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Molekulargenetische Verfahren zur Untersuchung der Patienten- und Umgebungsmikrobiom-assoziierten Krankheitsentstehung und -dynamik

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1. **Neidhöfer C**, Buechler C, Neidhöfer G, Bierbaum G, Hannet I, Hoerauf A, Parčina M. Global Distribution Patterns of Carbapenemase-Encoding Bacteria in a New Light: Clues on a Role for Ethnicity. Front Cell Infect Microbiol. 2021 Jun 29;11:659753. doi: 10.3389/fcimb.2021.659753. (IF: 5.7)

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Abkürzungsverzeichnis

ADLA	
AMR	Antimikrobielle Resistenz
CC	Zervixkarzinom
CEB	Carbapenemase-kodierende Bakterien
cfDNA	Zellfreie Desoxyribonukleinsäure
CIN	Cervikale intraepitheliale Neoplasie
DNA	Desoxyribonukleinsäure
HPV	Humane Papillomviren
hrHPV	High-Risk Human Papillomavirus
MIC	Minimale Hemmkonzentration
MRSA	Methicillin-resistenter Staphylococcus aureus
NDM	“New Delhi” Metallo-Beta-Laktamase
NGS	Next-Generation Sequencing
OXA	“Active on oxacillin” Beta-Laktamase
PAP	Papanicolaou-Test
ST	Sequenztyp
VIM	“Verona integron-encoded“ Metallo-Beta-Laktamase
VRE	Vancomycin-resistente Enterokokken
WGS	Whole-Genome-Sequencing

2. Einleitung

2.1 Ein sich durch neue Technologien stetig wandelndes Verhältnis zu Bakterien

Am frühen Morgen des 28. September 1928 stolperte Alexander Fleming, ganz durch Zufall, über eine Entdeckung, die als einer der größten Wendepunkte in der Geschichte der Medizin gelten sollte. In seinem Labor fand er eine Petrischale, die er offen gelassen hatte und in der sich ein Schimmelpilz der Gattung *Penicillium* ausgebreitet hatte. Um diesen Schimmelpilz herum bemerkte er eine klare Zone, in der das Wachstum von Staphylokokken gehemmt wurde. Dies war der erste Blick auf einen verborgenen Mikrokosmos, in dem Mikroorganismen seit Äonen einen unerbittlichen Krieg führten. Fleming erkannte, dass er vielleicht eine Methode gefunden hatte, um diese Kämpfe zu Gunsten der Menschheit zu beeinflussen.

Durch seine weiteren Forschungen und die anschließende Entwicklung und Verfeinerung durch andere Wissenschaftler wurde aus dem Schimmelpilz das erste echte Antibiotikum, Penicillin, gewonnen. Diese Substanz revolutionierte den Kampf gegen bakterielle Infektionen und rettete bis zum heutigen Tag unzählige Leben. Fleming hatte der Menschheit nicht nur ein mächtiges Werkzeug gegen lange gefürchtete Krankheiten in die Hand gegeben, sondern auch ein neues Zeitalter der medizinischen Behandlung eingeläutet. Jedoch, wie von Fleming selbst vorausgesagt, dauerte es nicht lange, bis die Bakterien begannen, sich an die neue Bedrohung anzupassen.

Zu Zeiten von Alexander Fleming wurden Bakterien hauptsächlich als Krankheitserreger wahrgenommen, die eine unmittelbare Bedrohung für die menschliche Gesundheit darstellten. Diese Sichtweise war damals weit verbreitet, da die medizinische Forschung und die öffentliche Wahrnehmung stark von den negativen Auswirkungen bakterieller Infektionen geprägt waren, wie etwa Tuberkulose oder Pest, die verheerende Epidemien verursachten. Erst in jüngerer Zeit beginnen wir zu realisieren, dass die große Mehrheit der Bakterien harmlos oder sogar förderlich für unsere Gesundheit und Umwelt ist.

Bakterien sind allgegenwärtige einzellige Lebewesen. Sie repräsentieren die zahlenmäßig vorherrschende Lebensform unseres Planeten und gedeihen in einer Vielzahl unterschiedlichster Umgebungen. Dazu gehören sowohl das Innere als auch die

Oberfläche zahlreicher mehrzelliger Organismen, einschließlich des menschlichen Körpers. Diese mikroskopisch kleinen Organismen sind nicht nur einfache Bewohner unseres Planeten, sondern spielen eine entscheidende Rolle in den Ökosystemen, in denen sie existieren. Sie sind essentiell für den Abbau organischer Materialien und tragen dadurch zur Nährstoffkreisläufen bei, welche die Grundlage für das Leben größerer Organismen bilden. Weiterhin sind Bakterien zentral für die biologische Diversität und die Stabilität von Habitaten, von den Tiefen der Ozeane bis zu den extremsten Bedingungen, wie sie in heißen Quellen und arktischen Eisfeldern vorkommen. Im menschlichen Körper tragen sie durch ihre Präsenz auf der Haut, im Verdauungstrakt und in anderen Bereichen wesentlich zur Gesundheit bei. Sie helfen z.B. bei der Verdauung von Nahrungsmitteln und trainieren unser Immunsystem, effektiv zu funktionieren.

Die komplexe Symbiose zwischen menschlichen Zellen und ihren bakteriellen Mitbewohnern ist ein faszinierendes Feld der Wissenschaft, das aufgrund vorher nicht verfügbarer Technologien erst in den letzten Jahrzehnten tiefergehend erforscht wurde. Diese Erkenntnis führt allmählich zu einem Wandel im Verhältnis und Bewusstsein gegenüber Bakterien. Die heutige und zukünftige Generationen entwickeln ein nuancierteres Verständnis, das die essentiellen positiven Beiträge dieser Mikroorganismen anerkennt. Die Herausforderung liegt nun darin, dieses Wissen in öffentliche Gesundheitsstrategien und alltägliche Praktiken zu integrieren, um sowohl die positiven Aspekte der bakteriellen Existenz zu nutzen als auch die Risiken zu mindern.

Unser Verhältnis zu Bakterien hat sich durch technologische Fortschritte und wissenschaftliche Entdeckungen erheblich gewandelt. Mit der Einführung von Antibiotika verloren Bakterien ihren Schrecken als unkontrollierbare Krankheitserreger, und die Menschen empfanden weniger Angst vor bakteriellen Infektionen. Später veränderte die Mikrobiomforschung unsere Sichtweise erneut grundlegend, indem sie aufzeigte, dass Bakterien nicht nur potenzielle Feinde, sondern auch unverzichtbare Verbündete unserer Gesundheit sind.

Vereinzelte Bakterienpopulationen besitzen jedoch wie erwähnt das Potenzial, für bestimmte Arten pathogen zu sein. Diese Bakterien sind in der Lage, Wirtszellen zu schädigen, indem sie in deren Funktionen eingreifen oder Immunreaktionen auslösen, die ebenfalls zu einer Schädigung der Wirtszellen führen. Pathogene Bakterien haben im

Laufe der Menschheitsgeschichte unzählige Todesfälle verursacht und stellen bis heute in vielen weniger entwickelten Regionen der Welt eine erhebliche Bedrohung dar. Opportunistische Pathogene sind Mikroorganismen, die normalerweise in oder auf dem menschlichen Körper existieren, ohne Krankheiten zu verursachen, jedoch die Fähigkeit besitzen, Infektionen auszulösen, wenn sich die Gelegenheit bietet – beispielsweise wenn das Immunsystem geschwächt ist oder wenn sie in eine normalerweise sterile Körperregion gelangen. Im Gegensatz zu obligaten Pathogenen, die gesunde Individuen infizieren und Krankheiten verursachen können, sind opportunistische Pathogene in der Regel nur unter bestimmten Umständen gefährlich.

In den letzten Jahrzehnten sind insbesondere in entwickelten Ländern opportunistische Pathogene zunehmend in den Vordergrund getreten (Allegranzi et al. 2011; Byarugaba 2004), fast wichtiger als die klassischen Pathogene, die durch Impfungen und öffentliche Gesundheitsmaßnahmen größtenteils kontrolliert warden (Excler et al. 2021; Minor 2002). Dieser Bedeutungszuwachs ist teilweise darauf zurückzuführen, dass durch medizinische Fortschritte, wie die erhöhte Nutzung von Immunsuppressiva und die längere Lebenserwartung von Personen mit chronischen Krankheiten, mehr Menschen anfällig für Infektionen durch opportunistische Pathogene sind (Sepkowitz 2002; Singh 2001). Darüber hinaus können die breite Anwendung von Antibiotika und die daraus resultierende Veränderung der natürlichen Mikroflora des Körpers ebenfalls das Risiko für Infektionen durch opportunistische Pathogene erhöhen (Sullivan et al. 2001). Die wachsende Bedeutung dieser Mikroorganismen unterstreicht die Notwendigkeit, unser Verständnis ihrer Pathogenitätsmechanismen zu vertiefen und neue Strategien zur Vorbeugung und Behandlung der von ihnen verursachten Infektionen zu entwickeln. Dies erfordert eine kontinuierliche Überwachung und Anpassung der öffentlichen Gesundheitsstrategien, um den Schutz der Bevölkerung sicherzustellen.

2.2 Das Krankenhaus im Zentrum der Resistenzproblematik

Bakterien besitzen die beachtliche Fähigkeit, Gene aus verschiedenen Bakterienpopulationen zu rekombinieren, was es ihnen ermöglicht, sich schnell an Umgebungen anzupassen, die ihr Wachstum eigentlich behindern würden. Diese

Resistenz nimmt zunehmend zu und schränkt die therapeutischen Möglichkeiten ein, sodass bestimmte menschliche Infektionen nicht mehr behandelbar sind. Die Empfindlichkeit verschiedener Bakterien gegenüber Antibiotika kann stark variieren. Beispielsweise reagieren Bakterien der Spezies *Staphylococcus aureus* bereits auf geringe Konzentrationen von Methicillin empfindlich, während andere Spezies viel höhere Dosen benötigen würden, die im menschlichen Körper nur schwer zu erreichen sind. Diese Unterschiede in der Empfindlichkeit führen zum Konzept der minimalen Hemmkonzentration (MIC) und der klinischen Resistenz, die nicht nur von der Art des Bakteriums und dem Antibiotikum, sondern auch von der Lokalisation der Infektion im Körper, dem Immunstatus des Patienten und der Verteilung des Medikaments im Körper abhängen (Andrews 2001). Einige Bakterien besitzen eine angeborene Resistenz gegen bestimmte Antibiotika, das größere Problem in der medizinischen Praxis ist jedoch die erworbene Resistenz, die sich in ursprünglich empfindlichen Bakterienpopulationen entwickelt. Auch wenn hochresistente Bakterienstämme theoretisch durch hohe Antibiotika-konzentrationen abgetötet werden könnten, sind solche Konzentrationen für Patienten ggfs. nicht verträglich.

Seit der Einführung von Antibiotika in die medizinische Praxis haben Bakterien, die häufige oder schwere Infektionen verursachen, in unterschiedlichem Ausmaß Resistenzen gegen jedes neu auf den Markt kommende Antibiotikum entwickelt. Das Aufkommen von Multiresistenzen bei pathogenen Bakterien gefährdet den Wert der Antibiotika, die zuvor die medizinischen Wissenschaften revolutioniert haben. Die zunehmende Antibiotikaresistenz stellt eine ernsthafte globale Bedrohung dar, die zunehmend Anlass zur Sorge gibt und eine erhebliche wirtschaftliche Belastung für die ganze Welt darstellt. Mehrere Bereiche der modernen Medizin sind auf die Verfügbarkeit wirksamer Antibiotika angewiesen; Chemotherapie zur Krebsbehandlung, Organtransplantationen, Hüftgelenkersatzoperationen, die Intensivpflege von Frühgeborenen und viele andere medizinische Maßnahmen könnten ohne wirksame Antibiotika nicht durchgeführt werden. Tatsächlich sind Infektionen, die durch multiresistente Bakterienstämme verursacht werden, einer der Hauptfaktoren, die Morbidität und Mortalität bei Patienten beeinflussen, die diese Verfahren durchlaufen (Giamarellos-Bourboulis et al. 2006; Tacconelli et al. 2014).

Die Situation der antimikrobiellen Resistenz (AMR) variiert erheblich in verschiedenen Teilen der Welt, was größtenteils auf Unterschiede in der Verfügbarkeit von Antibiotika, den lokalen Einsatz von antimikrobiellen Substanzen und die spezifischen Gesundheitsinfrastrukturen zurückzuführen ist. In vielen Entwicklungsländern, wo der Zugang zu medizinischer Versorgung und die Kontrolle des Antibiotikagebrauchs oft unzureichend sind, treten besonders hohe Raten an antimikrobieller Resistenz auf (Boeckel et al. 2019; Collignon et al. 2018). Studien zeigen, dass in Regionen wie Südasien und Afrika die Prävalenz von resistenten Bakterienstämmen besonders hoch ist, was die Behandlungsmöglichkeiten erheblich einschränkt und die Sterblichkeitsraten steigert (Bell & Turnidge 2002; Ingle et al. 2018; Leopold et al. 2014). In Industrieländern sind trotz besserer hygienischer Standards und strengerer Regulierung des Antibiotikaeinsatzes ebenfalls zunehmende AMR-Probleme zu verzeichnen, insbesondere bei Krankenhauskeimen wie MRSA (Methicillin-resistenter Staphylococcus aureus) oder VRE (Vancomycin-resistente Enterokokken) (Frost et al. 2019; Neely & Maley 2000) . Die Verfügbarkeit und der häufige Gebrauch von Breitbandantibiotika haben in diesen Ländern zu einer Selektion resistenter Stämme geführt.

Krankenhäuser stehen im Zentrum der AMR-Problematik, da hier hochvirulente Pathogene auf eine vulnerable Patientenpopulation treffen. In Krankenhäusern werden oft invasive Verfahren durchgeführt, die das Risiko für Infektionen erhöhen, und es werden regelmäßig Antibiotika eingesetzt, was die Selektion resistenter Bakterien fördert. Darüber hinaus tragen die hohe Patientendichte und der manchmal unzureichende Einsatz von Infektionskontrollmaßnahmen zur schnellen Verbreitung resistenter Keime bei. Diese Faktoren machen Krankenhäuser zu einem Brennpunkt für die Entwicklung und Ausbreitung von AMR (Goldmann & Huskins 1997), was die Notwendigkeit unterstreicht, wirksame Strategien zur Infektionskontrolle und zum Antibiotikamanagement in diesen Einrichtungen zu implementieren.

Die Integration der Whole-Genome-Sequencing-Technologie (WGS) hat das Verständnis und Management von Ausbrüchen im Krankenhausumfeld revolutioniert. WGS wird zunehmend eingesetzt, um die genetischen Profile von Krankheitserregern bei Ausbrüchen genau zu bestimmen und zu verfolgen. Diese Technik ermöglicht es, den Übertragungsweg von Infektionen innerhalb von Krankenhäusern detailliert

nachzuvollziehen, was für die Eindämmung von Infektionsketten entscheidend ist. Bei Ausbrüchen ermöglicht WGS eine schnelle und präzise Identifizierung von Infektionsclustern, was besonders in komplexen Krankenhausumgebungen von unschätzbarem Wert ist. Durch die Analyse der vollständigen Genome von Bakterien können Forscher die spezifischen Mutationen identifizieren, die für Antibiotikaresistenzen verantwortlich sind. Dies verbessert nicht nur unser Verständnis darüber, wie Resistenzen sich entwickeln und verbreiten, sondern auch die Entwicklung gezielter Strategien zur Bekämpfung resistenter Stämme. Ein weiterer entscheidender Vorteil von WGS ist die Fähigkeit, den genetischen Kontext von Resistenzgenen zu bestimmen, was hilft zu verstehen, wie sich diese Gene zwischen verschiedenen Bakterienstämmen und -arten ausbreiten (Ellabaan et al. 2021). Diese Informationen sind entscheidend für die Entwicklung effektiver Kontrollmaßnahmen in Krankenhäusern und können zur Formulierung von Richtlinien beitragen, die auf die Verhinderung der Verbreitung resistenter Bakterien abzielen.

Die Anwendung der 16S rRNA-Sequenzierung und Metagenomik hingegen hat neben der Erforschung des Mikrobioms auch die des Krankenhausmikrobioms erheblich vorangetrieben (Lax et al. 2017; Lax & Gilbert 2015; Rampelotto et al. 2019). Diese Techniken haben es ermöglicht, die Zusammensetzung und Funktion mikrobieller Gemeinschaften in einer Vielzahl von Umgebungen tiefgreifend zu verstehen, ohne auf traditionelle kulturbasierte Methoden angewiesen zu sein. 16S rRNA-Sequenzierung wird speziell zur Identifizierung und Klassifizierung von Bakterien genutzt, da der 16S rRNA-Genabschnitt in allen Bakterien vorhanden ist, aber genügend Variation aufweist, um unterschiedliche Bakterienarten zu unterscheiden. Metagenomische Ansätze gehen noch einen Schritt weiter, indem sie die gesamte genetische Information innerhalb einer Umweltprobe sequenzieren. Dies ermöglicht nicht nur die Identifikation von Bakterien, sondern auch von Viren, Pilzen und anderen Mikroben sowie deren genetischen Funktionen, einschließlich Resistenzgenen. Metagenomik wurde verwendet, um das "Resistom" verschiedener Umgebungen zu charakterisieren, also die Sammlung aller Resistenzgene in einem bestimmten Mikrobiom. Diese Technik hilft dabei, die Verbreitung und Dynamik von AMR-Genen in Krankenhäusern zu verstehen und kann auch für die Überwachung der Effektivität von Sanierungsmaßnahmen eingesetzt werden (Lanza et al. 2017; Willmann & Peter 2016; Lax et al. 2017).

2.3 NGS-Anwendungen verbessern Infektionskontrolle und Diagnostik

Die fortschreitende Entwicklung und Verbreitung von Next-Generation Sequencing (NGS) Technologien hat einen signifikanten Einfluss auf die klinische Diagnostik. NGS-Technologien wie Whole-Genome Sequencing (WGS), Targeted Gene Sequencing und Metagenomics werden zunehmend in der klinischen Praxis eingesetzt. Diese Methoden bieten eine hohe Auflösung und Sensitivität für die Identifikation und Charakterisierung von Pathogenen, was besonders in der Diagnostik von Infektionskrankheiten und in der personalisierten Medizin von Bedeutung ist.

Whole-Genome Sequencing bietet in der mikrobiologischen Praxis umfassende Möglichkeiten, da es das gesamte Genom eines Mikroorganismus entschlüsseln kann. Dies ermöglicht es, nicht nur die Art des Erregers präzise zu identifizieren, sondern auch dessen genetisches Potenzial für Resistenzmechanismen, Virulenzfaktoren und epidemiologische Verknüpfungen zu analysieren. In der Praxis wird WGS bereits erfolgreich für die Überwachung von Krankenhausausbrüchen eingesetzt, wo es hilft, Übertragungswege zu klären und Infektionsketten zu unterbrechen (Nutman et al. 2019; Quainoo et al. 2017) und unter Umständen sogar Kosten zu senken (Kumar et al. 2020). In der Tuberkulose-Diagnostik ermöglicht das WGS eine schnelle und genaue Erkennung von Resistenzmustern (Gygli et al. 2018; Papaventsis et al. 2017), was besonders wichtig ist, da die Resistenz gegen die üblichen Erstlinienmedikamente ein wachsendes Problem darstellt. Traditionelle Methoden, wie die kulturelle Anzüchtung und Empfindlichkeitstests, können Wochen dauern, während WGS Ergebnisse innerhalb weniger Tage liefern kann. Dies verbessert die Geschwindigkeit, mit der individuell angepasste Behandlungspläne entwickelt werden können, und trägt dazu bei, die Ausbreitung resistenter Mycobakterien-Stämme zu verhindern (Eigenbrod et al. 2019; Walker et al. 2017).

Targeted Gene Sequencing konzentriert sich auf spezifische, klinisch relevante Genabschnitte eines Pathogens. Diese Technik ist besonders wertvoll, wenn schnelle diagnostische Antworten benötigt werden, wie zum Beispiel die Bestimmung der Antibiotikaresistenz oder die Bestätigung pathogener Varianten. Der Hauptvorteil von TGS liegt in der Geschwindigkeit und Kosteneffizienz im Vergleich zu WGS, besonders wenn nur bestimmte Genregionen von Interesse sind. Zu den Nachteilen gehört, dass es weniger umfassend ist und keine Informationen über das gesamte Genom des Pathogens

liefert, was bei komplexeren epidemiologischen oder diagnostischen Fragen erforderlich sein kann.

Metagenomik hat das Potenzial, die mikrobiologische Diagnostik durch die umfassende Analyse aller genetischen Materialien in einer Probe zu revolutionieren. Diese Technologie kann besonders in der Diagnostik von polymikrobiellen Infektionen oder in der Analyse des Mikrobioms von Bedeutung sein, ohne dass eine vorherige Kultivierung der Mikroorganismen notwendig ist. Trotz des enormen Potenzials wird Metagenomik in der klinischen Praxis noch zurückhaltend eingesetzt, hauptsächlich aufgrund der hohen Kosten und der komplexen Datenanalyse, die spezialisierte bioinformatische Fähigkeiten erfordert (Greninger 2018; Martin et al. 2018). Die Ergebnisse der Metagenomik können auch durch die immense Datenmenge und die Notwendigkeit, diese effektiv zu interpretieren, kompliziert sein.

Die Sequenzierung zellfreier DNA (cfDNA) bietet in der Diagnostik von Sepsis signifikante Vorteile, da sie eine schnelle und nicht-invasive Methode zur Erkennung und Charakterisierung von pathogenen Mikroorganismen im Blut darstellt. Bei Sepsis ist eine rasche Diagnose entscheidend, da die Erkrankung schnell fortschreiten kann und eine unverzügliche Behandlung erfordert. Die cfDNA-Sequenzierung ermöglicht es, spezifische bakterielle oder virale DNA im Kreislauf zu identifizieren, was eine gezielte Behandlung erleichtert. Die Gewinnung von cfDNA aus dem Blut ist minimal-invasiv im Vergleich zu anderen diagnostischen Verfahren (Blauwkamp et al. 2019; Camargo et al. 2019; Grumaz et al. 2020). Allerdings führt die hohe Sensitivität der Methode manchmal zur Detektion von DNA, die klinisch nicht relevant ist, was die Ergebnisinterpretation erschweren kann.

