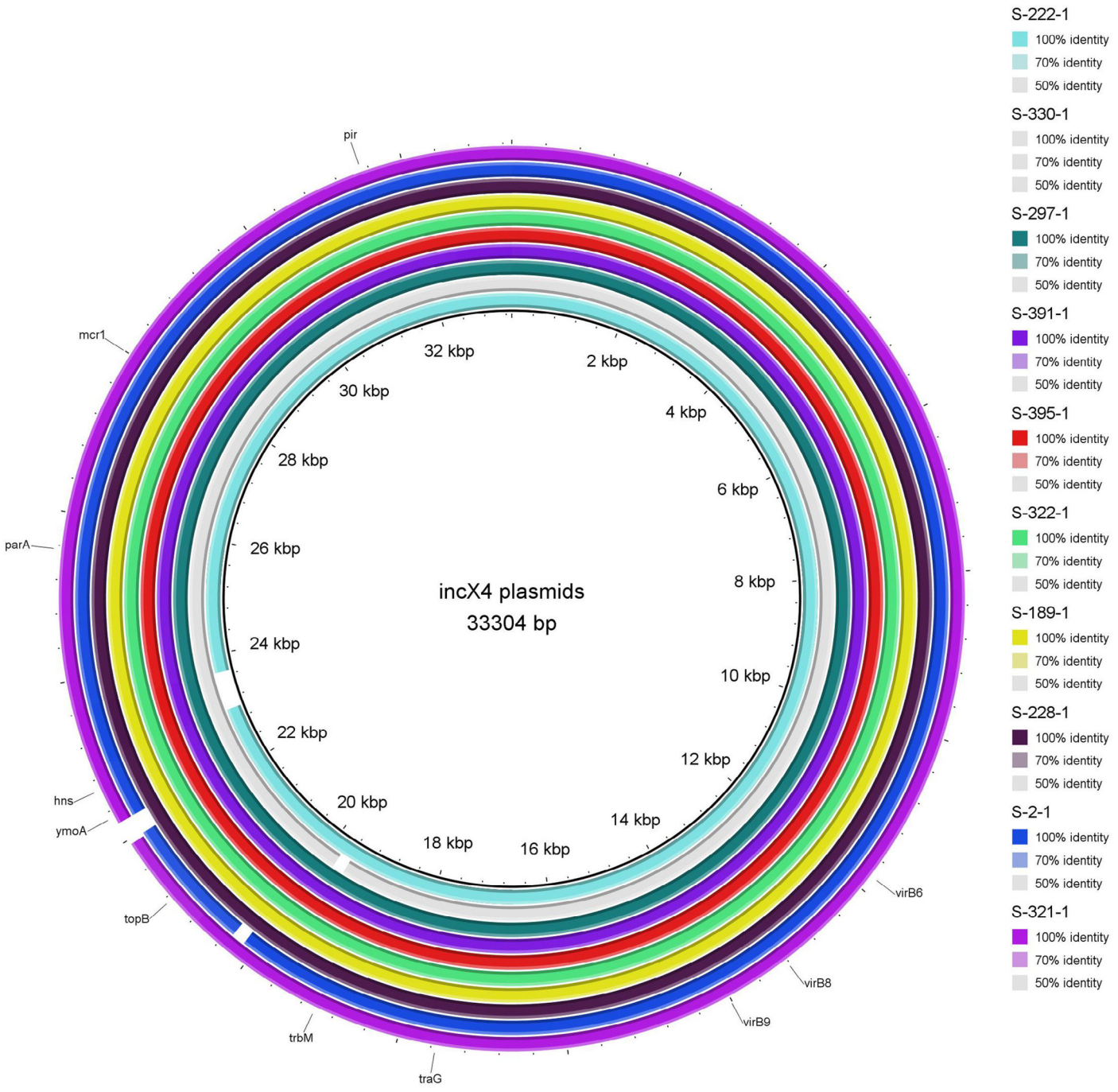

Fig. 1. Comparison of 10 *mcr-1*-containing IncX4 plasmids with pICBEC72Hmcr derived from a hospitalised Brazilian patient using BLAST Ring Image Generator (BRIG) [22].