2.4 Zielsetzung

Die nachfolgenden Kapitel präsentieren die Ergebnisse aus insgesamt sechs umfangreichen Studien, die sich mit der Anwendung moderner molekulargenetischer Techniken zur Untersuchung der Krankheitsdynamik in Verbindung mit dem Mikrobiom von Patienten und deren Umgebung im Krankenhauskontext befassen. Ziel dieser Untersuchungen ist es, die Rolle des Mikrobioms bei der Entwicklung und dem Verlauf

von Infektionen zu analysieren, wobei ein besonderes Augenmerk auf die Risikoprofile bestimmter Infektionskrankheiten und deren Entwicklungen gelegt wird. Diese Risikoprofile werden sowohl auf der Basis individueller Patientendaten als auch im Kontext ihrer unmittelbaren Umgebung während des Krankenhausaufenthalts erstellt. Ein wesentlicher Bestandteil der Studien ist der Einsatz der Ganzgenomsequenzierung, um auffällige Mikroorganismen zu identifizieren und deren genetische Merkmale detailliert zu analysieren. Diese Technologien ermöglichen eine präzise, an den Patienten und die spezifische Umgebung angepasste Überwachung. So werden etwa in der ersten Arbeit die globalen Verbreitungsmuster von Carbapenemase-produzierenden Bakterien, wichtiger Resistenzgene, unter die Lupe genommen, um Einflüsse wie Wohnort und Ethnizität sowie die Länge und Art- der Krankenhausaufenthalte auf das Auftreten dieser Erreger zu untersuchen. Die zweite Arbeit konzentriert sich auf das Mikrobiom in Krankenhaussanitärbereichen, um zu verstehen, wie diese Bereiche als Reservoir für resistente Pathogene dienen und wie sich dies auf das Risikoprofil und die Krankheitsentwicklung von Patienten auswirkt. Die dritte Arbeit untersucht spezifisch, wie das Mikrobiom in sanitären Einrichtungen von Krankenhäusern zur langfristigen Erhaltung von Reservoirs resistenter Pathogene beiträgt, sowie die Bedeutung der Krankenhausumgebung für die Besiedlung und das Risikoprofil bestimmter Erreger, insbesondere in Bezug auf Patientengruppen, die einer hohen Exposition gegenüber Antibiotika ausgesetzt sind. Durch die Analyse von Sanitärbereichen auf Stationen mit hohem Antibiotikaeinsatz zeigt diese Studie, dass solche Umgebungen eine Schlüsselrolle bei der Aufrechterhaltung von Resistenzen spielen, selbst über viele Jahre hinweg. In der vierten Studie nutzten wir das WGS auf breiter Ebene, um die klonalen Beziehungen und von Erregern und Assoziationen von Virulenz- und Resistenzgenen in Blutstrominfektionen zu analysieren um auch hier die genetischen Ursachen von Infektionen präzise zu identifizieren und Einblicke in die Übertragungswege sowie die Entwicklung von Resistenzmechanismen zu liefern. In der fünften und sechsten Arbeit gingen wir einen Schritt weiter und nutzten schließlich über das WGS hinausgehende experimentelle Technologien um in klinischen Studien Patientenmikrobiome zu analysieren um anhand von gesamten Patientenmikrobiomen Aussagen zu Krankheitsprogression und individuellen Risiken untersuchen zu können.

3. Ergebnisteil

3.1 **Neidhöfer C**, Buechler C, Neidhöfer G, Bierbaum G, Hannet I, Hoerauf A, Parčina M. Global Distribution Patterns of Carbapenemase-Encoding Bacteria in a New Light: Clues on a Role for Ethnicity. Front Cell Infect Microbiol. 2021 Jun 29;11:659753

Zielsetzung der Arbeit – Die Antibiotikaresistenz stellt weltweit ein großes Problem dar. Die rasche Ausbreitung opportunistisch pathogener Carbapenemase-kodierender Bakterien (CEB) verlangt von Klinikern, Forschern und politischen Entscheidungsträgern, rasch Lösungen zu finden, um die Übertragungsraten und die damit verbundene Gesundheitsbelastung zu verringern. Epidemiologische Daten sind der Schlüssel zur Planung von Kontrollmaßnahmen. Unsere Studie soll dazu beitragen, indem sie eine Analyse von 397 einzigartigen CEB-Isolaten liefert, die in einem tertiären Krankenhaus in Deutschland entdeckt wurden. Wir schlagen neue Erkenntnisse über demografische Variablen vor, um präventive Hygienemaßnahmen in der klinischen Routinepraxis zu unterstützen.

Methoden und Ergebnisse – Daten über nachgewiesene CEB wurden mit den demografischen und klinischen Informationen der Patienten für jedes Isolat kombiniert. Mit Hilfe multipler Regressionstechniken wurde die Vorhersagekraft der beobachteten Unterschiede geschätzt. Mithilfe von WGS analysierten wir die Unterschiede zwischen zwei wichtigen Erregergruppen. Unsere Ergebnisse bestätigen die Rolle von Alter und Geschlecht bei den CEB-Kolonisierungsmustern und deuten auf eine Rolle der ethnischen Zugehörigkeit und des Wohnsitzes hin. Außerdem wurden Carbapenemase-kodierende *Acinetobacter baumannii* Isolate, am häufigsten in das Krankenhaus eingeschleppt, während das Risiko einer Besiedlung mit VIM-kodierenden *Pseudomonas aeruginosa* mit der Dauer des Krankenhausaufenthalts anstieg.

Schlussfolgerungen – *P. aeruginosa* bleibt eine wichtige Komplikation bei längeren Krankenhausaufenthalten. Die enge Verbindung zum Krankenhausabwasser kann Auswirkungen auf die Krankenhausumgebung haben. Die Ausbreitung von *A. baumannii* bei der Aufnahme ins Krankenhaus kann wirksam bekämpft werden. Da OXA-kodierende

CEB bei Routineuntersuchungen schwieriger zu erkennen sind, wären gezielte Präventivmaßnahmen, wie z. B. für Carbapenem-resistente Bakterien selektive Nährböden, für Patienten aus ausgewählten Regionen sinnvoll. Die in unserer Studie festgestellten CEB-Unterschiede im Zusammenhang mit der ethnischen Zugehörigkeit könnten die Anpassung diagnostischer Ansätze sowie gesundheitspolitischer Maßnahmen unterstützen, sobald sie durch weitere Studien bestätigt werden und ein besseres Verständnis ihrer weltweiten Verbreitung vorliegt.

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Global Distribution Patterns of Carbapenemase-Encoding Bacteria in a New Light: Clues on a Role for Ethnicity

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Antibiotic resistance represents a major global concern. The rapid spread of opportunistically pathogenic carbapenemase-encoding bacteria (CEB) requires clinicians, researchers, and policy-makers to swiftly find solutions to reduce transmission rates and the associated health burden. Epidemiological data is key to planning control measures. Our study aims to contribute by providing an analysis of 397 unique CEB isolates detected in a tertiary hospital in Germany. We propose new findings on demographic variables to support preventive sanitary precautions in routine clinical practice. Data on detected CEB was combined with patient's demographic and clinical information for each isolate. Multiple regression techniques were applied to estimate the predictive quality of observed differences. Our findings confirm the role of age and gender in CEB colonization patterns and indicate a role for ethnicity and domicile. Also, carbapenemase-encoding *A. baumannii* was most frequently introduced to the hospital, while the risk of colonization with VIM-encoding *P. aeruginosa* rose with the length of hospital stay. *P. aeruginosa* remains an important complication of prolonged hospital stays. The strong link to hospital-wastewater may have implications for hospital-built environments. *A. baumannii* can be efficiently controlled from spreading at hospital admission. OXA-encoding CEB being harder to detect in routine screening, targeted preventive measures, such as culture media selective for carbapenem-resistant bacteria, would be opportune for patients from selected regions. The CEB differences linked to ethnicity found in our study may further be supporting the tailoring of diagnostic approaches, as well as health policies upon confirmation by other studies and a better understanding of their global distribution.

Keywords: carbapenemases, carbapenem-resistant Enterobacterales, carbapenem-resistant *Acinetobacter baumannii*, carbapenem-resistant *Pseudomonas aeruginosa*, carbapenem-resistant Enterobacteriaceae, carbapenem-resistant Gram negative bacteria, antibiotic resistance screening

INTRODUCTION

Bacteria excel at swiftly acquiring the genetic resources to thrive in environments that intend to inhibit their growth. Increasing anti-microbial resistance (AMR) continues to limit treatment options to where there may be no more cure. This growing threat to human health is a serious global concern with a significant health-economic burden. WHO considers the growing AMR issue one of the three major public health challenges of the 21st century, responsible for rising healthcare costs, extended hospital stays, treatment failures, and often death (Naylor et al., 2018; Dadgostar, 2019). The World Economic Forum, devoting a chapter to the growing public health challenge of AMR in its 2013 Global Risk Report, reported an intensified situation in 2018 and finally in 2020 predicted AMR to become the worldwide leading cause of death by 2050 if no action is taken.

Carbapenemase-encoding bacteria (CEB) have spread worldwide (Queenan and Bush, 2007; Lee et al., 2016; Logan and Weinstein, 2017). The most relevant carbapenemases include KPC, NDM, IMP, VIM, and OXA family enzymes, often carried on plasmids. Bacteria producing these enzymes are normally resistant not only to carbapenems but actually to nearly all antibiotics, often due to additional resistance genes carried on those plasmids (Johnning et al., 2018; Sawa et al., 2020). Previous studies have shown an overall rise of the prevalence of CEB in Europe and have highlighted the need for enhanced containment efforts at both country and European levels (Albiger et al., 2015; David et al., 2019).

Epidemiological data is of primary importance to understand the scope of the problem and to design effective control measures. We analyzed demographic and clinical data on 397 CEB detected at our University Hospital (Bonn, Germany) between September 2014 and December 2019 and offer new insights on factors that may be taken into account when optimizing preventive sanitary precautions and developing safe hospital environments. The collected data included variables such as gender, age, ethnicity, residency, CEB specimen type, co-detection of CEB with other multidrug-resistant bacteria, and length of hospital stay.

MATERIALS AND METHODS

Data

We analyzed retrospective data on CEB isolates that could be retrieved from the laboratory and hospital information systems of our institute, which is part of the University Hospital of Bonn, Germany (UKB). The UKB is a tertiary referral and maximum care hospital with 1,300 beds. Every year about 50,000 inpatients and 35,000 emergencies are treated, and over 350,000 outpatient procedures are provided. The UKB serves mainly German residents but also attracts patients living outside of Germany, mainly in the Arabian Peninsula. Our microbiological diagnostic unit services the University Hospital Bonn and other hospitals in the area and receives an average of 178,000 clinical samples each year. All CEB isolates from September 2014 till December 2019 were traced in the laboratory information system using species and resistance keywords by one operator. Only first isolates were selected for each pathogen–patient combination.

The isolate information was complemented by accessible clinical patient information including gender, age, co-colonization by methicillin-resistant *Staphylococcus aureus* (MRSA) or vancomycin-resistant *Enterococcus* (VRE), date of hospital admission, hospitalization stay, oncological or intensive-care ward (ICU) stay, place of residency, and ethnicity. We constructed a database that was password protected and accessible by only three operators who ensured that all patient data were delinked from any results other than CEB and were fully de-identified prior to analysis after the establishment of residency and ethnicity. To infer patient ethnicity, we utilized the free version of the validated and data protection compliant software tool Onolytics (Version 2020, San Jose, California, US). Onolytics classifies names into 189 cultural ethnic and linguistics groupings. Where available, the results were further controlled for plausibility and accuracy using additional information such as patients' nationality.

All data relevant to the study are included in the article or uploaded as Supplementary Information.

The ethics committee of the University Hospital Bonn confirmed that no ethics approval was required for this study.

Screening and Surveillance Policy

Following German guidelines, multi-resistance of gram-negative rods (MRGNs) was defined on the basis of resistance of a pathogen against three (3MRGN) or four (4MRGN) of the following antibiotic groups: acylureidopenicillins, third and fourth generation cephalosporins, carbapenems, and fluoroquinolones. At our UKB and serviced hospitals primary MRGN screening is performed on all patients who had been hospitalized abroad within the past year, on all transfers without current MRGN screening as well as on all patients that were in the same room with a patient with a 4MRGN. Screening and surveillance body sites include the anal region, the inguinal region, the throat as well as wounds if present. Surveillance after admission is ward-dependent but generally performed at least weekly. Screening and surveillance samples for CEB were routinely cultured by the laboratory on selective ESBL-media, identified *via* MALDI-TOF MS (VITEK MS, Biomerieux, Marcy-l'Etoile, France) and susceptibility-tested with the VITEK 2 system (Biomerieux, Marcy-l'Etoile, France). Carbapenem-resistant *Enterobacterales*, *A. baumannii* and *P. aeruginosa* or isolates with an unusual carbapenem-susceptibility profile (ertapenem/imipenem/meropenem) are routinely genotyped in-institute for the presence of common resistance genes, using the Allplex Entero-DR (Seegene, Seoul, South Korea) and eazyplex SuperBug Acineto Assays (AmplexBiosystems, Giessen, Germany).

Statistical Analysis

We used regression techniques to assess the association between age, sex, ethnicity, place of residency, and other parameters with the occurrence and/or type of CEB colonization in patients. This

allowed construction of patient and/or hospitalization driven risk profiles. First, we report the average prevalence of different types of CEB in various patient populations. Then, using multivariate regressions, we test whether the uncovered differences between groups persist when we take into account the differentials in observable characteristics (*e.g.* age, sex, days of hospitalization, *etc.*) among individuals in these groups. To run the regressions we use the statistical software package Stata.

Our main results showing the relationship between demographic and hospitalization related characteristics with CEB colonization are shown in Tables B1–B4 in Appendix B (Supplemental Material). Figures showing the absolute number and share of patients with a certain type of CEB colonization are shown throughout the text.

The p-values, marked with an asterisk (*) in these figures and throughout the text, refer to the significance (at the 0.05 or 0.01 level) of the point estimates obtained in the multivariate regression analysis (shown in Tables B1–B4).

To test the robustness of our results, we performed some additional sensitivity analyses. Results from univariate regression analysis (*i.e.* including only ethnicity or place of residence as independent variables and excluding all other covariates) are shown in Tables B9–B12 in Appendix B. Furthermore, due to the binary nature of the dependent variables (*e.g.* the prevalence of specific CEB in the patient), we also estimated the same specifications as in the multivariate analysis applying logistic models. The results of this application are shown in Tables B13–B16 in Appendix B. These sensitivity analyses show the same patterns of statistical significance and, hence, confirm our baseline results obtained with multivariate linear models.

TABLE 1 | Summary demographics for patients with detected CEB isolates.

Demographic parameters	Patients n = 317 (%)	
Age (years)		
Mean (Min, Max)	56.95 (0, 97)	
Sex		
Female	96	(30.28%)
Male	221	(69.72%)
Ethnicity		
German	205	(64.67%)
Arabic	33	(10.41%)
Turkish	15	(4.73%)
Punjabi	13	(4.10%)
Somalian	11	(3.47%)
Kashmiri	4	(1.26%)
Other/Not available	36	(11.36%)
Residency		
Germany	209	(65.93%)
Arabian Peninsula	32	(10.09%)
Other/Not avaiable	76	(23.98%)
Ward		
ICU (but not oncological)	76	(23.98%)
Oncological (but not ICU)	40	(12.62%)
Oncological ICU	13	(4.10%)
Other	188	(59.31%)

Clonality Analysis

We had access to the genomes of ten KPC-encoding *Enterobacter cloacae* complex isolates and MLST and OXA variant data of 12 OXA-48-encoding *K. pneumoniae* isolates from 2019 [see Tables C1, C2 in Appendix C (Supplemental Material)]. Genome analysis was performed with software tools (ResFinder, MLST, PlasmidFinder) of the CGE Server (Update June 8th 2020, Center for Genomic Epidemiology, DTU, Denmark).

RESULTS

Detected CEB From 2014 to 2019

Between September 2014 and December 31st, 2019, 1,917 isolates from 1,384 patients were genotyped with the Allplex Entero-DR and eazyplex SuperBug Acineto Assays [see Table A1 in Appendix A (Supplemental Material)], and revealed 301 CEB isolates from UKB patients and 96 CEB isolates from patients of neighboring clinics. Table 1 shows the demographic and clinical summary information for patients with detected CEB. Assuming that there is no gender imbalance regarding the overall amount of specimens received by our diagnostic unit, male patients (69.72%; 221/317) were more than twice as likely to be colonized with clinically relevant CEB compared to female patients (30.28%; 96/317). There were more CEB isolates during June, August, and October compared to the other months. However, clusters were mostly linked to small outbreaks rather than to seasonality.

Species, Carbapenemases, and Specimens

Detected species and encoded carbapenemases are displayed in Table 2. Two hundred and fifty-three patients carried a single CEB species, and 64 patients carried multiple CEB species. Of these 64 patients, fifty-one, eleven, one, and one patients carried two, three, four and five different CEB, respectively, in 144 species in total. Among patients colonized by only one CEB,

TABLE 2 | Number and type of carbapenemases the most prevalent species encoded.

Species	Total	KPC	VIM	NDM	OXA-23	OXA-48	Others
K. pneumoniae	117	12	7	28	2	85	0
Enterobacter cloacae complex	46	11	21	6	0	7	1
E. coli	40	3	9	11	0	18	0
Other *Enterobacterales*	55	4	13	11	0	27	0
P. aeruginosa	74	0	63	5	1	3	4
A. baumannii	58	1	0	5	42	0	12
Total	390	31	113	66	45	140	17

P. aeruginosa made up 26% (66/253) of all. Among patients that were detected to carry two or more CEB, *P. aeruginosa* constituted only 8% (11/142) (p <.01), and *Enterobacteriaceae* made up more. The OXA-48- and OXA-23-encoding *P. aeruginosa* and OXA-23-encoding *K. pneumoniae* isolates that appear in our statistic all occurred in 2014 and 2015 and were not cryopreserved in a sufficiently reliable traceable manner for their validity to now be confirmed in retrospect, given their rarity; DNA contaminations can, hence, not be excluded for these six isolates.

The number of KPC, VIM, NDM, OXA-23, and OXA-48 like carbapenemases that had been detected as colonizers fluctuated each year between 2015 and 2019 (see Figure A1 in Appendix A). The majority of the KPC enzymes were detected in 2019 between June and August, due to an outbreak. The source of the outbreak had been timely traced, and no patients developed clinical CEB infections. The outbreak was mainly characterized by *Enterobacter cloacae* complex ST 419 [see Table C1 in Appendix C (Supplemental Material)]. In contrast, all KPC-encoding bacterial isolates detected between 2015 and 2018 were *K. pneumoniae* isolates and were detected only sporadically. Of all KPC-encoding isolates, 84% (26/31) were detected in screening and surveillance samples. Of VIM-encoding isolates, 31% (36/116) belonged to oncological and 35% (40/116) to ICU patients, making them the most frequently detected carbapenemases in both patient populations. The number of NDM carbapenemases grew steadily until 2018. ICU patients carried 26% (18/67) of the isolates, and oncological patients carried 29% (20/67). Numbers of OXA-23 carbapenemases showed only minimal variations, and only 22% (10/46) of the OXA-23-encoding bacterial strains belonged to female patients.

K. pneumoniae made up 61% (85/140) of OXA-48-encoding isolates. It is known that *bla*$_{\text{OXA-48}}$ is on an IncL-type plasmid defective for the *tir* gene involved in the regulation of conjugation rendering this plasmid highly conjugative. Thus it has the ability to be transferred *in vivo* within different members of Enterobacterales. Genetic resistance profile and MLST-type of OXA-48-encoding *K. pneumoniae* isolates detected between January and October 2019 are displayed in Table C2 in Appendix C (Supplemental Material). Of interest was that, every 10th patient colonized by an OXA-48-encoding isolate was colonized by at least two different OXA-48-encoding species, all belonging to the family of Enterobacteriaceae. Sixteen isolates, including 14 K. *pneumoniae* isolates, encoded OXA-48-like carbapenemases in addition to other carbapenemases. Fourteen percent (19/140) of the OXA-48-encoding isolates belonged to oncological patients and 24% (34/140) to ICU patients.

Figure 1 shows relative and absolute quantities of carbapenemase-encoding species that were isolated from the respective types of clinical specimen. Urine samples made up the majority (104) of all the specimen types in which CEB isolates were detected, followed by inguinal swabs (77), anal swabs (77), stool samples (53), wound swabs (49), throat swabs (41), tracheal secretions (36), and blood cultures (14). Nearly half (46%, 183/397) of all CEB were detected in screening and surveillance samples. The frequency with which CEB species were identified differed substantially by specimen type (see Figure 1). In our setting, inguinal swabs were the most efficient specimen for detecting carbapenemase-encoding *A. baumannii*. *P. aeruginosa* was more evenly found in the various specimen types, and the least efficient recovery was in anal and inguinal swabs.

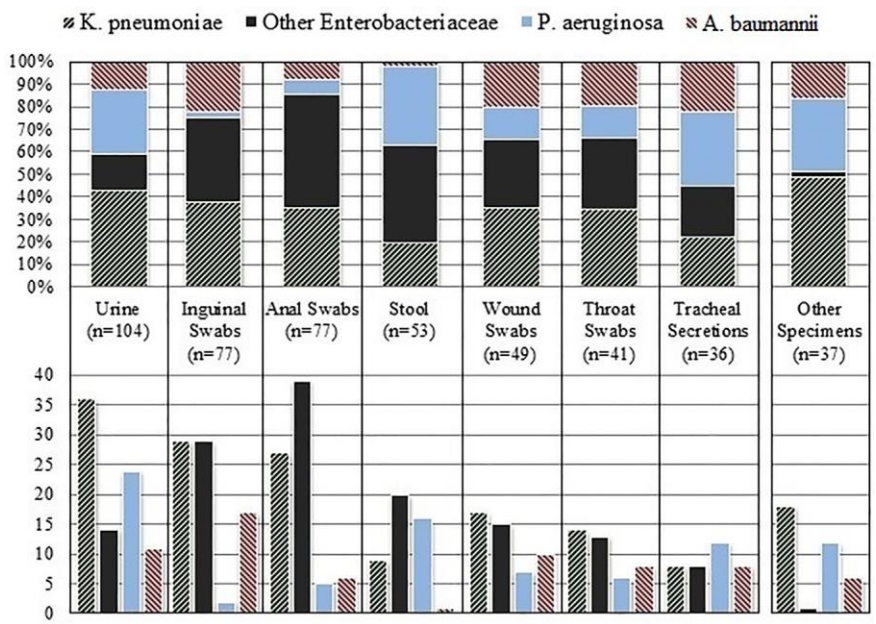


FIGURE 1 | Relative (above) and absolute (below) quantities of carbapenemase-encoding species that were isolated from the respective type of clinical specimen.

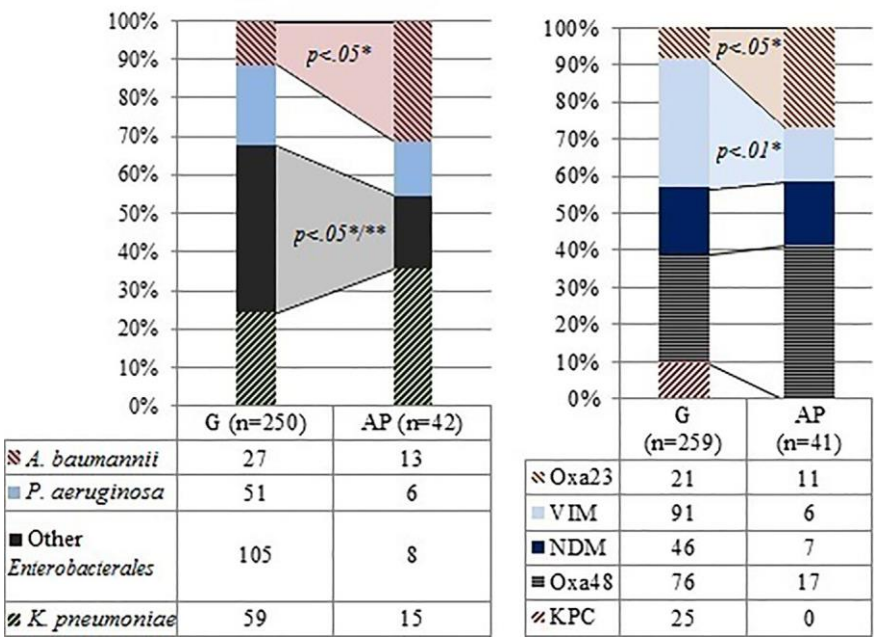
Age, Gender and Co-colonization With Other Resistant Bacteria

The total and relative frequencies with which certain carbapenemases and species were detected across different age groups are shown in Figure A2 in Appendix A. More than 83% (265/323) of the patients colonized by CEB were aged 40 or older, 51% (163/323) were 60 or older. No carbapenemase-encoding *P. aeruginosa* isolates were detected in patients below the age of 25 (n = 25). Among patients aged 25–29 years, including four oncological patients, a disproportionately high number (n=8/19) was found. While there are no gender differences in the average age, we found significant differences in the occurrence of CEB isolates in male and female patients. As the results of the multivariate regressions in Tables B1–B4 show, VIM carbapenemases (p <.05*) and *Citrobacter* isolates (p <.05*) were significantly more common in female patients.

Thirty-four and sixty-seven patients were co-colonized with methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE), respectively, and five of these patients with both. There were significantly less women co-colonized with MRSA, 5/34 (14.7%) (*vs* male, p = .04), compared to co-colonization with VRE, 22/67 (32.8%) (*vs* male p = .61).

Role of Residency and Ethnicity

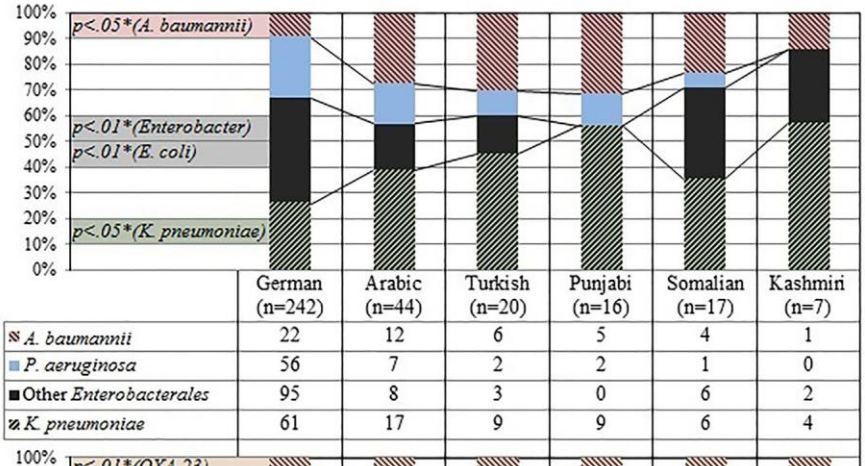
The human microbiome is very individual and has been correlated with factors such as ethnicity and geography (Brooks et al., 2018; Gaulke and Sharpton, 2018; Reinheimer et al., 2019). Hence, we investigated the presence of certain types of CEB in particular groups within our sample, identified by their ethnicity and country of residency. Two groups were considered for residence, patients residing in Germany (G-residents) and patients residing in the Arabian Peninsula (AP-residents) (see Table 1). AP-residents colonized with CEB were substantially younger (average age, 39 years) compared to G-residents (average age, 61 years) (t = 7.62, p <.01). Forty-four percent (14/32) of AP-residents were detected to carry CEB on admission, compared to only 23% (48/209) G-residents (p = .01). Figure 2 shows the relative frequencies of species and carbapenemases among the two groups. The indicated p-values derive from the multivariate regressions shown in Tables B1–B4. *Enterobacter* isolates (p <.05*) and VIM enzymes (p <.01*) were more frequent among G-residents, while KPC enzymes were solely detected in patients in this group. In contrast, *A. baumannii* isolates (p <.05*) and OXA-23 carbapenemases (p <.05*) were more frequent among AP-residents. The ethnicity of patients had also been retrieved, as explained in the section *Materials and Methods* (see Table 1). Figure 3 shows the relative frequencies of species and carbapenemases for each group. Again, p-values derive from the multivariate analysis. Although we report the uncovered differences among all ethnic groups in our sample, only German and Arabic ethnicities have a sufficiently high number of observations. Hence, results for the other groups should be read with caution. Controlling for all differential characteristics among groups, as age, sex, hospital stay *etc.*, patients of German ethnicity were less frequently colonized by *A. baumannii* than patients of Arabic ethnicity (p <.05*), and by *K. pneumoniae* than patients of Punjabi ethnicity (p <.05*). Conversely, they were more frequently colonized by *Enterobacter* isolates than patients of Arabic



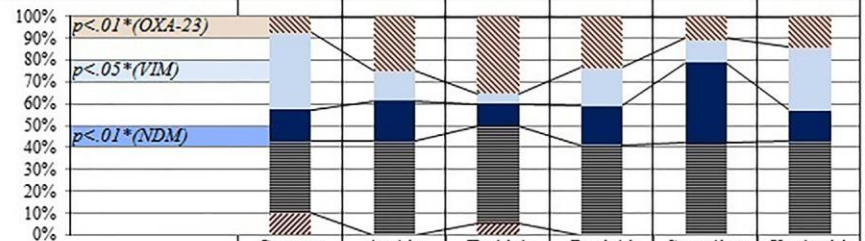
	G (n=250)	AP (n=42)
A. baumannii	27	13
P. aeruginosa	51	6
Other *Enterobacterales*	105	8
K. pneumoniae	59	15

	G (n=259)	AP (n=41)
Oxa23	21	11
VIM	91	6
NDM	46	7
Oxa48	76	17
KPC	25	0

FIGURE 2 | Species and carbapenemases patients resident in Germany (G) and on the Arabian Peninsula (AP) were detected with. *P-values refer to the significance of the point estimates in the regression analysis (see Appendix B). **P-value refers to Enterobacter isolates.



	German (n=242)	Arabic (n=44)	Turkish (n=20)	Punjabi (n=16)	Somalian (n=17)	Kashmiri (n=7)
A. baumannii	22	12	6	5	4	1
P. aeruginosa	56	7	2	2	1	0
Other *Enterobacterales*	95	8	3	0	6	2
K. pneumoniae	61	17	9	9	6	4



	German (n=249)	Arabic (n=44)	Turkish (n=20)	Punjabi (n=17)	Somalian (n=19)	Kashmiri (n=7)
OXA-23	18	11	7	4	2	1
VIM	88	6	1	3	2	2
NDM	36	8	2	3	7	1
OXA-48	82	19	9	7	8	3
KPC	25	0	1	0	0	0

FIGURE 3 | Summary of patient ethnicity and frequency of species and carbapenemases.*P-values refer to the significance of the point estimates in the regression analysis, baseline category for ethnicity is German (see Appendix B).

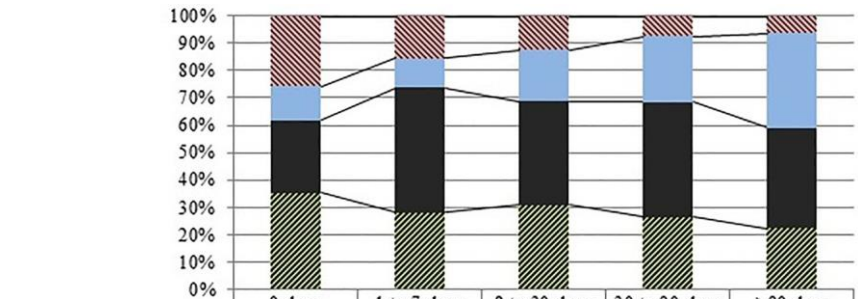
ethnicity (p <.01*). VIM enzymes were more frequently detected among patients of German ethnicity rather than among patients of Arabic (p <.05*), Somali (p <.05*), and Turkish ethnicity (p <.05*). On the other hand, patients of German ethnicity were less frequently colonized with bacteria harboring OXA-23 carbapenemases than patients of Arabic (p <.01*) and Turkish ethnicity (p <.01*) and by bacteria harboring NDM carbapenemases than patients of Somali ethnicity (p <.01*).

Our data suggests that residency and ethnicity play a role in terms of frequency with which certain carbapenemase-encoding species were detected in patients. Hence, we performed two further analyses. Firstly, we compared isolates of patients of German ethnicity residing in Germany with isolates of patients of Arabic, Somali, Turkish, Punjabi, or Kashmiri ethnicity residing in Germany (Tables B5, B6 in Appendix B). Secondly, we compared isolates of patients of Arabic ethnicity residing in Germany with isolates of patients of Arabic ethnicity residing on the Arabian Peninsula (Tables B7, B8 in Appendix B).

German ethnicity patients residing in Germany were less frequently colonized by OXA-23-encoding isolates compared to Turkish (p <.05*) and Arabic ethnicity patients residing in Germany (p <.05*), by OXA-48-encoding isolates compared to Somali ethnicity patients residing in Germany (p <.05*), and by VIM-encoding isolates compared to Kashmiri ethnicity patients. At the same time, they were associated more frequently with VIM enzymes than patients of Turkish ethnicity residing in Germany (p <.05*). Patients of Arabic ethnicity residing in Germany were more frequently colonized with carbapenemase-encoding *E. coli* isolates compared to patients of Arabic ethnicity residing in the Arabian Peninsula (p <.05*). Although the substantially smaller sample size challenges the consistency of these last results, they provide further suggestive evidence that confirms the significant role of ethnicity.

Introduced vs. Hospital-Acquired

In order to differentiate CEB that are frequently introduced into the hospital from those that are more likely hospital-acquired, we studied CEB detection and the length of stay in the hospital (LOS). Figure 4 demonstrates that carbapenemase-encoding *A. baumannii* isolates were more frequently detected in patients on hospital admission. Carbapenemase-encoding *P. aeruginosa* isolates on the other hand were more frequently detected with longer LOS.



	0 days	1 to 7 days	8 to 30 days	30 to 90 days	>90 days
A. baumannii	31	7	11	6	3
P. aeruginosa	15	5	17	20	17
Other *Enterobacterales*	32	21	34	35	18
K. pneumoniae	43	13	28	22	11

FIGURE 4 | Total and relative amounts of species by LOS.

In more than half of the cases *P. aeruginosa* was detected after more than four weeks LOS. The results of the multivariate analysis confirmed the existence of a statistically significant and negative relationship between the LOS and *P. aeruginosa*, *Proteus, Serratia, Klebsiella oxytoca*, and *Klebsiella aerogenes* as well as with production of OXA-48-like enzymes. *P. aeruginosa* was the most frequently isolated CEB from oncological and ICU patients. Especially oncological patients were colonized significantly more frequently by carbapenemase-encoding *P. aeruginosa* than by *A. baumannii* ($p < .01$).

DISCUSSION

As in other studies on multidrug-resistant gram-negative bacteria (GNB) (Kaase et al., 2016; Nicolas-Chanoine et al., 2019), in our study male patients were more prominently colonized with CEB. Methicillin-resistant *Staphylococcus aureus* (MRSA), but not vancomycin-resistant *Enterococcus* (VRE), more frequently colonized male patients (Humphreys et al., 2015; Markwart et al., 2019). The higher incidence of each, CEB and MRSA, in male patients explains the intensified gender imbalance in patients co-colonized by both. Higher MRSA-prevalence among men has been postulated to be linked to hand-hygiene and other behavioral factors as well as to immunological and endocrinological differences (Humphreys et al., 2015). Whether the same mechanisms apply for the higher occurrence of multidrug resistant gram-negative bacteria in males, too, remains uncertain.

The 2019 KPC outbreak highlights how explosively KPC-encoded resistance genes can spread in hospital settings despite surveillance and preventive measures. On the other hand, the quantity of carbapenemase-encoding bacteria (CEB) in screening and surveillance samples demonstrates the importance of screening and surveillance practices (Luebbert et al., 2013) to prevent and contain outbreaks. In our study OXA-48-like and VIM carbapenemases were the first and second most prevalent carbapenemases. However in 2018 NDM-carbapenemases were firstly detected more commonly than VIM-enzymes. The steadily growing number of NDM carbapenemases follows a countrywide trend (Robert-Koch-Institute, 2019). The interchangeable and compatible nature of OXA-48-like enzymes has been reported before (Pulss et al., 2018; Hamprecht et al., 2019). In our study these enzymes were encoded by many *Enterobacterales* and even by *P. aeruginosa. K. pneumoniae* confirmed its versatility in taking up resistance genes (Navon-Venezia et al., 2017). That *P. aeruginosa* is more frequently the only carbapenemase-encoding species carried by patients compared to *Enterobacteriaceae*, might be caused by the fact that the plasmid-subtypes generally carried by *P. aeruginosa* are less conjugative to other species, whereas plasmid-subtypes encoded by *Enterobacteriaceae* are more readily transferable to other *Enterobacteriaceae* (Pulss et al., 2018; Hamprecht et al., 2019; Sawa et al., 2020).

We hypothesize that several factors influence the different spectrum of CEB detected in anal swabs and stool specimen. In our experience, anal swabs are frequently erroneously collected by only swabbing the superficial area surrounding the anal sphincter. Also, only stool samples of oncological patients are routinely cultured on media on which CEB grow. Among the clinical specimens that we received, inguinal swabs were the best for detecting carbapenemase-encoding *A. baumannii*. The likelihood with which different CEB may be detected in various specimen types should be considered when screening patients in clinical practice.

The apparent decline in occurrence of CEB after the age of 70 observed in our study is solely due to the decreasing number of people in that age group. Colonization with multidrug-resistant bacteria is generally antibiotic- and healthcare associated (Logan and Weinstein, 2017; Nicolas-Chanoine et al., 2019; Segagni Lusignani et al., 2020) and hence more prevalent among the older population. Also, patients residing in foreign countries in need of medical attention coming to Germany for treatment

likely more frequently have a long history of hospital stays and antimicrobial therapies. This also explains the higher amount of patients already colonized with CEB at hospital admission among AP-residents compared to G-residents, even though endemicity might as well play a role to a certain degree (van der Bij and Pitout, 2012). The age difference between the two groups may also be linked to the more than 15 years higher life expectancy in Germany compared to several countries on the Arabian Peninsula.

Our data suggests that besides the place of residence, ethnicity plays a role in terms of frequency with which certain CEB are detected in patients. Given the fact that the variability in the microbial species colonizing the human body has been shown to be linked to ethnicity as well as geography (Brooks et al., 2018; Gaulke and Sharpton, 2018; Reinheimer et al., 2019), we are not surprised by this result. Even though statistically significant in our study, confirmation by further studies is required due to the possibility of bias with smaller sample sizes. If confirmed, global distribution patterns of carbapenemases should be considered with these new insights. A higher likelihood of certain CEB in certain patient groups would offer the possibility to further tailor screening and diagnostic approaches as well as patient care, aiming for personalized and efficient precision medicine. On the other hand, there might currently be patients disadvantaged in healthcare settings because of their ethnicity, OXA-enzymes being the most challenging carbapenemases to detect with routine screening processes (Koroska et al., 2017). Culture media selective for carbapenem-resistant bacteria might for example be opportune for the above mentioned patient groups. The result is, however, challenged by the potentially different socioeconomic characteristics of patients. In particular, German residents of Arabic ethnicity in our sample are likely to be in a very different economic situation than residents of the Arabian Peninsula. Unfortunately, with the data at our disposal we are not able to control for the socioeconomic characteristics of patients, but the topic should be addressed carefully in future research.

A. baumannii has been documented to swiftly spread within hospital environments if effective preventive measures are inadequate (Teare et al., 2019). We therefore assume hospital-acquired cases in our study to be low due to effective screening, hygiene, and isolation measures (Luebbert et al., 2013). *P. aeruginosa* is documented to predominantly infect critically ill as well as immuno-suppressed patients (Aloush et al., 2006; Tsao et al., 2018) and its infection risk increases with longer LOS. Our study confirms these findings. A previous study showed that carbapenemase-encoding *P. aeruginosa* isolates detected in the siphons of the oncological ward mostly belong to the rare sequence type ST823 (Sib et al., 2019). Future studies should address if this and other detected sequence types match those detected in patients.

Given its frequent presence in hospital-wastewater, hotspots such as the drains, traps, sinks, faucets and toilets have undergone extensive remodeling. Nevertheless, our results and previous findings of the biofilms in the wastewater networks (Sib et al., 2019) further highlight the need to continuously rethink hospital-built environments for safety (Kizny Gordon et al., 2017; Hopman et al., 2019).

CONCLUSIONS

We analyzed 397 carbapenemase-encoding isolates detected during a 5 year period with the associated demographic and clinical patient information. We found evidence of a role for ethnicity in the type of CEB colonization, and confirmed the important role of *P. aeruginosa* among complications of prolonged hospital stays. Continued research will further elucidate the observed gender differences in the frequency of MDR-GNB colonization/infection and the various microbial spectra linked to ethnicity and residency. The way global distribution patterns of carbapenemases are seen and studied may be substantially influenced and new screening, diagnosis, and patient care may be offered. These include more effective screening for challenging resistance enzymes linked with ethnicity/residence and the awareness of the optimal specimen for the various carbapenemase-encoding bacteria. Finally, our study has shown that even when extensive safety precautions are in place, not all hospital-acquired pathogens can be equally well contained, which prompts to continuously rethink hospital-built environments and further optimize all precautions according to risk factors and the spectrum of expected pathogens.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

CN and MP contributed to study design, data collection, data analysis, data interpretation, the literature search, and writing of the report. CB, GN, and IH contributed to data analysis, data interpretation, and writing of the report. GB contributed to the literature search, data interpretation, and writing of the report. AH contributed to data interpretation and writing of the report. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.659753/full#supplementary-material

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Conflict of Interest: IH was employed by the company H.I.M.A. Consulting.

The remaining 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.

3.2 **Neidhöfer C**, Sib E, Benhsain AH, Mutschnik-Raab C, Schwabe A, Wollkopf A, Wetzig N, Sieber MA, Thiele R, Döhla M, Engelhart S, Mutters NT, Parčina M. Examining Different Analysis Protocols Targeting Hospital Sanitary Facility Microbiomes. Microorganisms. 2023 Jan 11;11(1):185

Zielsetzung der Arbeit – Innenräume weisen eine mikrobielle Zusammensetzung auf, die sich deutlich voneinander und von Außenräumen unterscheidet. Einzigartig in dieser Hinsicht und ein Thema, das erst kürzlich in den Fokus gerückt ist, ist das Mikrobiom von Krankenhäusern. Obwohl es zweifellos von Vorteil ist, genau zu wissen, welche Mikroorganismen sich wie und wo in Krankenhäusern vermehren, um Krankenhausinfektionen zu verhindern, gibt es bisher keine standardisierten Verfahren, wie das Krankenhausmikrobiom am besten untersucht werden kann. Ziel unserer Studie war es, das Mikrobiom von Krankenhaussanitäranlagen zu untersuchen und zu zeigen, inwieweit sich Krankenhausmikrobiomanalysen aufgrund der Neuartigkeit der Technologie und des Fehlens von Standardverfahren je nach Probenaufbereitungsprotokoll unterscheiden.

Methoden und Ergebnisse – Zu diesem Zweck wurden fünfzig Proben aus zwei verschiedenen Krankenhäusern - von drei Stationen und einem Krankenhauslabor - unter Verwendung von zwei verschiedenen Aufbewahrungsmedien entnommen, aus denen die DNA mit zwei verschiedenen Extraktionskits extrahiert und mit zwei verschiedenen Primerpaaren (V1-V2 und V3-V4) sequenziert wurde. Es gab keine erkennbaren Unterschiede zwischen den Probenaufbewahrungsmedien, geringe Unterschiede bei den nachgewiesenen Taxa zwischen den DNA-Extraktionskits (hauptsächlich bei Propionibacteriaceae) und große Unterschiede bei den nachgewiesenen Taxa zwischen den beiden Primerpaaren V1-V2 und V3-V4. Diese Analyse zeigte auch, dass das Vorkommen und die Zusammensetzung von Mikroorganismen in Toiletten, Waschbecken und Duschen sowie in verschiedenen Abteilungen und Krankenhäusern sehr unterschiedlich sein können. In chirurgischen Abteilungen schienen die Patiententoiletten durch einen geringeren Artenreichtum und eine geringere Diversität gekennzeichnet zu sein als die Personaltoiletten.

Schlussfolgerungen – Welche Probeentnahmestellen für die jeweilige Untersuchung am geeignetesten sind, sollte eingehender analysiert werden. Die Tatsache, dass die von uns untersuchten Probenaufbereitungsmethoden (abgesehen von der Wahl der Primer) die Ergebnisse nur geringfügig verändert zu haben scheinen, legt nahe, dass die Gegenüberstellung von Studien zum Krankenhausmikrobiom eine durchaus realistische Perspektive darstellt. Die beobachteten Unterschiede im Artenreichtum und in der Diversität zwischen Patienten- und Personaltoiletten sollten weiter untersucht werden, da diese, falls sie sich bestätigen, auf die Ausscheidung von antimikrobiellen Substanzen zurückzuführen sein könnten.

microorganisms

MDPI

Article

Examining Different Analysis Protocols Targeting Hospital Sanitary Facility Microbiomes

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Abstract: Indoor spaces exhibit microbial compositions that are distinctly dissimilar from one another and from outdoor spaces. Unique in this regard, and a topic that has only recently come into focus, is the microbiome of hospitals. While the benefits of knowing exactly which microorganisms propagate how and where in hospitals are undoubtedly beneficial for preventing hospital-acquired infections, there are, to date, no standardized procedures on how to best study the hospital microbiome. Our study aimed to investigate the microbiome of hospital sanitary facilities, outlining the extent to which hospital microbiome analyses differ according to sample-preparation protocol. For this purpose, fifty samples were collected from two separate hospitals—from three wards and one hospital laboratory—using two different storage media from which DNA was extracted using two different extraction kits and sequenced with two different primer pairs (V1–V2 and V3–V4). There were no observable differences between the sample-preservation media, small differences in detected taxa between the DNA extraction kits (mainly concerning *Propionibacteriaceae*), and large differences in detected taxa between the two primer pairs V1–V2 and V3–V4. This analysis also showed that microbial occurrences and compositions can vary greatly from toilets to sinks to showers and across wards and hospitals. In surgical wards, patient toilets appeared to be characterized by lower species richness and diversity than staff toilets. Which sampling sites are the best for which assessments should be analyzed in more depth. The fact that the sample processing methods we investigated (apart from the choice of primers) seem to have changed the results only slightly suggests that comparing hospital microbiome studies is a realistic option. The observed differences in species richness and diversity between patient and staff toilets should be further investigated, as these, if confirmed, could be a result of excreted antimicrobials.