Compared with the detected *mcr-2* prevalence of 20.75% in Belgium in the years 2011–2012 [6], none of the German samples was positive for *mcr-2* in the same time period. However, it has to be considered that the Belgian samples were derived from diseased piglets, whilst the cultures investigated in the present study were initially taken from asymptomatic fattening pigs and their surroundings.

A comparison of the 43 *mcr-1*-positive *E. coli* isolates derived from 15 farms indicated broad heterogeneity. Overall, 26 different PFGE patterns have been detected and even among *E. coli* isolates derived from same farms differences occurred (Table 1). However, the *E. coli* isolates from farms 55 and 56 (south of Germany) as well as farms 14 and 16 (middle of Germany) displayed the same or similar PFGE patterns. Similar results were obtained by the whole-genome analyses of 15 selected *mcr-1*-positive *E. coli* isolates. A possible explanation for these findings can be the local distribution of the *mcr-1*-positive farms. Farms 14 and 16 were located within the same rural district, and farms 55 and 56 were located in neighbouring districts (distance ca. 50 km). Although the presently described data showed great heterogeneity among the *mcr-1*-positive isolates, it has been shown that the *mcr-1* gene was plasmid-located in 14 of 15 investigated samples; 12 of them were also proved to be self-transmissive. In addition, in 10 of the 14 cases, and therefore most frequently, the *mcr-1* gene was located on an IncX4 plasmid. S1-nuclease PFGE and BRIG alignments of the *mcr-1*-containing IncX4 plasmids showed a high similarity among these ten plasmids, which indicates that a highly conserved *mcr-1*-containing IncX4 plasmid is widespread among German pig-fattening farms. This assumption

was also shown in a quite recently published study [24]. The plasmid sequences were compared with the already reported *mcr-1*-containing IncX4 plasmids and the results indicate a high degree of similarity with pICBEC72Hmcr, which was derived from a human patient hospitalised in Brazil [21] (Fig. 1). Recently, IncX4 plasmids containing the colistin resistance gene *mcr-1* have been isolated from *Salmonella* and *E. coli* isolates derived from different sources (e.g. livestock and human samples, meat, ready to cook guinea fowl pie) all over the world [21]. Therefore, the current results emphasise the already voiced concerns for public health.

The question of whether these results may support a reduction in colistin use in animals to decrease the risk of antimicrobial resistance as claimed by the European Medicines Agency (EMA) in July 2016 [25] remains to be demonstrated, considering that spread of the *mcr-1* gene is not exclusively connected to the colistin supply within stables. As colistin belongs to one of the most important antibiotics in veterinary practice, and in consideration of the claimed reduction of colistin sales for veterinary use, these measures will only be effective if colistin is not going to be replaced by an increased usage of antibiotics such as third- and fourth-generation cephalosporins or fluoroquinolones. Therefore, further monitoring programmes and intervention strategies, addressing the reduction of *mcr-1* and also helping to contain its transmission, should be considered.

5. Conclusion

The current data show that *mcr-1*-containing *E. coli* isolates were present in 25.9% of the investigated pig-fattening farms in the years 2011–2012. These numbers are quite alarming and, as colistin belongs to the critically important antibiotics for the treatment of serious infections in humans, the current situation has to be assessed carefully. As distribution via the plasmid route appears to play a greater role than clonal spread of *mcr-1*-harbouring bacteria, ongoing studies addressing the detection of *mcr-1* in animal and environmental samples as well as cases of human infection are highly recommended. The generated data should be used for the evaluation of intervention strategies consisting of the prudent use of antibiotics in livestock farming as well as human medicine and the containment of the environmental spread of antibiotic-resistant bacteria.

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