Keywords: NGS; high-throughput DNA sequencing; 16S rRNA gene sequencing; microbiome; microbial ecology; built environment; hospital environment; hospital-acquired infections

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1. Introduction

The fight against antibiotic-resistant pathogens has led to an ever-increasing burden on the healthcare system in recent decades. A lack of effective treatment inevitably leads to a vicious cycle of prolonged hospitalization, which in turn fosters hospital-acquired infections with pathogens that may also be highly resistant to therapy, and ultimately, to a concomitant surge in morbidity and mortality [1]. In 2021, the WHO emphasized the importance of global surveillance of antimicrobial resistance to effectively implement

strategies to combat MDR pathogens [2]. Next-generation sequencing has been widely used for this purpose over the past decade and shows great promise [3]. The obtained data are generally used to determine antimicrobial resistance, virulence patterns, and clonality [3–5]. In contrast, the idea of studying the totality of microorganisms in patients and their hospital environments is a more recent one and opens up the possibility of better understanding the emergence of niches of multidrug-resistant organisms in the patient and the hospital [6–8].

At any time, we are surrounded by microbes that can positively and negatively influence our health. The totality of microorganisms we carry in and on us is often categorized as our microbiome and thus distinguished by definition from microorganisms that merely surround us. To what extent some should be called our own and others not remains part of many ongoing investigations [8–12]. Throughout modern human evolution, built environments—and hospitals in particular—have changed in ways that make them increasingly inhospitable to microbial life, with largely dry surfaces often covered with antimicrobial materials [13]. While this has undoubtedly contributed to reducing the spread of communicable diseases, it has also changed our microbial relationship with the environment [13,14]. Research concerning the microbiome of indoor environments such as hospitals, houses, or buildings could have several implications for human health [7–14]. The hospital microbiome consists of complex, nested systems with a multilayered transmission of strains, plasmids, and smaller genetic elements between patients, medical staff, hospital surfaces, and water networks [15]. Although metagenomic analyses can be used beyond bacterial community relations to obtain information on non-bacterial microorganisms and resistance genes, amplicon sequencing of genes such as the 16S rRNA gene offers the best cost–benefit ratio for assessing indoor microbiome profiles to date [16]. The currently available data suggest that NGS bacteriome analysis (together with a similar assessment of the mycobiome and the resistome) provides valuable additional information about the microbiome contaminating the hospital environment, resulting in a subsequent improvement in protocols and measures to combat the increasing prevalence of antimicrobial resistance [13,15,17]. These molecular analyses could ideally be integrated into ongoing surveillance programs. Further research and technological advances are needed before these approaches can be routinely used for hospital surveillance; however, their ability to track outbreaks of multidrug-resistant bacteria and the spread of antimicrobial resistance, identify persistent environmental reservoirs, and assess future risks [7,8,12–17] is promising.

In our study, we compared different sample collection sites in hospital-patient bathrooms and different sample preparation and sequencing protocols regarding the number and type of detected taxa. This was performed to investigate the microbiomes of hospital sanitary facilities and evaluate the degree to which hospital microbiome analyses vary with different protocols.

2. Materials and Methods

2.1. Sample Collection

Two hospitals were included in this study. One is a tertiary referral and maximum-care facility (TCH), and the other a military hospital (MH). The two analyzed hospitals are approximately 60 km apart. Altogether, 50 sites were sampled. Each site was swabbed and preserved in 1 mL eNAT medium tubes (Copan, Brescia, Italy) and 1 mL DNA/RNA Shield Collection Tubes (Zymo Research Europe GmbH, Freiburg, Germany), totaling 100 samples. The 50 sample sites included biofilms along the water level of 24 toilets (12MH/12TCH) and in siphons of 14 wash basins (12MH/2TCH) and 12 showers (all MH).

2.2. DNA Extraction

Samples were stored for less than 14 days at 4 °C before highly purified DNA was extracted using both the column-based PureLink Microbiome DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA), henceforward referred to as the PMP kit, and the ZymoBIOMICS DNA Miniprep Kit (Zymo Research Europe GmbH, Freiburg, Germany),

henceforward referred to as the ZBM kit, according to the manufacturers' instructions. At the end of the extraction process, the DNA was qualitatively and quantitatively evaluated using the NanoDrop OneC (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Library Preparation

The 16S rRNA gene sequencing libraries were constructed using the Quick-16S NGS Library Prep Kit (Zymo Research Europe GmbH, Freiburg, Germany) with its included optimized primer pairs. All samples extracted with the PMP kit and 91 of those extracted with the ZBM kit were sequenced with the V1–V2 primer pairs, whereas 89 samples extracted with the PMP kit and 20 of those extracted with the ZBM kit were sequenced with the V3–V4 primer pairs. Each run included 94 samples, the positive control included in the kit, and a negative control. For quantitative PCR, quality control, and normalization purposes, the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was utilized.

2.4. Sequencing

After pooling, the DNA was quantified with the QuantiFluor dsDNA System on the Quantus Fluorometer (Promega GmbH, Walldorf, Germany) and diluted strictly according to the Illumina protocol for MiSeq sample preparation. For the final library, a loading concentration of 10 pM was chosen and a 10% Illumina v3 PhiX spike-in control added before running it on the Illumina MiSeq platform. Libraries prepared using the V1–V2 primer pair were sequenced with 500cycle v2 Illumina MiSeq Reagent Kits, and libraries prepared using the V3–V4 primer pair with 600cycle v3 Illumina MiSeq Reagent Kits. All reagents and equipment for sequencing samples were obtained from Illumina, San Diego, CA, USA.

2.5. Bioinformatic Analysis

The bioinformatics analysis included three main parts, starting with the preprocessing of raw paired-end reads. Following preprocessing, the sequences were assigned to taxonomies. Finally, a statistical and graphical evaluation was performed on the resulting taxa. QIIME2 (2022.8) [18] was used for both preprocessing and classification of the data. With the plugin tool DADA2 (2022.8.0) [19], forward and reverse reads were trimmed from the 3′ end at position 249, while shorter reads and low-quality reads were discarded. DADA2 was also used to perform error correction, the merging of forward and reverse reads if there was an overlap of at least 12 base pairs, and chimera removal. The processed sequences were clustered into operational taxonomic units (OTUs) of 100% sequence identity and assigned to taxa using a classifier trained on full-length sequences of SILVA [20]. The trained classifier was provided by QIIME2 using scikit-learn 0.24.1 and the plugin tool q2-feature-classifier [21,22]. Based on the quantified OTUs and taxa, different diversity indices were calculated using Python and the skbio.diversity library: the richness, Shannon, Simpson, and Fisher indices as a measurement for alpha diversity, and the Bray–Curtis and Jaccard indices as a measurement for beta diversity.

All data relevant to this study are included in this article.

3. Results

In total, 300 sequenced hospital microbiome profiles (which generated a total of 29,774,051 reads with a mean read count of 99,247 per microbiome) passed our set minimum quality criteria of >4000 reads and >1400 merged reads each. At the phylum-to-species level, all taxa with an average prevalence of >0.3% were considered for the statistical analysis. These included 13 phyla, 15 classes, 34 orders, 47 families, 50 genera, and 3 species. Swabs from 50 sites, each in 2 different storage media, yielded 100 samples. DNA was extracted from 92 samples using both the ZBM and PMP kits and from the remaining 8 with only the PMP kit, which resulted in 192 extracted DNA eluates. Of these, 108 were sequenced with the V1–V2 and V3–V4 primer pairs. Of the remaining 84, 83 were sequenced

with only the V1–V2 primer pairs and 1 with only the V3–V4 primer pairs (resulting in 300 microbiome profiles). (See Figure 1.) This analysis showed, and the manufacturer subsequently confirmed, that 10 of the Shield tubes were affected by the contamination of a raw chemical during production, which ultimately limited the final evaluation to 262 microbiome profiles. The remaining Shield tubes belonged to another batch.

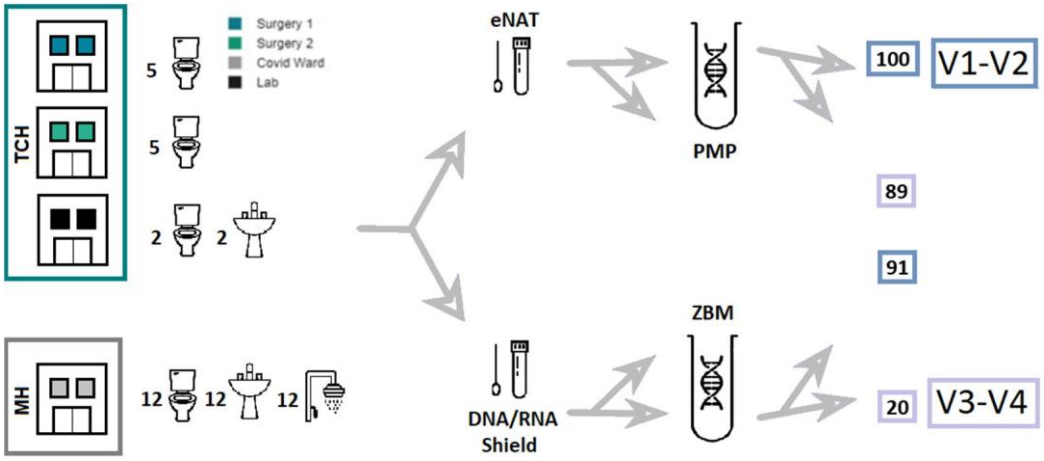

Figure 1. Specimen collection, preservation, and processing flow. The color of the boxes on the right of the image indicates how many samples from each workflow were ultimately sequenced with the corresponding primers.

3.1. Present Taxa

Altogether, 99.99% of the reads were of bacterial origin, and 0.01% were archaeal. The latter were only found in one toilet (with both primer pairs) and one shower (only V1–V2). The prevalence of the 30 most important taxa is displayed in Figure 2A. Only three taxa with an average prevalence of >0.3% were identified at the species level—*Acinetobacter ursingii*, *Lactobacillus iners,* and *Microbacterium lacticum*—the first of which was only detected in one hospital (MH) ($t(212) = -3.33$, $p = 0.001$). In terms of detected phyla, richness, and diversity, the most striking differences were observed between the two hospitals and the institutes/wards (see Figure 2B–E).

3.2. Collection and Preservation Systems

Given that among swabs obtained in surgical wards, only those collected with eNAT were valid and taxa and diversity differed distinctively between institutes, these wards were not considered for comparing the two collection and storage systems. This left 221 microbiomes for comparison (111 Shield, 110 eNAT). No statistically significant differences in terms of detected phyla, classes, orders, families, or species were detected between the two different systems. No statistically significant differences were detected with respect to richness or any of the three diversity indices (Shannon, Fisher-alpha, Simpson).

3.3. DNA Extraction

Because many of the samples extracted with the PMP kit were also sequenced with the V3–V4 primer pair in addition to the V1–V2 primer, only samples sequenced with the latter were selected for a first comparison of the two kits, resulting in an analysis that included 164 microbiomes (82 PMP, 82 ZBM). Among the samples extracted with the PMP kit, a significantly higher prevalence of the phylum *Actinobacteriota* ($t(162) = -2.1$, $p = 0.037$), the class *Actinobacteria* ($t(162) = -2.04$, $p = 0.043$), the order *Propionibacteriales* ($t(162) = -2.01$, $p = 0.046$), and the family *Propionibacteriaceae* ($t(162) = -1.99$, $p = 0.048$) was observed. No statistically significant differences were detected concerning richness or diversity.

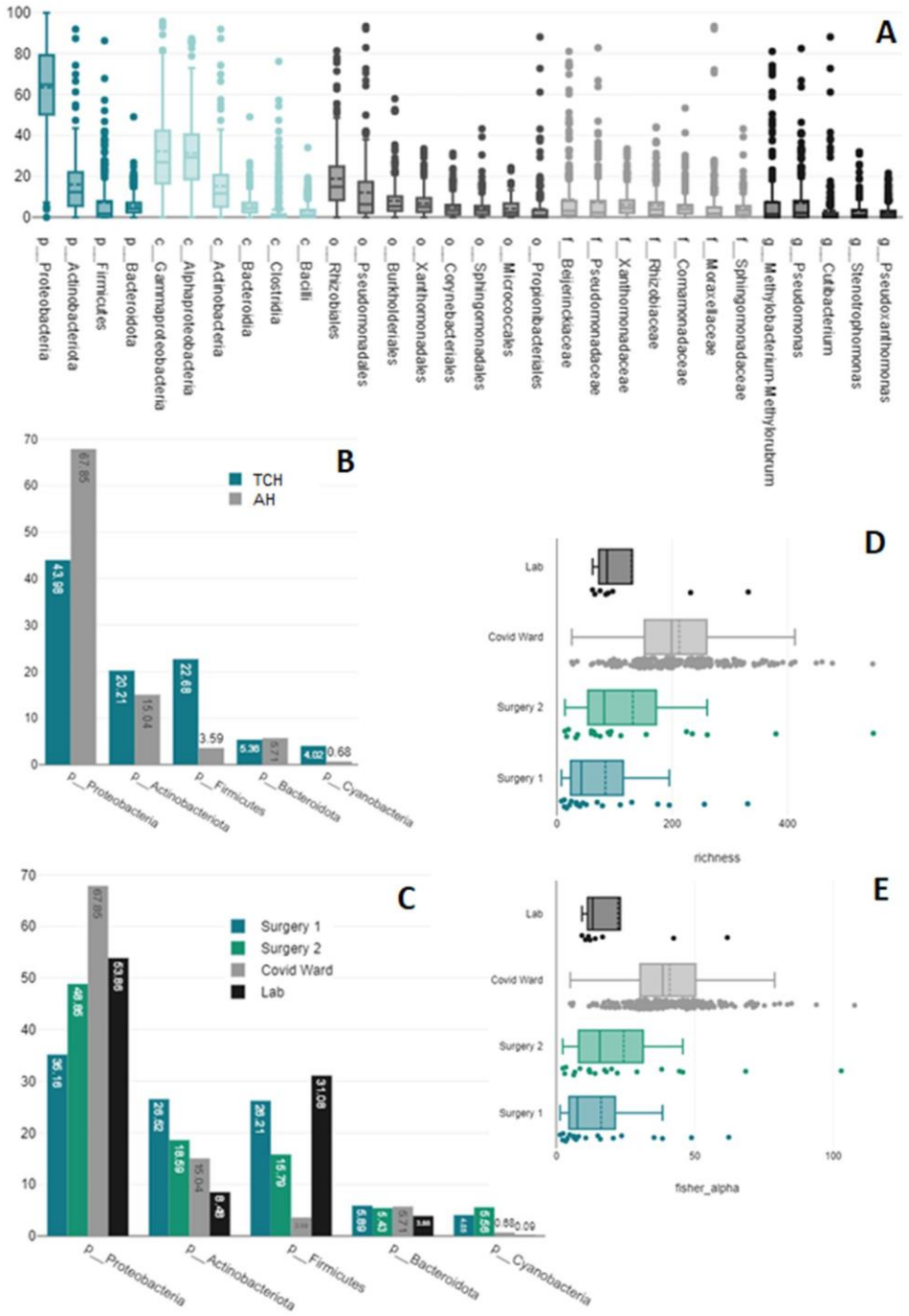

Figure 2. (**A**) Overall prevalence of the 30 most important taxa. Colors allow a more straightforward distinction between the different levels of taxonomic resolution. (**B**) Prevalence of the five most important phyla in the two hospitals and (**C**) institutes/wards. (**D**) Differences in richness and (**E**) Fisher-alpha diversity across the different institutes/wards.

A second analysis of the two kits included samples sequenced with the V3–V4 primer pair and extracted with both extraction kits, resulting in an analysis that included 20 microbiomes (10 PMP, 10 ZBM). Among the samples extracted with the PMP kit, a significantly higher prevalence of the phyla *Actinobacteriota* ($t(18) = -2.71$, $p = 0.014$) and *Verrucomicrobiota* ($t(9) = -2.38$, $p = 0.041$), the class *Actinobacteria* ($t(18) = -2.23$, $p = 0.039$), the order *Propionibacteriales* ($t(18) = -2.11$, $p = 0.049$), the family *Propionibacteriaceae* ($t(18) = -2.43$, $p = 0.026$), and the genus *Mycobacterium* ($t(9) = -2.55$, $p = 0.031$) was

observed. On the contrary, a lower prevalence was observed of the order *Pseudomonadales* (t(12.29) = 2.34, p = 0.037), the family *Pseudomonadaceae* (t(9.45) = 2.35, p = 0.042), and the genus *Pseudomonas* (t(9.45) = 2.35, p = 0.042).

3.4. Primer Pairs

Of all 262 microbiomes, 172 were sequenced with V1–V2 primer pairs and 89 with V3–V4 primer pairs. On average, microbiomes sequenced with V1–V2 primers were found to be richer (t(259) = 2.09, p = 0.038) and more diverse in terms of their Shannon Diversity Index (t(259) = 2.4, p = 0.017). To analyze differences in the detection and prevalence of specific taxa, we restricted the comparison to microbiomes sequenced with V1–V2 and V3–V4 primers. This left us with 181 microbiomes (92 V1–V2, 89 V3–V4), among which the differences in terms of richness and diversity were not confirmed. Which taxa were detected more or less with which primer pair is shown in Table 1.

Table 1. Prevalence of taxa when sequenced with primer pair V1–V2 compared to V3–V4.

	Higher Prevalence	**Lower Prevalence**
Phylum	*Actinobacteriota* (t(126.06) = 6.44, p = < 0.001)	*Bacteroidota* (t(124.82) = −4.75, p = < 0.001)
		Bdellovibrionota (t(128.87) = −3.95, p = < 0.001)
		Verrucomicrobiota (t(103.03) = −5.55, p = < 0.001)
		Acidobacteriota (t(116.99) = −2.33, p = 0.021)
		Chloroflexi (t(106.62) = −3.78, p = < 0.001)
Class	*Actinobacteria* (t(119.62) = 6.73, p = < 0.001)	*Bacteroidia* (t(124.82) = −4.72, p = < 0.001)
		Bdellovibrionia (t(132.42) = −3.78, p = < 0.001)
		Verrucomicrobiae (t(104.34) = −4.5, p = < 0.001)
		Plactomycetes (t(137.82) = −2.28, p = 0.024)
Order	*Pseudomonadales* (t(141.41) = 2.21, p = 0.029)	*Enterobacterales* (t(108.68) = −4.71, p = < 0.001)
	Corynebacteriales (t(135.31) = 4.2, p = < 0.001)	*Flavobacteriales* (t(161.51) = −2.59, p = 0.011)
	Propionibacteriales (t(95.63) = 4.62, p = < 0.001)	*Cytophagales* (t(163.96) = −2.22)
	Micrococcales (t(156.14) = 2.23, p = 0.027)	*Chitinophagales* (t(118.58) = −3.31, p = 0.001)
	Pseudonocardiales (t(91.89) = 2.48, p = 0.015)	*Sphingobacteriales* (t(121.64) = −4.09, p = < 0.001)
		Bdellovibrionales (t(139.31) = −2.89, p = 0.005)
		Acetobacterales (t(120.77) = −3.45, p = 0.001)
		Legionellales (t(132.55) = −2.28, p = 0.024)
Family	*Pseudomonaceae* (t(129.75) = 2.38, p = 0.019)	*Enterobacteriaceae* (t(108.26) = −4.64, p = < 0.001)
	Propionibacteriaceae (t(94.67) = 4.74, p = < 0.001)	*Chitinophagaceae* (t(116.69) = −3.13, p = 0.002)
	Hyphomicrobiaceae (t(109.04) = 2.65, p = 0.009)	*Bdellovibrionaceae* (t(139.31) = −2.89, p = 0.005)
	Microbacteriaceae (t(114.06) = 2.93, p = 0.004)	*Flavobacteriaceae* (t(131.52) = −2.59, p = 0.011)
	Mycobacteriaceae (t(105.44) = 3.65, p = < 0.001)	*Sphingobacteriaceae* (t(120.42) = −3.41, p = 0.001)
	Pseudonocardiaceae (t(91.89) = 2.48, p = 0.015)	*Acetobacteraceae* (t(120.77) = −3.45, p = 0.001)
		Legionellaceae (t(132.55) = −2.28, p = 0.024)
Genus	*Pseudomonas* (t(130.47) = 2.31, p = 0.022)	*Escherichia-Shigella* (t(95.52) = −5.29, p = < 0.001)
	Cutibacterium (t(92.62) = 4.66, p = < 0.001)	*Sphingomonas* (t(179) = −2.25, p = 0.026)
	Hyphomicrobium (t(126.76) = 2.35, p = 0.02)	*Bdellovibrio* (t(139.19) = −2.87, p = 0.005)
	Mycobacterium (t(105.44) = 3.65, p = < 0.001)	*Flavobacterium* (t(131.16) = −2.71, p = 0.008)
	Microbacterium (t(105.42) = 3.5, p = 0.001)	*Legionella* (t(132.46) = −2.25, p = 0.026)
	Pseudonocardia (t(91.79) = 2.43, p = 0.017)	*Mesorhizobium* (t(88.44) = −3.09, p = 0.003)
	Ochrobactrum (t(95.55) = 2.55, p = 0.013)	
	Acidovorax (t(95.7) = 3.67, p = < 0.001)	
	Shinella (t(145.01) = 2.46, p = 0.015)	
	Delftia (t(152.05) = 2.18, p = 0.031)	
	Amaricoccus (t(112.84) = 2.28, p = 0.024)	
	Ottowia (t(99.21) = 2.43, p = 0.017)	
Species	*Lactobacillus iners* (t(92.48) = 2.61, p = 0.011)	
	Microbacterium lacticum (t(91) = 2.48, p = 0.015)	

3.5. Sampling Sites

In order to assign the prevalence of different taxa to specific swab sites (toilets/sinks/showers), we compared the microbiomes of different sites in the most suitable ward (COVID ward).

Our analysis was limited to one ward due to considerable discrepancies between the other wards, previously shown in Figure 1, and because it was the only ward in which toilets, sinks, and showers were sampled. We also limited our analysis in each case to only samples stored in one of the two media (eNAT), extracted by one of the two extraction methods (PMP), and sequenced with one of the two primer pairs (V1–V2) and then confirmed the results with those of the other combinations (all groups n = 34 to 36). The reported *p*-values refer to the significance of the analysis of variance in the group of samples stored in eNAT, extracted with the PMP kit, and sequenced with the V1–V2 primer pair. Where ANOVA found significant differences, a Bonferroni post hoc test was used to compare the groups in pairs. The results, confirmed across all groups of samples, included a significantly higher prevalence of the family *Pseudomonadaceae* (F = 7.79, p = 0.002) and the genus *Pseudomonas* (F = 7.64, p = 0.002) in showers; a higher prevalence of the genera *Acinetobacter* (F = 8.45, p = 0.001) and *Phenylobacterium* (F = 4.18, p = 0.024) in sinks; and a higher prevalence of the families *Hyphomicrobiaceae* (F = 11.58, p = < 0.001) and *Beijerinckiaceae* (F = 3.87, p = 0.031) and the genera *Hyphomicrobium* (F = 13.19, p = < 0.001) and *Methylobacterium/Methylorubrum* (F = 3.89, p = 0.03) in toilets. No significant differences were detected regarding taxa richness or diversity. Figure 3 depicts the differences and similarities between the samples linked to the sampling site.

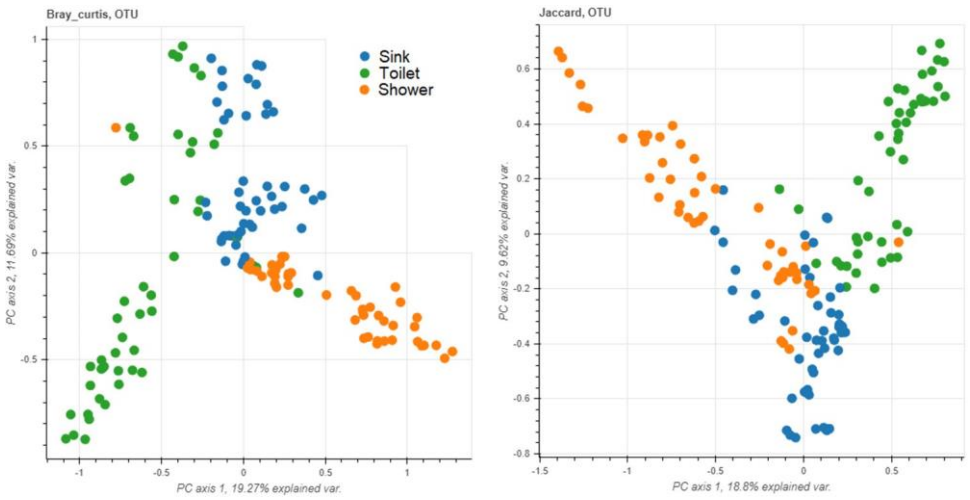

Figure 3. Principal coordinate analysis (PCoA) plot with Bray–Curtis (**left**) and Jaccard (**right**) dissimilarity highlighting operational taxonomic unit (OUT) differences and similarities between MH samples linked to the sampling site.

3.6. Staff vs. Patient Toilets

Almost one-tenth (9.16%, 24/262) of all microbiomes evaluated were from the staff sanitary inventory rather than from patients. These were exclusively samples collected from the TCH. They were 9.24% of those sequenced with the V1–V2 primer pair (16/172) and 8.99% of those sequenced with V3–V4 (8/89). In terms of species richness and diversity indices, large differences were evident between the two groups when limiting the analysis to the surgical wards. A significantly lower species richness (t(31.3) = −2.77, p = 0.009), and Shannon (t(46) = −2.53, p = 0.015) and Fisher-alpha diversity (t(32) = −2.52, p = 0.017) were observed across patient toilets. To confirm this observation without the bias introduced by quadruplication, we matched only samples stored in the same medium, extracted in the same way, and sequenced with the same primer pairs. The results are listed in Table 2. Although all analyses confirmed these results in their tendencies, only two were statistically significant. It should be considered that in all four analyses, the sample size did not exceed ten.

Table 2. Prevalence of taxa when sequenced with primer pair V1–V2 compared to V3–V4; significant differences highlighted in green.

	V1–V2		V3–V4	
	ZBM n = 10	**PMP n = 10**	**ZBM n = 10**	**PMP n = 10**
Richness	t(3.17) = −0.95, p = 0.408	t(8) = −2.39, p = 0.044	t(3.02) = −1.5, p = 0.229	t(3.14) = −1.74, p = 0.177
Shannon diversity	t(3.11) = −0.97, p = 0.402	t(8)=−2.65, p = 0.029	t(8) = −1.63, p = 0.142	t(8) = −1.85, p = 0.101
Simpson diversity	t(8) = −0.37, p = 0.719	t(8) = −1.96, p = 0.086	t(8) = −1.12, p = 0.297	t(8) = −1.09, p = 0.308
Fisher-alpha diversity	t(3.1) = −1.04, p = 0.373	t(8) = −2.23, p = 0.056	t(3.02) = −1.46, p = 0.24	t(3.16) = −1.7, p = 0.183

4. Discussion

Any microbiome analysis intends to reflect the microbial composition of the sample as faithfully as possible. The best, but not always the most practical, approach is to immediately process the sample or immediately freeze the sample until it is further processed [23,24]. In practice, preservation media are often used, chemically producing the effect otherwise achieved by freezing. Numerous studies have compared the performance of different preservation media against each other, against immediate freezing, and against native storage at room temperature [24]. However, while the vast majority have focused on stool samples, to our knowledge, no environmental swabs of indoor hospital environments have yet been studied in this regard. Given that the intent of our study was, among other things, to develop a protocol for collecting and processing hospital microbiome specimens, we only compared two preservation media with each other since it is not considered an option to routinely freeze samples upon collection or store them without preservation media. However, it would certainly be useful to compare preservation media with immediate freezing in a follow-up study to determine the extent of any potential differences.

It is difficult to determine which of the extraction kits with their respective minor differences better reflects the actual conditions [25]. A welcome discovery was that the results differed only slightly. Further extraction kits and extraction modalities should be compared. To determine which one of the two primer pairs better reflects the actual composition of the sample, an additional metagenomic analysis should be performed. Currently, it would be difficult to choose between the V1–V2 primer, which seems to better detect *Pseudomonas*, and the V3–V4 primer, which seems to better detect *Escherichia-Shigella* and *Legionella*. If neither performs well when compared to metagenomics, other primer pairs would be needed for further investigation. Full-length 16S rRNA gene amplification analyses (16S-longreads) or metagenomic analyses would certainly deliver additional relevant information beyond the short-reads-based 16S amplificon analysis. Based on the cost–benefit ratio, it would have to be investigated in which cases they would bring more obvious advantages [6,26–28]. Additionally, it would be desirable to investigate which detected taxa are still viable [29], something for which culture-based methods are still vital in routine practice.

All in all, it seems reasonably viable to compare analyses of differently processed samples up to a certain extent. It seems more challenging to compare microbiomes from different hospitals and wards. Here, which taxa or variables are predictive of what must be investigated in more detail. Further investigations should also clarify which sampling locations are best for which applications.

Most interesting is the species richness and diversity differences observed between patient and staff toilets on the surgical wards despite the small sample size. It should be confirmed forthwith whether patient toilets in wards where large amounts of antibiotics are prescribed are indeed characterized by lower species richness and diversity and that the lower diversity does not simply derive from an altogether worse health state of patients

compared to staff. This would suggest that the emergence and maintenance of multidrug-resistant monocultures in hospital wastewater are linked to excreted antibiotics [30,31]. In a hospital setting, where patients are often immunosuppressed, it may be good to be exposed to an ecosystem with minimal microbial diversity. However, it is also possible that the lack of a rich, diverse microbiome may negatively impact patient outcomes. Without a diverse microbial community, pathogens that would otherwise be displaced could thrive [9,12–17,32,33].

5. Conclusions

The fight against antibiotic-resistant pathogens demands intensified monitoring of antimicrobial resistance. Next-generation sequencing has been used extensively for this purpose. Amplicon sequencing of genes such as the 16S rRNA gene offers excellent monetary value for the study of hospital microbiome profiles, leading to subsequent improvements in protocols. These molecular analyses could be integrated into ongoing surveillance programs. Our study provides evidence that protocol-related variability can be kept to a minimum and allows follow-up studies to address identified challenges.

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Zielsetzung der Arbeit – Krankenhäuser mit ihrem hohen antimikrobiellen Selektionsdruck stellen das vermutlich wichtigste Reservoir für multiresistente menschliche Krankheitserreger dar. Antibiotika, die im Rahmen der Behandlung verabreicht werden, werden ausgeschieden und in das Abwassersystem eingeleitet. Nicht nur in Patienten, sondern auch in der Kanalisation üben antimikrobielle Substanzen einen Selektionsdruck auf vorhandene Bakterien aus und fördern die Entstehung und Verbreitung multiresistenter Klone. In früheren Studien wurden in allen untersuchten Abschnitten des Krankenhausabwassernetzes zwei Hauptcluster identifiziert, ein *K. pneumoniae* ST147-Cluster, das für NDM- und OXA-48-Carbapenemasen kodiert, und ein VIM-kodierender *P. aeruginosa* ST823-Cluster. In der aktuellen Studie untersuchten wir, ob NDM- und OXA-48-kodierende *K. pneumoniae*- und VIM-kodierende *P. aeruginosa*-Isolate, die zwischen 2014 und 2021 von onkologischen Patienten gewonnen wurden, zu denselben Clustern gehörten.

Methoden und Ergebnisse – Die 32 Isolate wurden rekultiviert, das gesamte Genom sequenziert, phänotypisch auf ihre Empfindlichkeit gegenüber antimikrobiellen Mitteln getestet und in silico auf Klonalität und Resistenzgene untersucht. Von diesen Stämmen gehörten 25 zu den beiden Clustern, die im Abwasser vorherrschend waren, während zwei andere zu einem Sequenztyp gehörten, der weniger häufig in den Abflüssen der Patientenzimmer nachgewiesen wurde.

Schlussfolgerungen – Patienten, die ständig Antibiotika ausgesetzt sind, können in Wechselwirkung mit ihren ständig antibiotikaexponierten sanitären Einrichtungen eine Nische bilden, die aufgrund des antibiotikabedingten Selektionsdrucks die Entstehung, Entwicklung, Verbreitung und Aufrechterhaltung bestimmter nosokomialer Erregerpopulationen im Krankenhaus begünstigen kann. Technische Lösungen und

Lösungen zur Infektionskontrolle könnten dazu beitragen, die Übertragung von Mikroorganismen aus dem Abwassersystem auf den Patienten und umgekehrt zu verhindern, insbesondere in Bezug auf die Dusch- und Toilettenabflüsse. Eine wichtige treibende Kraft könnte jedoch auch der antibiotikabedingte Selektionsdruck sein, so dass parallele Bemühungen um antimikrobielles "Stewardship" von wesentlicher Bedeutung sein könnten.

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Antimicrobial Resistance and Infection Control

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Hospital sanitary facilities on wards with high antibiotic exposure play an important role in maintaining a reservoir of resistant pathogens, even over many years

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Abstract

Background Hospitals with their high antimicrobial selection pressure represent the presumably most important reservoir of multidrug-resistant human pathogens. Antibiotics administered in the course of treatment are excreted and discharged into the wastewater system. Not only in patients, but also in the sewers, antimicrobial substances exert selection pressure on existing bacteria and promote the emergence and dissemination of multidrug-resistant clones. In previous studies, two main clusters were identified in all sections of the hospital wastewater network that was investigated, one *K. pneumoniae* ST147 cluster encoding NDM- and OXA-48 carbapenemases and one VIM-encoding *P. aeruginosa* ST823 cluster. In the current study, we investigated if NDM- and OXA-48-encoding *K. pneumoniae* and VIM-encoding *P. aeruginosa* isolates recovered between 2014 and 2021 from oncological patients belonged to those same clusters.

Methods The 32 isolates were re-cultured, whole-genome sequenced, phenotypically tested for their antimicrobial susceptibility, and analyzed for clonality and resistance genes in silico.

Results Among these strains, 25 belonged to the two clusters that had been predominant in the wastewater, while two others belonged to a sequence-type less prominently detected in the drains of the patient rooms.

Conclusion Patients constantly exposed to antibiotics can, in interaction with their persistently antibiotic-exposed sanitary facilities, form a niche that might be supportive for the emergence, the development, the dissemination, and the maintenance of certain nosocomial pathogen populations in the hospital, due to antibiotic-induced selection pressure. Technical and infection control solutions might help preventing transmission of microorganisms from the wastewater system to the patient and vice versa, particularly concerning the shower and toilet drainage. However, a major driving force might also be antibiotic induced selection pressure and parallel antimicrobial stewardship efforts could be essential.

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Keywords Multidrug-resistant bacteria, Carbapenemase-encoding bacteria, Antibiotic-resistance, CRKP, CRPsA, *K. pneumoniae* ST147, *P. aeruginosa* ST823, Bacterial colonization, Hospital hygiene, Hospital wastewater

Introduction

Antibiotic resistance is a major global concern, a complex public health issue and is accelerated by improper use of antibiotics as well as a growing population and increased networking and travelling. It depends on many interconnected factors and is far from limited to the clinical setting [1–3]. Resistance is usually acquired through the uptake of resistance genes by bacterial conjugation or other horizontal transmission pathways, spontaneous mutation of genes, upregulation of efflux pumps or intrinsic resistance genes, which subsequently allow the spread of resistant clones by vertical propagation [4]. After administration, antibiotics and their metabolites are released through environmental matrices, such as the sewage system, and exert selection pressure on the bacteria in this environment, favoring the occurrence of the mechanisms described above, multiplying resistance in the environment [1, 5–8]. Among antibiotic-resistant bacteria, carbapenemase-encoding Gram-negative bacteria are currently the most critical microorganisms [9, 10]. Carbapenems are antibiotics of last resort and administered in life-threatening infections caused by Gram-negative bacteria. Because of the large number of high-risk patients in hemato-oncology departments, these only intravenously administered substances are used there to an inordinately high extent, and as a result are released in excessively large quantities into the same sanitary facilities. Such concentrations were found to be in therapeutic concentrations [11, 12]. Because of this massive use of last-resort antibiotics, these highly resistant and critical bacteria are spread primarily through hospital wastewater, as opposed to municipal wastewater or agricultural process water [13]. Furthermore antibiotic resistant bacteria in wastewater are often associated with the ability to form biofilms in which they are able to survive even when confronted with high levels of antibiotics or disinfectants [14].

Klebsiella pneumoniae and *Pseudomonas aeruginosa* are two of the most important opportunistic and nosocomial pathogens worldwide and are known for the ability to produce biofilms to escape treatment with antibiotics [3, 15, 16]. In this study, we compared NDM and OXA-48 encoding *K. pneumoniae* and VIM encoding *P. aeruginosa* isolates recovered from clinical specimens of patients in oncology wards of a tertiary care center to the highly resistant strains that most prominently colonized patient bathrooms or were recovered from different sampling points of the same sewer system. Environmental *K. pneumoniae* ST147 and *P. aeruginosa* ST823 were analyzed when encoding the mentioned carbapenemases [17]. *K. pneumoniae* ST147 is a well-known high-risk clone that likely emerged in the 1990s and swiftly became a prominent global pathogen [18–20]. Different ST147 clusters are associated with different carbapenemases [18]. On the other hand, *P. aeruginosa* ST823 is mostly associated with bla_{VIM} and only few publications exist [21–23]. Reports of this strain in Europe are limited [17, 24].

Methods

Isolates

In the period under study Gram-negative bacterial pathogens recovered from clinical specimens were routinely identified via MALDI-TOF MS (VITEK MS, Biomerieux, Marcy-l'Etoile, France) and susceptibility-tested with the VITEK 2 system (Biomerieux, Marcy-l'Etoile, France). Carbapenem-resistant *K. pneumoniae* and *P. aeruginosa* isolates, or such with an unusual carbapenem-susceptibility profile (ertapenem/imipenem/meropenem) were routinely analyzed for the presence of common resistance genes, using the Allplex Entero-DR Assay (Seegene, Seoul, South Korea).

All VIM-encoding *P. aeruginosa* isolates and all NDM- and OXA-48-encoding *K. pneumoniae* isolates recovered from patients of oncology wards between September 2014 and November 2021 were traced in the laboratory information system, thawed and sub-cultured twice on Columbia 5% sheep blood agar (Becton Dickinson, Heidelberg, Germany) prior to testing and DNA extraction. Only first isolates were selected for each pathogen–patient combination. The environmental strains used for comparison had been isolated between Nov. 2016 and Sept. 2018 [17]

Susceptibility testing

Antimicrobial susceptibility of all isolates after re-cultivation from cryo stocks was determined thrice by broth microdilution. Susceptibility tests were employed strictly according to the manufacturer's instruction. From each isolate, a bacterial suspension in 0.9% saline solution was prepared. The suspension was adjusted to a McFarland value of between 0.48 and 0.52 using a DensiCHEK plus photometer (bioMerieux, Marcy-l'Etoile, France). For broth microdilution, Micronaut-S MDR MRGN-Screening MIC-Plates (Merlin, Bornheim, Germany) were utilized (tested antibiotics are listed in the legend of Table 2

in the "Results" section). Tests were performed with Mueller–Hinton broth (Merlin, Bornheim, Germany) and read with a BioTek ELx808 Absorbance Microplate Reader (now: Agilent Technologies Inc., Santa Clara, CA, USA). MICs were interpreted according to EUCAST 2022 v12 breakpoints (version 01.01.2022) for *Enterobacterales* and *Pseudomonas*, respectively.

Whole genome sequencing

Highly purified DNA was extracted from all strains using the column-based DNeasy UltraClean Microbial Kit (Qiagen GmbH, Hilden, Germany). The isolation was performed according to the manufacturer's instructions. Obtained DNA was qualitatively and quantitatively evaluated using the NanoDrop OneC from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Dual-indexed Illumina sequencing libraries were constructed from each sample using the Illumina Nextera XT DNA Library Preparation Kit, pooled, and sequenced on the Illumina MiSeq platform with the Illumina MiSeq Reagent Kit v3, 600 cycles (all three: Illumina, San Diego, CA, USA). All steps were carried out following the manufacturer's instructions. Raw reads have been uploaded to the Sequence Read Archive (SRA); accession PRJNA845217.

Assembly and genome analysis

Genome assembly and analysis were carried out independently in two different ways. On the one hand, paired-end reads were trimmed and filtered with BBDuk Trimmer with a Q value of 20 and de novo assembled using Geneious Prime (software version 2020.1 Biomatters, Auckland, New Zealand). Analysis of the de novo assembled contigs was then performed with online tools of the CGE-server, ResFinder-4.0 (https://cge.cbs.dtu.dk/services/ResFinder/) and PlasmidFinder-2.0 (https://cge.cbs.dtu.dk/services/PlasmidFinder/) [25–28] and the AMRFinderPlus v3.10.24 (https://www.ncbi.nlm.nih.gov/pathogens/antimicrobial-resistance/AMRFinder/) (with its according NCBI reference databases of 04-04-2022 [29]), which were used to identify the presence of antimicrobial resistance genes [30]. In addition to BLASTX, nucleotide sequences were translated into amino-acid sequences to identify corresponding proteins with ALLELEX and EXACTX. Genes with insertions for a stop codon were found with INTERNAL_STOP. POINTN considered strain-specific point mutations and refer to the majority of mentioned stress factors. PARTIALX took gene fragments with incomplete reference sequence into account. The minimum coverage value for PARTIALX was set to 60%. Only results with > 90% identity and > 90% coverage were accepted. On the other hand, genomes were assembled and analyzed with ASA^{3}P v1.3.0 [31].

Clustering

For epidemiological clustering Ridom SeqSphere + (version 6.0.2) (Ridom; Münster, Germany) (http://www.ridom.de/seqsphere) [32] was used. *K. pneumoniae* sensu lato was employed as cgMLST template for the *K. pneumoniae* strains and the cgMLST template for *P. aeruginosa* was used for the *P. aeruginosa* strains (www.ridom.de/seqsphere/u/Task_Template_Sphere.html). Minimum spanning trees were calculated after ignoring pairwise missing values and after exclusion of genes that were present only in the template strain.

Phylogenetic analysis

Raw sequencing reads for both *K. pneumoniae* and *P. aeruginosa* isolates were processed using ASA^{3}P v1.3.0 [30]. SNP-based maximum-likelyhood phylogenetic trees were calculated with FastTree within ASA^{3}P calculating 100 bootstraps using isolates CNK1 and A15448 [17] as reference genomes for the sets, respectively.

All data relevant to the study are included in the article or uploaded as Appendix. The precise source of each environmental sample can be located in the corresponding publication [17]. Environmental samples were collected in approximately equal quantities from drains of sinks, toilets, and showers of hospital rooms in the hemato-oncological ward, from the wastewater of the hemato-oncological clinic, and wastewater sampling locations downstream of it.

The ethics committee of the University Hospital Bonn confirmed that no ethics approval was required for this study.

Results

Isolate and patient information

From September 2014 until November 2021, twenty-two VIM-encoding *P. aeruginosa* isolates and ten NDM- and OXA48-encoding *K. pneumoniae* isolates from patients of the oncology clinic were included into this study. Typing revealed that the majority of *P. aeruginosa* isolates (17) belonged to ST823; two belonged to ST235, another two to ST111, and one to ST233 (see Table 1). All but two *K. pneumoniae* isolates (which belonged to ST78) belonged to ST147. In the majority of patients (18/32), the isolates were first detected after more than four weeks of hospitalization. In half of the patients (16/32), the isolates were obtained from stool samples. All but one, *K. pneumoniae* ST147 isolates, were recovered from the same ward.

Clustering

Fourteen sequenced *P. aeruginosa* ST823 isolates, fell into two different groups as clustering showed (cluster distance threshold was set to 12) when clustered with environmental isolates from 2016 to 2018 from a study

Table 1 General patient information of genotyped VIM-encoding *P. aeruginosa* isolates (CVP) (above) and NDM- and OXA48-encoding *K. pneumoniae* isolates (CNK) (below)

Patient	Age	**Year**	Ward	LOS	Previously in ICU	Localization	Species	Sequence type	**Isolate**
1	50–60	**2015**	A	30	Yes	Stool	*P. aeruginosa*	823	**CVP1**
2	60–70	**2015**	B	13	No	Stool	*P. aeruginosa*	111	**CVP2**
3	30–40	**2015**	B	17	No	Blood	*P. aeruginosa*	233	**CVP3**
4	70–80	**2015**	C	36	–	Skin swab	*P. aeruginosa*	235	**CVP4**
5	60–70	**2015**	C	17	–	BAL	*P. aeruginosa*	111	**CVP5**
6	60–70	**2016**	C	80	–	Urine	*P. aeruginosa*	823	**CVP6**
7	50–60	**2016**	A	2	No	TS	*P. aeruginosa*	823	**CVP7**
8	50–60	**2016**	C	26	–	Stool	*P. aeruginosa*	823	**CVP8**
9	20–30	**2016**	B	48	No	Stool	*P. aeruginosa*	823	**CVP9**
10	70–80	**2016**	A	8	No	Stool	*P. aeruginosa*	823	**CVP10**
11	60–70	**2016**	B	57	No	Stool	*P. aeruginosa*	823	**CVP11**
12	50–60	**2016**	C	17	–	TS	*P. aeruginosa*	823	**CVP12**
13	60–70	**2017**	A	324	Yes	Stool	*P. aeruginosa*	823	**CVP13**
14	70–80	**2017**	A	105	Yes	Stool	*P. aeruginosa*	823	**CVP15**
15	60–80	**2017**	C	101	–	TS	*P. aeruginosa*	823	**CVP16**
16	<20	**2017**	D	180	No	Wound	*P. aeruginosa*	823	**CVP17**
17	50–60	**2018**	B	99	No	Inguinal swab	*P. aeruginosa*	823	**CVP18**
18	20–30	**2019**	A	22	No	Stool	*P. aeruginosa*	235	**CVP19**
19	70–80	**2020**	E	1	No	Anal swab	*P. aeruginosa*	823	**CVP23**
20	70–80	**2020**	D	70	No	Stool	*P. aeruginosa*	823	**CVP24**
21	40–50	**2021**	A	80	No	Stool	*P. aeruginosa*	823	**CVP25**
22	60–70	**2021**	A	104	No	Urine	*P. aeruginosa*	823	**CVP27**
23	80–90	**2017**	B	1	No	Stool	*K. pneumoniae*	147	**CNK1**
24	40–50	**2017**	B	59	Yes	Stool	*K. pneumoniae*	147	**CNK2**
25	60–70	**2017**	C	22	–	Stool	*K. pneumoniae*	147	**CNK3**
26	60–70	**2017**	F	2	No	Urine	*K. pneumoniae*	78	**CNK4**
27	50–60	**2018**	B	12	No	Stool	*K. pneumoniae*	147	**CNK5**
28	70–80	**2020**	B	65	Yes	Wound	*K. pneumoniae*	147	**CNK6**
29	70–80	**2020**	B	1	No	Throat swab	*K. pneumoniae*	147	**CNK7**
30	70–80	**2021**	B	182	No	Stool	*K. pneumoniae*	147	**CNK8**
31	50–60	**2021**	C	65	–	Throat swab	*K. pneumoniae*	78	**CNK9**
32	70–80	**2021**	B	44	Yes	Urine	*K. pneumoniae*	147	**CNK10**

LOS: Length of Hospital Stay in days, previous to the date on which the positive sample was collected. Rows are color-coded according to isolate sequence type for better visualization, *P. aeruginosa* ST823 cluster in light blue, *P. aeruginosa* ST235 cluster in purple and *K. pneumoniae* ST147 cluster in red

on hospital drains and wastewater [17] (see Fig. 1). Three out of the four environmental isolates in cluster B, which was the cluster with the predominant number of clinical isolates, had been isolated from the drains of patient rooms. Of the two sequenced *P. aeruginosa* ST235 isolates, CVP4 did not cluster with environmental isolates but CVP19 was closely related to two environmental isolates (see Fig. 1). All eight sequenced *K. pneumoniae* ST147 isolates were closely related to each other and to the environmental isolates, forming

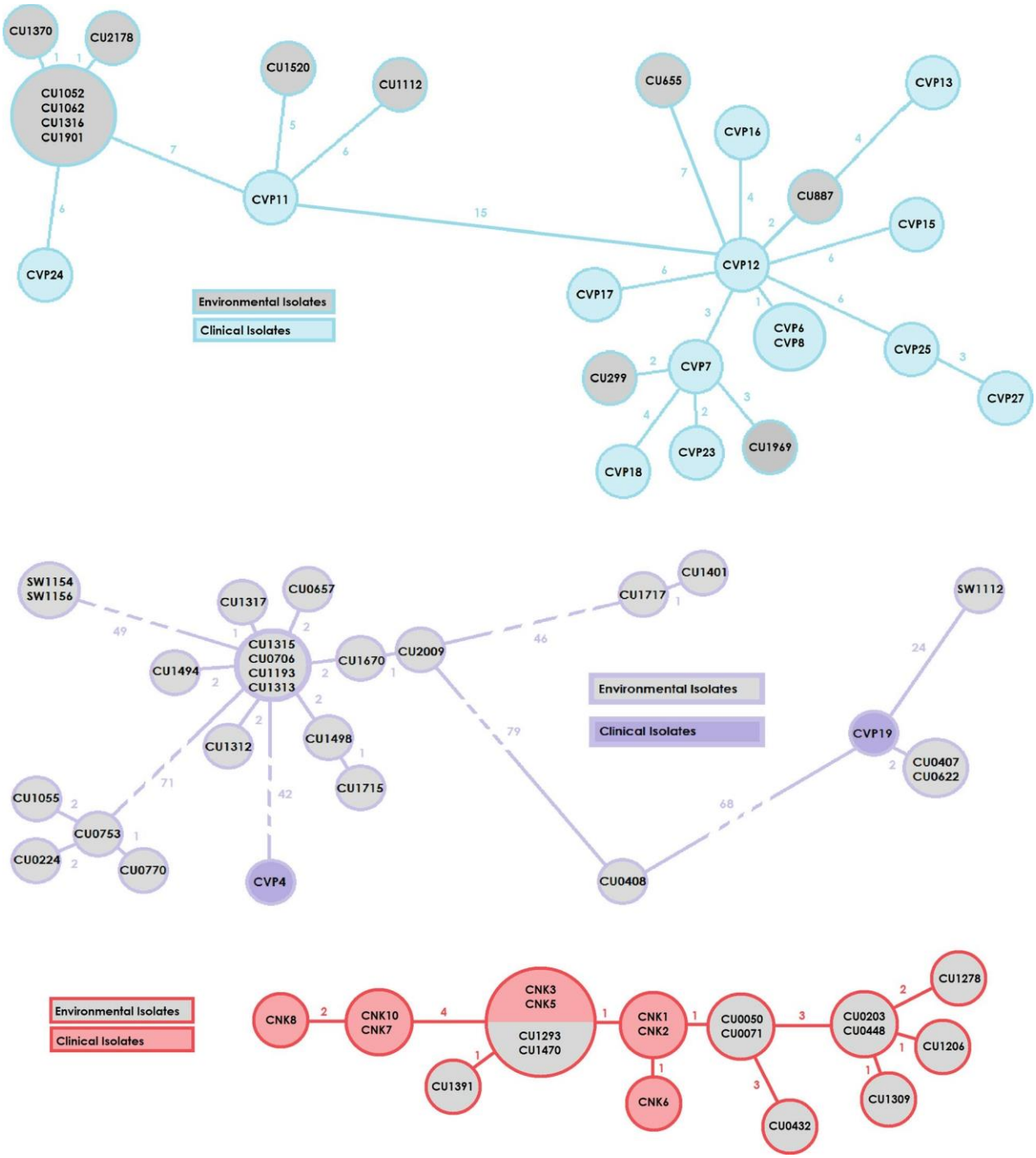

Fig. 1 VIM-encoding *P. aeruginosa* ST823 cluster in light blue, VIM-encoding *P. aeruginosa* ST235 cluster in purple, and NDM- and OXA48-encoding *K. pneumoniae* ST147 cluster in red, as minimum spanning tree, respectively from top to bottom. The lines and numbers show the number of allele differences between isolates

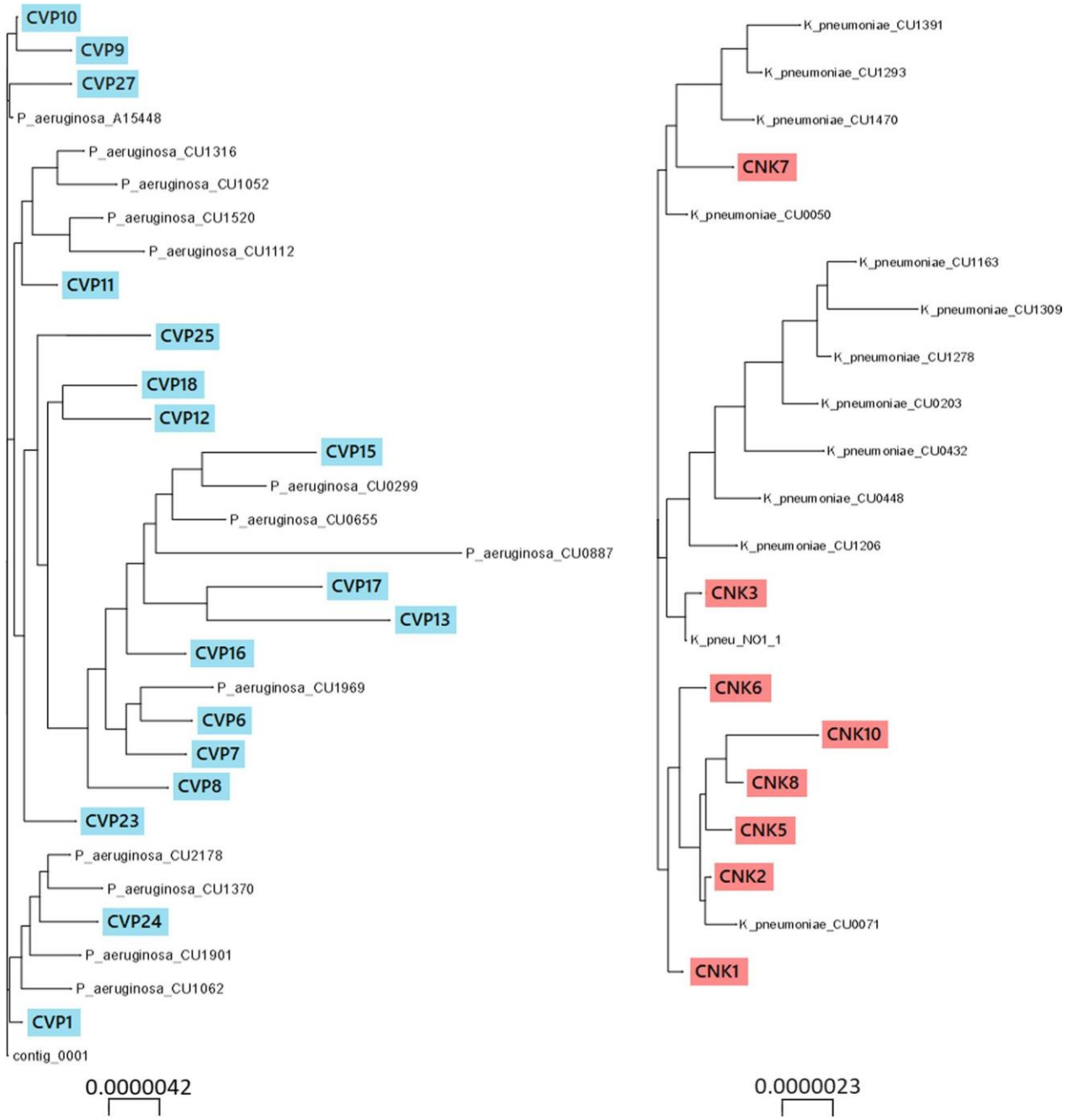

Fig. 2 SNP-based maximum-Likelyhood trees of *K. pneumoniae* ST147 and *P. aeruginosa* ST823 isolates (100 bootstraps). Clinical isolates are marked in light blue and red for *P. aeruginosa* and *K. pneumoniae*, respectively

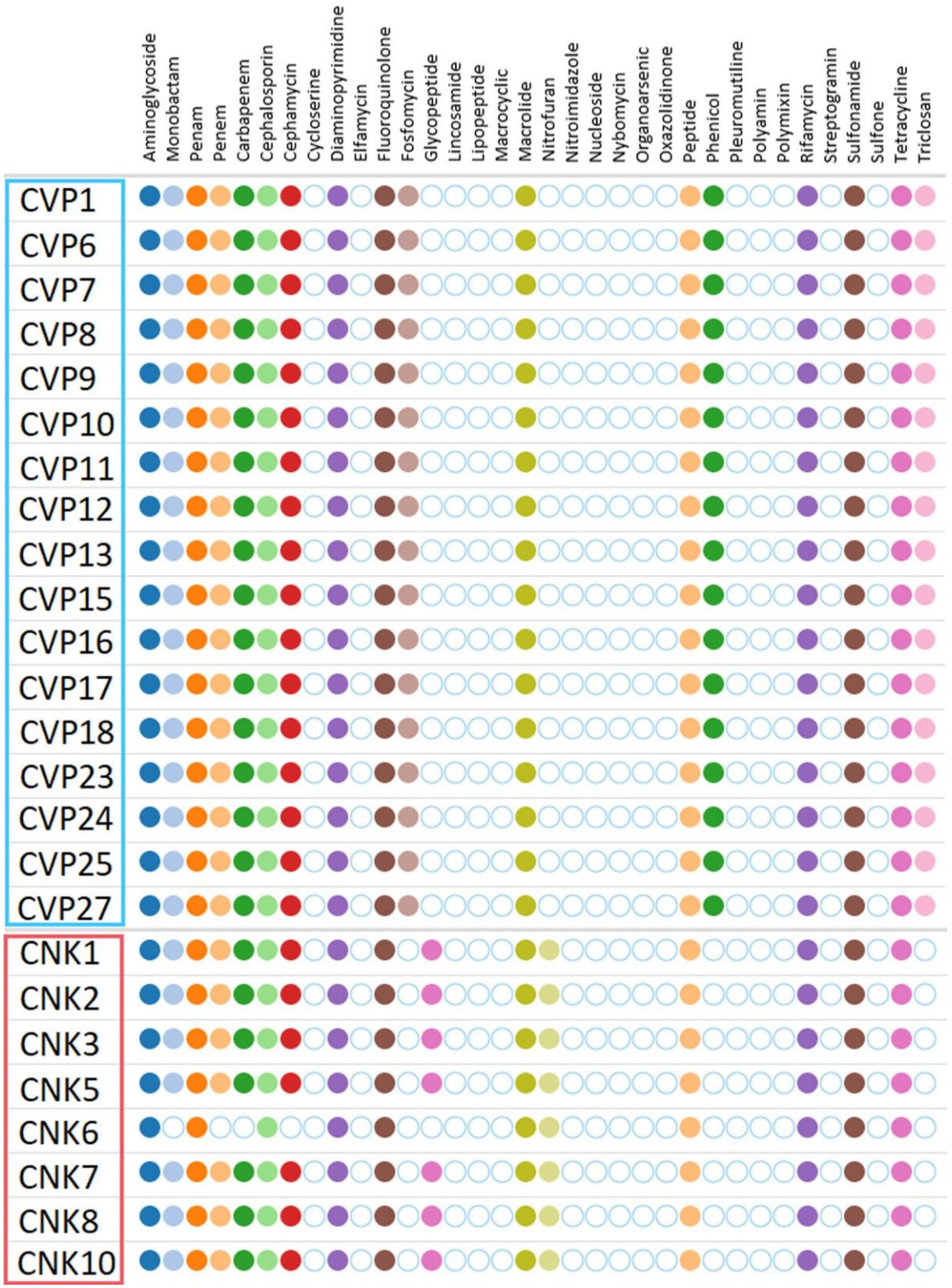

Fig. 3 Antibiotic resistances profile as predicted by the ASA^{3}P pipeline based on CARD annotations for *P. aeruginosa* ST823 and *K. pneumoniae* ST147 isolates. Colored circles refer to the isolate likely being resistant to the respective substance class, while empty circles refer to the isolate likely being susceptible to it

Table 2 Phenotypical susceptibility of *P. aeruginosa* (above) and *K. pneumoniae* (below) isolates determined by broth microdilution

	CIP	LVX	AMK	CST	CHL	FOF	TGC	SXT	PIP	TZP	CTX	CAZ	CAA	CTA	IPM	MEM	TMO
CVP1	> 2	> 2	32	2	> 16	32	> 4	> 4/76	> 16	16/4	> 2	< = 1	< = 1/4	> 8/4	> 8	32	> 128
CVP2	> 2	> 2	> 32	< = 1	> 16	> 64	> 4	> 4/76	> 16	64/4	> 2	32	16/4	> 8/4	> 8	64	> 128
CVP3	> 2	> 2	> 32	2	> 16	64	> 4	> 4/76	> 16	> 64/4	> 2	64	> 16/4	> 8/4	> 8	> 128	> 128
CVP4	> 2	> 2	< = 4	< = 1	> 16	32	> 4	> 4/76	> 16	> 64/4	> 2	64	> 16/4	> 8/4	> 8	64	> 128
CVP5	> 2	> 2	> 32	< = 1	> 16	> 64	4	> 4/76	> 16	64/4	> 2	16	8/4	> 8/4	> 8	32	> 128
CVP6	> 2	> 2	32	2	> 16	64	> 4	> 4/76	< = 8	8/4	> 2	4	4/4	> 8/4	> 8	16	128
CVP7	> 2	> 2	32	< = 1	> 16	64	2	> 4/76	> 16	8/4	> 2	8	8/4	> 8/4	> 8	32	128
CVP8	> 2	> 2	16	2	> 16	< = 16	> 4	> 4/76	16	8/4	> 2	4	4/4	> 8/4	> 8	64	128
CVP9	> 2	> 2	16	2	> 16	64	> 4	> 4/76	16	16/4	> 2	4	4/4	> 8/4	> 8	16	128
CVP10	> 2	> 2	> 32	< = 1	> 16	< = 16	> 4	> 4/76	> 16	> 64/4	> 2	4	4/4	> 8/4	> 8	128	> 128
CVP11	> 2	> 2	16	< = 1	> 16	32	> 4	> 4/76	> 16	> 64/4	> 2	8	8/4	> 8/4	> 8	32	> 128
CVP12	> 2	> 2	> 32	< = 1	> 16	64	4	> 4/76	> 16	> 16/4	> 2	8	8/4	> 8/4	> 8	16	128
CVP13	> 2	> 2	< = 4	< = 1	> 16	32	2	> 4/76	> 16	64/4	> 2	16	8/4	8/4	> 8	32	> 128
CVP15	> 2	> 2	> 32	2	> 16	64	> 4	> 4/76	16	16/4	> 2	16	16/4	> 8/4	> 8	64	128
CVP16	> 2	> 2	< = 4	< = 1	> 16	< = 16	> 4	> 4/76	> 16	32/4	> 2	8	< = 1/4	< = 1/4	> 8	16	128
CVP17	> 2	> 2	< = 4	< = 1	> 16	64	2	> 4/76	< = 8	8/4	> 2	8	8/4	8/4	> 8	32	128
CVP18	> 2	> 2	32	2	> 16	64	> 4	> 4/76	< = 8	8/4	> 2	4	4/4	> 8/4	> 8	32	128
CVP19	> 2	> 2	> 32	< = 1	> 16	32	> 4	> 4/76	> 16	64/4	> 2	> 128	16/4	> 8/4	> 8	8	128
CVP23	> 2	> 2	> 32	< = 1	> 16	64	2	> 4/76	16	16/4	> 2	16	16/4	> 8/4	> 8	64	128
CVP24	> 2	> 2	32	< = 1	> 16	32	4	> 4/76	> 16	64/4	> 2	32	> 16/4	> 8/4	> 8	> 128	128
CVP25	> 2	> 2	> 32	< = 1	> 16	64	> 4	> 4/76	< = 8	8/4	> 2	4	4/4	> 8/4	> 8	16	128
CVP27	> 2	> 2	> 32	4	> 16	64	> 4	> 4/76	16	8/4	> 2	4	4/4	> 8/4	> 8	32	128
CNK1	> 2	> 2	< = 4	< = 1	< = 8	64	0,5	> 4/76	> 16	> 64/4	> 2	> 128	> 16/4	> 8/4	> 8	64	> 128
CNK2	> 2	> 2	< = 4	< = 1	< = 8	64	1	> 4/76	> 16	> 64/4	> 2	> 128	> 16/4	> 8/4	> 8	128	> 128
CNK3	> 2	> 2	< = 4	< = 1	< = 8	128	1	> 4/76	> 16	> 64/4	> 2	> 128	> 16/4	4/4	> 8	128	> 128
CNK4	> 2	> 2	> 32	> 8	> 16	32	0,5	> 4/76	> 16	> 64/4	> 2	> 128	> 16/4	> 8/4	> 8	64	> 128
CNK5	> 2	> 2	< = 4	< = 1	< = 8	128	1	> 4/76	> 16	> 64/4	> 2	> 128	> 16/4	> 8/4	> 8	128	> 128
CNK6	> 2	> 2	8	< = 1	< = 8	64	1	> 4/76	> 16	> 64/4	> 2	> 128	> 16/4	> 8/4	> 8	32	> 128
CNK7	> 2	> 2	> 32	> 8	> 16	< = 16	2	> 4/76	> 16	> 64/4	> 2	> 128	> 16/4	> 8/4	> 8	128	> 128
CNK8	> 2	> 2	< = 4	> 8	< = 8	32	1	> 4/76	> 16	> 64/4	> 2	> 128	> 16/4	> 8/4	> 8	128	> 128
CNK9	> 2	> 2	< = 4	> 8	< = 8	64	1	> 4/76	> 16	> 64/4	> 2	> 128	> 16/4	> 8/4	> 8	128	> 128
CNK10	> 2	> 2	> 32	> 8	> 16	32	0,5	> 4/76	> 16	> 64/4	> 2	> 128	> 16/4	> 8/4	> 8	128	> 128

Isolate cells are colored according to sequence type for better visualization, MIC cells are colored according to isolate susceptibility as per EUCAST 2022 v12 breakpoints (version 01.01, 2022) (green: susceptible, yellow: susceptible at increased dosage, red: resistant)

CIP, ciprofloxacin; *LVX*, levofloxacin; *AMK*, amikacin; *CST*, colistin; *CHL*, chloramphenicol; *FOF*, fosfomycin; *TGC*, tigecycline; *SXT*, trimetoprim-sulfamethoxazole; *PIP*, piperacillin; *TZP*, piperacillin/tazobactam; *CTX*, cefotaxime; *CAZ*, ceftazidime; *CAA*, ceftazidime/avibactam; *CTA*, ceftolozan/tazobactam; *IPM*, imipenem; *MEM*, meropenem; *TMO*, temocillin

one large cluster. *P. aeruginosa* ST111 and ST233 isolates and *K. pneumoniae* ST78 isolates were not clustered due to the absence of environmental isolates with matching sequence types.

Phylogenetic analysis

As Fig. 2 shows, the isolates within the *K. pneumoniae* ST147 Cluster exhibited a high degree of relatedness to each other, as did the isolates within the *P. aeruginosa* ST823 cluster. Seven out of eight clinical *K. pneumoniae* ST147 isolates belonged to two very small but extremely tight clusters (CNK7 with environmental isolates CU1391, CU1293, CU1470, and CU0050 and CNK1, CNK2, CNK5, CNK6, CNK8 and CNK10 with the environmental isolate CU0071). The *P. aeruginosa* isolates, on the other hand, even more markedly formed one large cluster with the environmental isolates.

Resistance genes

Plasmid incompatibility groups detected in *K. pneumoniae* isolates by PlasmidFinder-2.0 are listed in Table 3 in the appendix. The table shows that several plasmids were shared with environmental ST147 isolates. *K. pneumoniae* ST78 isolates and ST147 isolates did not share any plasmids. All ST147 isolates carried the same plasmids, except for the three latest ST147 isolates, recovered in 2020 and 2021, which carried an additional IncHI2-type plasmid. Tables 4 and 5 in the appendix show the Antibiotic resistance genes detected on VIM-encoding *P. aeruginosa* genomes and on NDM- and OXA48-encoding *K. pneumoniae* genomes by AMRFinderPlus, respectively. There were only two internal stop codons found with INTERNAL_STOP (CVP2 *fosA* and CVP5 *fosA*) in resistance genes. The VIM-encoding gene that was detected in the course of routine diagnostics in CVP19 was not detected on its sequenced genome, but a repeated targeted PCR with the Allplex Entero-DR Assay (Seegene, Seoul, South Korea) from the isolate and its extracted genomic DNA confirmed the presence of the resistance gene. Predicted antibiotic resistances by the ASA^{3}P pipeline for *P. aeruginosa* ST823 and *K. pneumoniae* ST147 isolates are displayed in Fig. 3 in the appendix.

Phenotypical susceptibility

According to EUCAST 2022 v12 breakpoints (version 01.01, 2022) all tested isolates were resistant to fluoroquinolones and imipenem (see Table 2). Despite the metallo-beta-lactamases (MBL), one *P. aeruginosa* isolate (CVP19) was susceptible at high dosing regimen to meropenem, nine to piperacillin, eleven to piperacillin/tazobactam, and 13 to ceftazidime, as well as 15 at standard dosing regimen to ceftazidime/avibactam. While all *P. aeruginosa* were still susceptible to colistin and 15/22 to ceftazidime/avibactam, 5/10 K. *pneumonia* isolates were resistant to both. Both *K. pneumoniae* ST78 isolates and the three latest ST147 isolates were resistant to colistin. Analysis of the contigs of the IncHI2-type plasmid showed that it did not carry colistin resistance genes; however, the full sequence of the plasmid was not available for analysis. Among these, two ST147 and one ST78 isolate were also resistant to tigecycline. Two ST147 isolates and one ST78 were resistant to chloramphenicol.

Discussion

K. pneumoniae clones with high AMR risk represent a tremendous public health burden and have played a central role in the global spread of AMR [33]. *K. pneumoniae* ST147 has emerged as one of the most important AMR clones and clearly exhibits most of the essential characteristics that define a global high-risk AMR clone. Several studies have described efficient transmission between patients in the hospital setting [18] and even through drainage water from one room to another [24, 34]. *P. aeruginosa* is increasingly recognized for the ability of certain hospital populations to cause nosocomial infection outbreaks with significant morbidity and mortality. Both, *K. pneumoniae* and *P. aeruginosa,* form biofilms in toilet bowls, particularly behind the flushing rim of the toilet, and establish themselves in hospital water systems, which allow the pathogens to persist and potentially spread out of the toilet each time it is flushed [35, 36]. The two clustering methods exhibited differences in the exact relationships of the isolates, but both demonstrated that even over years, the patient and environmental isolates clustered very closely together, which supports the assumption that these were the same clones [37, 38].

Our study highlights that, consistently antibiotic-exposed patients might, in interaction with their constantly antibiotic-exposed sanitary facilities form a niche that could be supportive for the emergence of certain nosocomial pathogen populations in the hospital, due to antibiotic-induced selection pressure [11, 39]. These highly resistant clones subsequently survive particularly well in the sanitary facilities of those patients who are at highest risk of colonization or infection, i.e., patients who are frequently treated with broad-spectrum antibiotics due to their immunodeficiency, and who have

hardly any remaining healthy normal flora able to outcompete the highly resistant clones [40]. Once colonized, the patients in turn excrete the clones and thus distribute them to other premises and facilities.

In our study, we sequenced two selected sets of carbapenemase-encoding isolates which account only for a fraction of carbapenemase-encoding pathogens encountered in routine practice [41]. While the susceptibility profiles appear atypical for *P. aeruginosa* isolates that encode VIM-type carbapenemases, it should be mentioned that ST823 wastewater isolates exhibited the same kind of antibiograms [17]. Overall, as expected, the therapeutic options were very limited for all isolates [42] with a few newer antibiotics and combinations untested [43]. The effect of the phoQ and pmrB mutations in the strains is still unclear [44–47].Prevention, evidently, remains the much more potent means of combating this problem. Since biofilm is one of the most effective ways for bacteria to colonize the aquatic niche of sanitary facilities, its formation should be prevented. Therefore, several preventive hygienic measures were taken according to national recommendations [48]. As constructional measures, toilets in all newly build wards are designed rimless and in high-risk areas toilets were remodeled to fit the rimless standard. As additional routine hygienic measures, surfaces of toilets, sinks and shower basins are daily disinfected chemically, moreover, sink and shower drains are incubated with a solution of oxidizing disinfectants on a weekly basis, in order to minimize biofilm formation and to reduce high microbial burden. In high risk areas (e.g. bone marrow transplantation) disinfection devices on sink drains applying heat and electromechanical vibration [49] had been installed, however, regarding toilets and shower basins there exist no corresponding technical solutions [48]. With the implemented hygiene and prevention measures presumably colonization pressure is decreased due to reduced microbial load of showers and sinks. The present study did not involve an individualized, patient-specific evaluation of transmission routes or a clinical assessment. It is highly probable that all the following transmission routes existed: from patient to environment, from environment to patient, directly from patient to patient, and through cases that were imported and became detectable under antibiotic selection pressure. Without more detailed analyses, it is not possible to accurately determine which transmission route dominated, only that all of these possibilities exist.

As for the differences between genotype-predicted/expected and phenotypic resistance, it must be pointed out that the accuracy of such algorithms decreases with the number of resistance and virulence genes present, which the isolates analyzed here have an overabundance of. Moreover, it is particularly difficult to infer resistance from the genotype in *P. aeruginosa*, as resistance is usually porin-mediated rather than resistance gene-mediated [50]. For example, *fosA* was detected on all *P. aeruginosa* genomes, whereas a few were phenotypically susceptible, yet, these were not the two isolates in which the gene was determined to be non-functional. Thus, despite the many advantages of molecular antibiograms [51], conventional resistance testing appears to be indispensable, especially for such highly resistant pathogens that are increasingly being screened for molecularly.

Conclusions

Hospital drains continue to play a role in the spread of multidrug-resistant pathogens, as they might form favorable niches for the emergence of multidrug-resistant bacterial populations influenced significantly due to the constant patient-driven antibiotic selection pressure. Extensive technical (e.g. rimless toilets) and hygienic measures (i.e. chemical or technical disinfection of drains), constant monitoring and strict hygiene precautions help to prevent infections, however, further technical solutions are needed to prevent biofilm formation and selection pressure at the sanitary inventory level, as antibiotics are necessary for therapy, but their metabolites in the drains cause undesirable effects.

Appendix

(See Fig. 3 and Tables 3, 4, 5).

Table 3 Plasmids detected in NDM- and OXA48-encoding *K. pneumoniae* isolates by PlasmidFinder-2.0

Plasmid	CU0071	Others	CNK1	CNK2	CNK3	CNK4	CNK5	CNK6	CNK7	CNK8	CNK9	CNK10
IncFIB(pKPHS1)	**Present**	**5/11**	**98.57**	**98.57**	**98.57**		**98.57**	**98.57**	**98.57**	**98.57**		**98.57**
IncFIB(pQil)	**Present**	**7/11**	**100**	**100**	**100**		**100**	**100**	**100**	**100**		**100**
IncFIB(K)	**Absent**	**6/11**				**100**					**100**	
IncHI1B(pNDM-MAR)	**Absent**	**0/11**				**100**					**100**	
IncHI2	**Absent**	**0/11**							**100**	**100**		**100**

Numbers in cells refer to sequence identity and are colored for easier visualization (for CNK isolates: dark green for 100% sequence identity, light green for 98.57% sequence identitiy; for CU0071 dark green for presence, grey for absence of the plasmid; for others: light green for the presence of the plasmid in some of the isolates, grey for the absence in all). The closely related environmental isolates (others) and CU0071 in particular [17] are included in the table to visualize shared plasmids. The "others" column refers to how many of the "other" environmental isolates carried the respective plasmid

Table 4 Antibiotic resistance genes detected on VIM-encoding *P. aeruginosa* genomes by the AMRFinder

	1	2	3	4	5	6	7	8	9	10	11	12	13	15	16	17	18	19	23	24	25	27
aac(3)-Id	x		x			x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x
aac(6')-29a		x																				
aac(6')-29b		x			x																	
aac(6')-Ib				x																		
aac(6')-Ib'				x																		
aac(6')-Ib4																		x				
aac(6')-Il	x		x			x		x	x	x	x	x	x	x	x	x	x		x	x	x	x
aadA2			x																			
aadA6				x														x				
aph(3'')-Ib	x					x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x
aph(3')-IIb	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
aph(3')-XV																		x				
aph(6)-Id	x					x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x
blaGES-1																		x				
blaOXA	x					x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x
blaOXA-395		x			x																	
blaOXA-4			x																			
blaOXA-486			x																			
blaOXA-488				x														x				
blaPDC-3		x	x		x																	
blaPDC-35				x														x				
blaPDC-38	x					x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x
blaVIM-2	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x		x	x
blaVIM-6																				x		
catB7	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
chrA		x			x																	
cmlA6			x																			
crpP	x	x			x	x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x
dfrB5	x		x			x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x
floR2			x															x				

Table 4 (continued)

fosA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
gyrA_T83I	x	x	x	x	x	x	x	x	x	x		x	x	x	x	x	x	x	x	x	x	x
hdeD-GI		x		x	x																	
kefB-GI				x																		
merA		x	x	x	x													x		x		
merB	x					x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x
merD			x	x														x		x		
merE	x		x	x		x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
merP		x	x	x	x													x		x		
merR		x	x	x	x													x		x		
merT		x	x	x	x													x		x		
mexA	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
mexE	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
mexX	x	x	x		x	x	x	x	x	x	x	x		x	x		x	x				
parC_S87L	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
qacEdelta1		x	x	x	x													x		x		
shsP		x		x	x																	
sul1	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x		x	x
sul2	x					x	x	x	x	x	x	x	x	x	x	x	x		x	x	x	x
tet(G)			x															x				
trxLHR		x		x	x																	
yfdX1		x		x	x																	
yfdX2		x		x	x																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	15	16	17	18	19	23	24	25	27

Isolates are colored according to isolate sequence type as in previous tables and figures

Table 5 Antibiotic resistance genes detected on NDM- and OXA48-encoding *K. pneumoniae* genomes by the AMRFinder

	CNK1	CNK2	CNK3	CNK4	CNK5	CNK6	CNK7	CNK8	CNK9	CNK10
aac(3)-IId				x					x	
aac(3)-IIe	x	x	x		x	x	x	x		x
aac(6')-Ib-cr5	x	x		x	x	x	x	x	x	x
aadA1							x	x		x
aadA2				x			x	x	x	x
aph(3')-Ia							x	x		x
aph(3')-VI				x					x	
aph(6)-Id							x	x		x
armA				x					x	
arsA				x					x	
arsB				x					x	
arsD				x					x	
arsR				x					x	
blaCTX-M-15	x	x		x	x	x	x	x	x	x
blaNDM-1	x	x	x	x	x		x	x	x	x
blaOXA				x						
blaOXA-1	x	x			x	x	x	x	x	x
blaOXA-48	x	x	x	x	x	x	x	x	x	x
blaSHV	x	x	x		x	x	x	x		x
blaSHV-28				x					x	
blaTEM-1	x	x	x		x		x	x		x
ble	x	x	x	x	x		x	x	x	x
catA1				x					x	
catB3	x	x		x	x	x	x	x	x	x
dfrA1	x	x	x	x	x	x	x	x	x	x
dfrA12				x			x	x	x	x
emrD	x	x	x	x	x	x	x	x	x	x
fosA	x	x	x	x	x	x	x	x	x	x
gyrA_D87G				x					x	
gyrA_S83I	x	x	x		x	x	x	x		x
gyrA_S83Y				x					x	
hsp20				x					x	
merA				x					x	
merD				x					x	
merE				x					x	
merR				x					x	
merT				x					x	
mph(A)							x	x		x
mph(E)				x					x	
msr(E)				x					x	

Table 5 (continued)

ompK36_D135DGD	x	x	x	x	x	x	x	x	x	x
oqxA	x	x	x	x	x	x	x	x	x	x
oqxB	x	x	x	x	x	x	x	x	x	x
parC_S80I	x	x	x	x	x	x	x	x	x	x
pcoA				x			x	x	x	x
pcoB				x			x	x	x	x
pcoC				x			x	x	x	x
pcoD				x			x	x	x	x
pcoE				x			x	x	x	x
pcoR				x			x	x	x	x
pcoS				x			x	x	x	x
phoQ_R16C				x					x	
pmrB_R256G	x	x	x		x	x	x	x		x
qacE								x		x
qacEdelta1	x	x	x	x	x	x	x	x	x	x
qacL							x	x		x
qnrB1	x	x	x		x	x	x	x		x
sat2				x					x	
silA				x			x	x	x	x
silB				x			x	x	x	x
silC				x			x	x	x	x
silE				x			x	x	x	x
silF				x			x	x	x	x
silP				x			x	x	x	x
silR				x			x	x	x	x
silS				x			x	x	x	x
sul1	x	x	x	x	x	x	x	x	x	x
sul3							x	x		x
terB				x					x	
terC				x					x	
terD				x			x	x	x	x
terE				x					x	
terW							x	x		x
terZ							x	x		x
tet(A)	x	x	x	x	x	x	x	x	x	x
tet(M)							x	x		x
ybtP	x	x	x	x	x	x	x	x	x	x
ybtQ	x	x	x	x	x	x	x	x	x	x
	CNK1	CNK2	CNK3	CNK4	CNK5	CNK6	CNK7	CNK8	CNK9	CNK10

Isolates are colored according to isolate sequence type as in previous tables and figures

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CN and GB contributed to study design. CN, ES, TD, CB, NK, JB, KA, KS, TH, GF, PB and MP contributed to data collection. CN, MN, OS, TD, CB, NK, JB, KA, SE and GB contributed to data analysis. CN, ES, MN, OS, CB, KS, SE, NTM, GB, and MP contributed to data interpretation, CN, ES, MN, OS, CB, KA and GB contributed to the literature search. CN, ES, MN, OS, TD, CB, JB, KS, SE, NTM, GB and MP contributed to writing of the report. All authors reviewed and approved the final version of the manuscript.

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Availability of data and materials
All data relevant to the study are included in the article or uploaded as Appendix. Raw reads have been uploaded to the Sequence Read Archive (SRA); accession PRJNA845217.

Declarations

Ethics approval and consent to participate
The ethics committee of the University Hospital Bonn confirmed that no ethics approval was required for this study.

Consent for publication
Not applicable.

Competing interests
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Publisher's Note

3.4 **Neidhöfer C**, Neuenhoff M, Jozič R, Atangcho B, Unsleber S, Neder U, Grumaz S, Parčina M. Exploring clonality and virulence gene associations in bloodstream infections using whole-genome sequencing and clinical data. Front Cell Infect Microbiol. 2023 Nov 14;13:1274573.

Zielsetzung der Arbeit – Blutstrominfektionen (BSI) sind nach wie vor eine wichtige Ursache für Todesfälle weltweit. Die verursachenden Erreger werden routinemäßig identifiziert und auf Anfälligkeit getestet, aber nur sehr selten auf ihre Resistenzgene, Virulenzfaktoren und Klonalität untersucht. Unser Ziel war es, einen Einblick in die Klonalitätsmuster verschiedener Arten, die BSI verursachen, und die klinische Relevanz verschiedener Virulenzgene zu gewinnen.

Methoden und Ergebnisse – Für diese Studie haben wir über 400 zufällig ausgewählte wichtige Krankheitserreger, die zwischen 2016 und 2021 aus Blutkulturen in unserer diagnostischen Abteilung isoliert wurden, einer Ganzgenomsequenzierung unterzogen. Genomische Daten zu Virulenzfaktoren, Resistenzgenen und Klonalität wurden mit In-vitro-Daten sowie demografischen und klinischen Informationen verknüpft. Die Untersuchung ergab umfangreiche und informative Daten über die Verteilung von Genen, die bei BSI eine Rolle spielen, sowie über die Klonalität von Isolaten über verschiedene Arten hinweg.

Schlussfolgerungen – Assoziationen zwischen Überlebensraten und dem Vorhandensein spezifischer Gene müssen mit Vorsicht interpretiert werden, und die Durchführung von Replikationsstudien mit größeren Stichproben für jede Spezies scheint unerlässlich. Ein genauerer Blick auf Wirtsmerkmale könnte möglicherweise sogar aufschlussreicher sein als Erregerfaktoren für den Verlauf von Infektionen mit den heutzutage dominanten opportunistischen Erregern. Ebenso wird ein tieferes Wissen über Virulenz- und Wirtsfaktoren bei der Interpretation der Ergebnisse helfen und könnte zu gezielteren therapeutischen und präventiven Maßnahmen führen. Eine effizientere Überwachung der Übertragungsdynamik verspricht ein wertvolles Instrument zur Prävention insbesondere von durch nosokomiale Erreger verursachten BSI zu werden.

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Exploring clonality and virulence gene associations in bloodstream infections using whole-genome sequencing and clinical data

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Background: Bloodstream infections (BSIs) remain a significant cause of mortality worldwide. Causative pathogens are routinely identified and susceptibility tested but only very rarely investigated for their resistance genes, virulence factors, and clonality. Our aim was to gain insight into the clonality patterns of different species causing BSI and the clinical relevance of distinct virulence genes.

Methods: For this study, we whole-genome-sequenced over 400 randomly selected important pathogens isolated from blood cultures in our diagnostic department between 2016 and 2021. Genomic data on virulence factors, resistance genes, and clonality were cross-linked with *in-vitro* data and demographic and clinical information.

Results: The investigation yielded extensive and informative data on the distribution of genes implicated in BSI as well as on the clonality of isolates across various species.

Conclusion: Associations between survival outcomes and the presence of specific genes must be interpreted with caution, and conducting replication studies with larger sample sizes for each species appears mandatory. Likewise, a deeper knowledge of virulence and host factors will aid in the interpretation of results and might lead to more targeted therapeutic and preventive measures. Monitoring transmission dynamics more efficiently holds promise to serve as a valuable tool in preventing in particular BSI caused by nosocomial pathogens.

KEYWORDS

bloodstream infections, whole-genome sequencing, clonality patterns, virulence factors, resistance genes, genotype-phenotype correlation, genomic characterization of BSI, molecular epidemiology of BSI

Introduction

Bloodstream infections (BSIs) pose a substantial global health threat, leading to increased morbidity and mortality rates (McNamara et al., 2018). While identifying the causative pathogen and its antibiotic susceptibility remains a clinical priority, exploring additional factors such as genetic relatedness, virulence genes, and antibiotic resistance genes can significantly enhance patient outcomes and alleviate the overall burden (Wren, 2000; Leavis et al., 2006; Wyres et al., 2020; Allen et al., 2021). Whole-genome sequencing (WGS) studies have revolutionized our understanding of pathogen identification, antibiotic resistance, and epidemiology (Wren, 2000). Insights into clonality patterns can guide interventions to curtail the dissemination of specific strains and inform targeted prevention strategies (Wren, 2000; Leavis et al., 2006; Wyres et al., 2020). Furthermore, studying pathogen clonality provides valuable insights into the evolutionary dynamics of BSI pathogens, facilitating the prediction of future trends in antibiotic resistance and virulence (Wren, 2000; Allen et al., 2021).

Virulence factors play a crucial role in the colonization, invasion, and evasion of the host immune system by pathogens. Understanding the specific virulence factors associated with BSI-causing bacteria provides insights into disease mechanisms, severity, and potential therapeutic targets (Wren, 2000; L Thomas and Lee, 2012; Wyres et al., 2020). In-depth investigations of bacterial virulence factors associated with BSI have yielded significant findings concerning pathogenesis and host–pathogen interactions (Wren, 2000; Leavis et al., 2006; L Thomas and Lee, 2012; Wyres et al., 2020). Studying resistance genes and correlating them with phenotypical susceptibility are of paramount importance for addressing key research questions in the field of antimicrobial resistance leading to a comprehensive understanding of the interplay between genotype and phenotype (Wren, 2000; L Thomas and Lee, 2012; Mahfouz et al., 2020; Allen et al., 2021). It enables the validation and verification of resistance mechanisms and provides a more accurate assessment of the clinical implications of specific genetic variants.

In this study, we conducted a comprehensive whole-genome sequencing of over 400 randomly selected common BSI-causing pathogens to elucidate clonality patterns and assess the clinical significance of virulence and resistance genes, including their association with mortality. Our study findings advance the understanding of BSI pathogenesis and hold implications for more targeted therapeutic interventions.

Methods

Study design and data collection

For this study, over 400 bacterial isolates of common pathogens detected in blood cultures between January 2019 and December 2021 in our microbiological diagnostic unit, which services a tertiary referral and maximum care hospital and other hospitals in the area, were randomly selected for the genera *Acinetobacter*, *Bacteroides*, *Citrobacter*, *Enterobacter*, and *Serratia* and the species *Enterococcus faecalis*, *Enterococcus faecium*, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* from cryo-storage. These were thawed, subcultured twice on Columbia 5% sheep blood agar (Becton Dickinson, Heidelberg, Germany), and inspected by two experienced operators prior to sequencing. Isolate information was complemented by accessible laboratory information on phenotypical susceptibility, growth of additional bacteria in the same blood culture, and routine diagnostic resistance gene detection, as well as accessible patient information on age, sex, hospitalization, 30-day mortality, and outcome. We constructed a database that was password-protected and accessible by only three operators who ensured that all patient data were fully de-identified prior to analysis. Unless specifically mentioned or reported, the minimum inhibitory concentrations (MICs) were determined by the VITEK 2 system (bioMérieux, Marcy-l'Etoile, France). Isolate susceptibility was inferred based on EUCAST Cl. Br. Tables v. 13.0.

DNA preparation and sequencing

DNA isolation, library preparation, sequencing, and sequence assembly were carried out by Noscendo GmbH, Germany. Genomic DNA was prepared from pellets obtained from 5 ml of culture in a brain heart infusion (BHI) medium (Becton Dickinson, Heidelberg, Germany). Cell pellets were prepared, shipped on dry ice, and stored at −80°C until further processing. DNA was isolated with the ZymoBIOMICS DNA Miniprep Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions with a Vortex Genie 2 device equipped with an SI-H524 horizontal tube holder (Scientific Industries, Bohemia, NY, USA) to perform mechanical cell disruption for 10 min. DNA concentration was measured with the Qubit 1X dsDNA Assay-Kit on a Qubit 2.0 instrument (Thermo Fisher Scientific, Waltham, MA, USA), and size distribution was checked with the Agilent Genomic DNA 50 kb Kit on a 5200 Fragment Analyzer System (Agilent Technologies, Santa Clara, CA, USA). The libraries were prepared using the Ligation Sequencing Kit SQK-LSK109 with Native Barcoding Expansion 1–12 (PCR-free) EXP-NBD104 and Native Barcoding Expansion 13–24 (PCR-free) EXP-NBD114 according to the manufacturer's instructions (Oxford Nanopore Technologies, Oxford, UK) together with NEB Blunt/TA Ligase Master Mix and NEBNext Companion Module for Oxford Nanopore Technologies Ligation Sequencing (New England Biolabs, MA, USA). Then, the libraries were prepared, pooled in equimolar ratio, quality checked, loaded on a MinION Flow Cell (R9.4.1), and finally sequenced on a MinION benchtop sequencer (Mk1B). For each batch preparation, a pellet of a 1.5-ml overnight culture (BHI medium) of DSM 1576–*Escherichia coli* was used as an internal quality control during the whole process, starting with DNA isolation.

Bioinformatics analysis

After sequencing, fast5 data were basecalled using the Oxford Nanopore Technologies neural-network-based basecalling software Guppy (version 5.0.7) applying a high accuracy mode (config dna_r9.4.1_450bps_hac.cfg). Fastq statistics on read length distributions, expected coverages, and N50 values were calculated. Following closely the manual of the tool Trycycler (Wick et al., 2021) (https://github.com/rrwick/Trycycler, version 0.4.2), long-read consensus assemblies were produced from multiple input assemblies of the same input data set by using the following assemblers: Flye (https://github.com/fenderglass/Flye, version 2.8.3), Miniasm and Minipolish (https://github.com/rrwick/Minipolish, version 0.1.3), and Raven (https://github.com/lbcb-sci/raven, version 1.4.0). Before assembly, reads below a length of 1,000 bp were removed using the tool Filtlong (https://github.com/rrwick/Filtlong, version 0.2.0), at most removing 5% of the original data set. Nine subsamples were created, providing a minimum read depth of 100×. In cases in which average coverages were below 100×, the minimum read depth after subsampling was lowered to 50×. Subsampled data sets were then forwarded to the assemblers, producing nine assemblies in total, three of every assembler. The Trycycler then clustered similar contigs to detect spurious, incomplete, or misassembled contigs. After manual inspection, the conspicuous clusters were removed. The remaining clusters were further processed to result in circular chromosomes and plasmids. Consensus sequences were polished using Medaka (https://github.com/nanoporetech/medaka, version 1.4.3) to increase base accuracy and minimize assembly and sequencing errors.

Assembly quality was finally evaluated by Busco (https://busco.ezlab.org/, https://gitlab.com/ezlab/busco/-/releases/5.2.1, version 5.2.1). Furthermore, reads were mapped to the consensus sequence by using Minimap2 (https://github.com/lh3/minimap2, version 2.18) and Flye-samtools to get coverage values of every consensus base. Assemblies having a Busco completeness value above 95% and every consensus base covered with at least 5× coverage were regarded as high quality. All isolates with valid results and sufficient coverage were included in the analysis.

Detection of antimicrobial resistance (AMR) genes, stress response genes, and virulence factors was detected with AMRFinderPlus (3.11.4) "–plus" option/NCBI reference gene database (2023-02-23.1) (Feldgarden et al., 2021; Feldgarden et al., 2022). If species were present in the list of curated organisms, the "–organism" option was used to ignore universal species mutations and resistance genes and to screen for known point mutations. Only results with identity and coverage >90% were included. For further analysis, assemblies were imported into bactopia (Shen, ; Ewels et al., 2020; Petit and Read, 2020). FastANI (v1.33) was used to calculate the average nucleotide identity within species (Jain et al., 2018). MLST types were determined with the PubMLST database using MLST (2.23.0, https://github.com/tseemann/mlst) (Jolley et al., 2018). For *Enterobacterales*, plasmids were detected using plasmidFinder (2.1.6) (Camacho et al., 2009; Carattoli et al., 2014).

Strain-specific tools were used to obtain more in-depth information: *E. coli*—ECTyper (1.0.0) (Iguchi et al., 2015), *K. pneumoniae*—Kleborate (2.3.2) (Lam et al., 2021), *P. aeruginosa*—pasty (1.0.2, https://github.com/rpetit3/pasty) (Camacho et al., 2009; Thrane et al., 2016), and *S. pneumoniae*—Seroba (1.0.2) (Epping et al., 2018).

Phylogenetic trees and ANI values in supplementary data were created with Anvi'o v7.1 (Eren et al., 2021) using "anvi'o pangenomics workflow" (Edgar, 2004; Hyatt et al., 2010; Buchfink et al., 2015; Pritchard et al., 2016; Chan and Lowe, 2019). All detected resistance genes, stress response genes, and virulence factors for each species can be found in the Supplementary Material. (Sequence names are mentioned yellow for findings with shared gene symbol but multiple possible alleles. These were merged for our purposes if the genes belong to the same subclass.)

All data relevant to the study are included in the article or uploaded as supplementary information. Due to collaboration agreements, genomes have not been uploaded to any publicly accessible platform but can be shared upon reasonable request.

The Ethics Committee of the University Hospital Bonn confirmed that no ethics approval was required for this study.

Results

Isolate and patient information

The 364 isolates for which genomes were available with satisfactory coverage and quality belonged to 364 different patients of which the majority were men (60.16%). Detailed information on patients and isolates is listed in Table 1. In 55 cases (15.11%), the pathogen under study was not the only one isolated from the respective blood culture. In 7, there were 2 additional pathogens, and in another three, 3. Fourteen isolates were methicillin-resistant *S. aureus* (MRSA), 10 were vancomycin-resistant *enterococci* (VRE), and 26 were gram-negative rods falling into the German guideline classification of multidrug-resistant gram-negative rods on the basis of resistance against three (3MRGN) or four (4MRGN) of the following antibiotic groups: acylureidopenicillins, third- and fourth-generation cephalosporins, carbapenems, and fluoroquinolones.

Species characteristics

The most frequently detected AMR genes were *fosA* (107), *oqxA* (73), and *emrD* (70). These were, however, only detected in *Enterobacterales* and *P. aeruginosa* that made up a large part of all isolates. An overview of detected virulence, stress response, and AMR genes can be found in the Supplementary Material. Figure 1 displays to what degree the average nucleotide identity could vary across different pathogens and the number of virulence genes, AMR genes, stress response genes, and plasmids that different pathogens carried. The 30-day and 90-day mortality did not correlate with the number of resistance, AMR, or stress response genes but with

TABLE 1 Patient and isolate information.

Age (years) Mean (min, max)	62.15 (0, 95)	
Sex		
Female	145	39.84%
Male	219	60.16%
Ward type		
Emergency center	92	25.28%
ICU	112	30.77%
Non-ICU ward	154	42.31%
Outpatient clinics	6	1.65%
Clinic		
Anesthesiology	20	5.49%
Emergency departments	89	24.45%
General surgery	19	5.22%
Rehabilitation	49	13.46%
Gynecology	8	2.2%
Heart surgery	10	2.75%
Internal medicine	77	21.15%
Neonatology	6	1.65%
Neurosurgery	8	2.2%
Neurology	17	4.67%
Oncology	40	10.99%
Orthopedics	5	1.37%
Pediatrics	10	2.75%
Urology	4	1.1%
Others	2	0.55%
Year		
2016	77	21.15%
2017	71	19.51%
2018	83	22.80%
2019	62	17.03%
2020	61	16.76%
2021	10	2.75%
Isolates		
Acinetobacter spp.	12	3.3%
Bacteroides spp.	4	1.1%
Citrobacter spp.	15	4.12%
Enterobacter spp.	45	12.36%
Enterococcus faecalis	19	5.22%
Enterococcus faecium	19	5.22%
Escherichia coli	40	10.99%
Klebsiella pneumoniae	40	10.99%
Proteus mirabilis	20	5.5%
Pseudomonas aeruginosa	38	10.44%
Serratia spp.	27	7.42%
Staphylococcus aureus	48	13.19%
Stenotrophomonas maltophilia	5	1.37%
Streptococcus pneumoniae	13	3.57%
Streptococcus pyogenes	18	4.95%

patient age (rpb = 0.22, n = 248, p = 0.001 and rpb = 0.25, n = 201, p = <0.001, respectively).

Enterococcus faecalis

The majority of the isolates originated from patients in intensive care units (ICUs) with eight cases, followed by the emergency center (EC) with four cases. None of the isolates were identified as VRE either phenotypically or genotypically. There were 11 unique multilocus sequence typing (MLST) types represented once and four types represented twice. The isolates showed an average nucleotide identity of 98.78% to each other, ranging from 98.40% to 98.97%. Notably, patients infected with *E. faecalis* isolates encoding *dfrF*, *gyrA_S83I*, and *parC_S80I* exhibited significantly lower 30-day (all p = 0.015) and 90-day survival rates (all p = 0.047), although the assumptions for the χ^2 test were not met due to low cell frequencies. Out of all the isolates, only two showed higher MICs for trimethoprim/sulfamethoxazole than the threshold of ≤10, and both of these isolates tested positive for the *dfrG* gene.

Enterococcus faecium

Out of the *E. faecium* isolates, 10 were classified as VRE, but their presence was not associated with a lower 30-day or 90-day survival rate. Among these isolates, the MLST types of seven could not be determined, while six belonged to ST117 and three to ST80. Five ST117 isolates and two ST80 isolates were identified as VRE. No significant differences were observed in the 30-day and 90-day survival rates based on the presence or absence of specific genes. However, a positive correlation was found between survival and younger age (rpb = 0.71, p = 0.001). The isolates displayed an average nucleotide identity of 98.84% to each other, with a range of 94.29% to 99.27%.

Staphylococcus aureus

Fourteen isolates were MRSA, of which all were *mecA*-positive. Two isolates were resistant to tetracycline, while *tet(k)* was only encoded by one of them and by one phenotypically susceptible isolate; *tet(38)* was encoded by both resistant ones but also by all but one of those susceptible. Out of four that were resistant to rifampicin, only one was found to have genes conferring resistance to it (*rboB_H481Y*, *rboB_L466S*, and *rpoB_S486L*). Resistance to levofloxacin well matched the presence of resistance genes *gyrA_S84L*, *parC_E84G*, *parC_E84K*, *parC_S80F*, and/or *parE_P585S*. While all isolates were susceptible to linezolid, in nine, *23S_C2220T* was found. Out of 23 resistant to erythromycin, 11 encoded *erm(A)*, 4 *erm(C)*, 2 *erm(T)*, and another 2 *msr(A)* and *mph(C)*; in 4, no macrolide resistance genes were detected. For 40 isolates, information on the 30-day survival could be retrieved, as well as for 37 on 90-day survival and 30-day and 90-day outcomes. The only significant difference that was found was that of a lower 30-day survival in patients with *S. aureus* isolates that encoded *splE* (p = 0.045; Cramér's V 0.32) but without meeting χ^2 test assumptions due to low cell frequencies and without significant Fisher exact test (p = 0.072). Isolates had an average nucleotide identity to each other of 98.36, ranging from 97.73 to 98.75 (see also Figure A1 in the Supplementary Material). The two most represented MLST types were 225 (10) and 22 (9).

Streptococcus pneumoniae

In 11 out of 13 cases in which *S. pneumoniae* grew, blood cultures were collected in the EC. The available data were insufficient to perform statistical analyses pertaining to hypotheses involving 30-day and 90-day outcomes and survival rates. Only three isolates had MICs to penicillin sufficiently high to be considered susceptible only at increased dosage (I). Of these, two

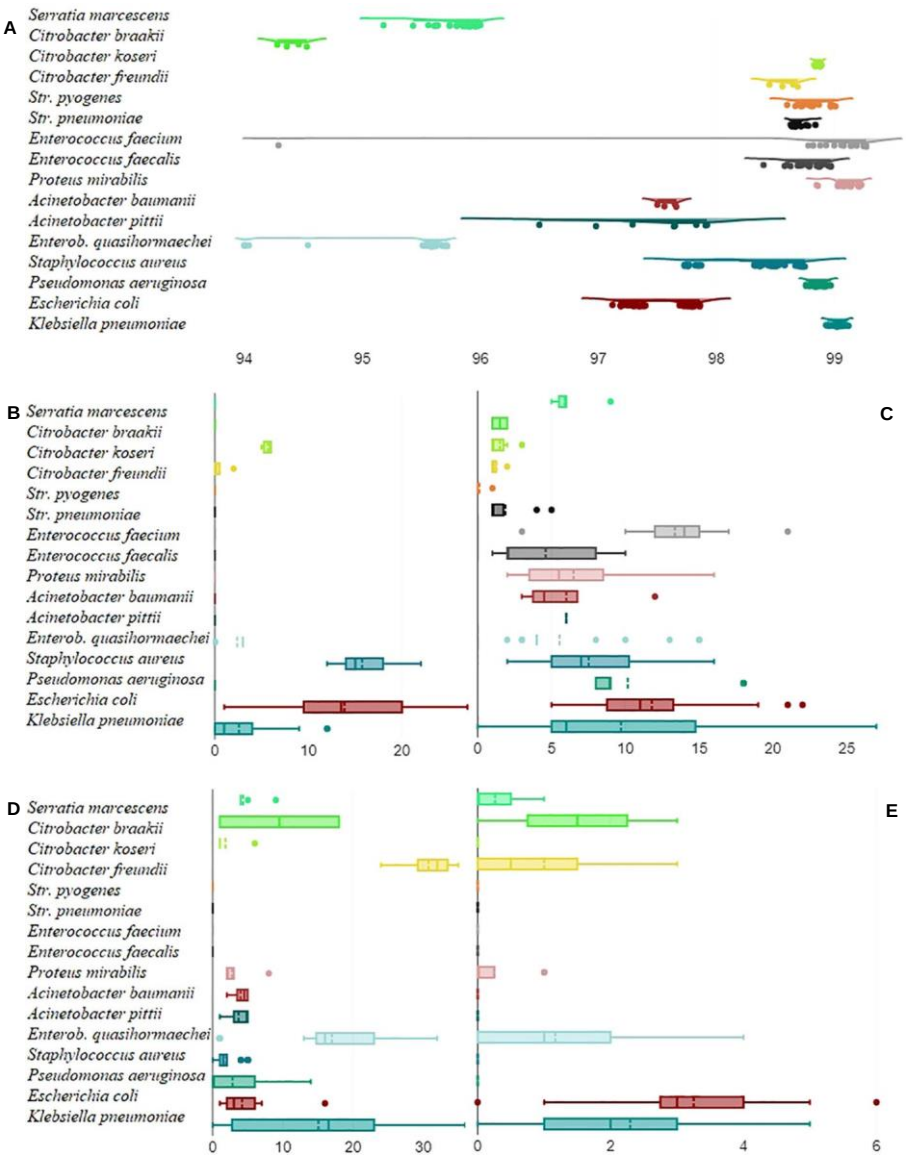

FIGURE 1
Mean average nucleotide identity by pathogen. For each isolate, the average nucleotide identity it had to all the other isolates within the same group in percent (*x*-axis) was calculated and displayed as a raincloud plot (A). Boxplots on the number (*x*-axis) of virulence genes (B), AMR genes (C), stress response genes (D), and plasmids (E) that pathogens carried.

encoded *pbp1a* and *pbp2b*. Two out of three isolates resistant to erythromycin encoded *erm(B)*. Three out of four isolates resistant to tetracycline encoded *tet(M)*. Isolates had an average nucleotide identity to each other of 98.71, ranging from 98.63 to 98.84. Isolates belonged to 10 different serotypes, the most frequent being 8, with 16F and 17F having two isolates each, and these isolates had 13 different MLST types.

Streptococcus pyogenes

In 12 out of 18 cases in which *S. pyogenes* grew, blood cultures were collected in the EC. Here, too, the available data were insufficient to perform statistical analyses pertaining to hypotheses involving 30-day and 90-day outcomes and survival rates. Both isolates resistant to tetracycline carried *tet(M)*. Isolates had an average nucleotide identity to each other of 98.82, ranging from 98.60 to 99.01. The two most represented MLST types were ST28 (5) and ST39 (4).

Bacteroides fragilis

The four *B. fragilis* isolates had an average nucleotide identity of 98.97 to each other that ranged from 98.91 to 99.03. One MLST type could not be determined, while the remaining were ST40, ST67, and ST129. The only isolate resistant to clindamycin was also the only one to encode *mef(A)*, as well as the only isolate to have an increased MIC to meropenem was the only one to encode *cfxA*.

Escherichia coli

The mean nucleotide identity of *E. coli* isolates ranged from 96.74 to 98.42 (see also Figure A2 in the Supplementary Material) with an average of 97.63, with MLST type ST69 being the most frequent (6). Four isolates belonged to ST12, ST73, and ST131 each, and two isolates to ST88 and ST95 each. Two isolates could not be assigned and the remaining isolates all belonged to different MLST types. The most represented O-antigen types were O4, O6, and O25, with four isolates each, and the most represented H-antigen type was H4 (9). The majority of isolates carried one or more plasmids with the most frequent being *IncFIB(APoo1918)* (32), *Col156* (17), *IncFll* (14), and *IncFlA* (13). The significant differences found were only that of a lower 30-day and 90-day survival in patients with isolates encoding *IncFll(pSE11)* and *p0111* ($p = 0.001$, Cramér's $V = 0.69$) but without c^2 test assumptions being met due to low cell frequencies.

Klebsiella pneumoniae

For three *K. pneumoniae* isolates, the MLST type could not be determined, and another three belonged to ST147. Six MLST types were represented twice (ST14, ST15, ST20, ST78, ST661, and ST3328), and another 22 just once. With Kleborate, the missing MLSTs were identified as ST307, ST223-1LV, and ST1013-1LV. Isolates had an average nucleotide identity of 99.03 to each other, ranging from 98.93 to 99.12 (see also Figure A3 in the Supplementary Material), and carried an average of 2.4 plasmids, ranging from two isolates carrying none to three carrying five. The number of encoded stress tolerance, AMR, and virulence genes varied from none to 36, from none to 27, and from none to 12, respectively, with only one isolate encoding neither of each. significant differences found were that of a lower 30-day and 90-day survival in patients with isolates encoding *iucA*, *iucB*, and *iucC* ($p = 0.039$, Cramér's $V = 0.35$) and *iroB* and *iroN* ($p = 0.039$, Cramér's $V = 0.35$), but all violating c^2 test assumptions due to low cell frequencies, and for *aac(3)-IId*, *aph(3′)-VI*, *gyrA_D87G*, and *phoQ_R16C* ($p = 0.046$, Cramér's $V = 0.34$) and *blaSHV*, *dfrA1*, *iucC*, and *ompK36_D135DGD* ($p = 0.039$, Cramér's $V = 0.35$), additionally without significant Fisher exact test. The four isolates encoding *blaOXA-48* and the three isolates encoding *blaNDM-1* were all correctly identified as such in routine diagnostics.

Enterobacter spp.

The sequenced *Enterobacter* isolates were all reported in routine diagnostics as *Enterobacter cloacae* complex isolates, but WGS revealed these to be *E. quasihormaechei* (3 1), *E. quasiroggenkampii* (4), *E. sichuanensis* (3), *E. cloacae* (2), *E. chengduensis* (2), and *E. wuhouensis* (1). Isolates either encoded no virulence genes or three (*iroB*, *iroC*, *iroN*), with all isolates encoding three being *E. quasihormaechei* isolates. The number of carried AMR and stress response genes varied significantly among the isolates, ranging from 1 to 15 for AMR genes and 1 to 35 for stress response genes. Isolates encoded between none and four plasmids, the most frequent being *IncFII(pECLA)* (12), *IncFIB (pECLA)* (10), and *Col440I* (8). The one *blaVIM-1*-positive isolate was phenotypically susceptible to imipenem and meropenem and was, hence, not investigated for carrying carbapenemases in routine diagnostics. MLST type could not be determined in 11 cases; in seven, ST50 was identified, and in three, ST118 was identified. Other MLST types were only represented once or twice. No significant differences were found regarding the 30-day and 90-day survival and the presence or absence of investigated genes.

Citrobacter spp.

Four *Citrobacter* isolates were *C. freundii*, another four were *C. braakii*, and seven were *C. koseri*. The average nucleotide identity of *C. koseri* (98.88) and *C. freundii* (98.59) isolates was higher than that of *C. braakii* (94.41) isolates, despite *C. freundii* isolates belonging to four different MLST types. Among all eight *C. freundii* and *C. braakii* isolates, there was only one isolate carrying virulence genes (*ybtP* and *ybtQ*), whereas all *C. koseri* isolates encoded the same virulence factors (*iucA*, *iucB*, *iucC*, *ybtP*, and *ybtQ*) with the exception of *senB*, which was only encoded by four. Isolates encoded between one and three AMR genes. The most significant variation was observed in the number of stress response genes encoded by the isolates. Two *C. braakii* isolates and six *C. koseri* isolates only encoded a single stress response gene, which was *fief*, while the remaining *C. koseri* isolates encoded six, the remaining *C. braakii* isolates encoded 17, and *C. freundii* isolates encoded between 24 and 35. Isolates carried from none to four plasmids with not a single plasmid carried by more than one isolate. The only two *Citrobacter* isolates encoding *qnrB38* were the only ones to have MICs to moxifloxacin above the lowest measurable level.

Proteus mirabilis

Proteus mirabilis isolates had an average nucleotide identity of 99.12 to each other, ranging from 98.83 to 99.25. The significant differences found were that of a lower 30-day survival when isolates encoded *aph(3′)-la* ($p = 0.028$; Cramér's V 0.63); however, c^2 test assumptions were violated due to low cell frequencies and the Fisher exact test was not significant ($p = 0.091$). All isolates carrying *blaTEM-1* were resistant to ampicillin and all but one isolate to ampicillin/sulbactam. All isolates resistant to trimethoprim/sulfamethoxazole carried *dfrA1* and in addition either *sul1* or *sul2*. Four isolates carried an *IncQ1* plasmid.

Serratia marcescens

More than half of the isolates were isolated from patients in the ICU, but isolates only had an average nucleotide identity to each other of 95.79, ranging from 95.18 to 96.00 (see also Figure A4 in the Supplementary Material). No significant differences were found in the 30-day and 90-day survival linked to the presence or absence of certain genes, age, or ward type. Four isolates carried a *pSM22* plasmid and another three isolates either *Col440I*, *IncX5*, or *IncX6*. The *IncX5* carrying isolate carried a *blaVIM-1* alongside it that was neither specifically investigated nor detected in routine diagnostics. That isolate was resistant to piperacillin–tazobactam, tested cephalosporins, and imipenem but had a MIC to meropenem of 0.19 as determined by the gradient strip test (Liofilchem, Roseto degli

Abruzzi, Italy). It was also the only one to be *sul1*-positive although it was just as susceptible to trimethoprim/sulfamethoxazole as all the other isolates. Isolates carried four or five out of 10 stress response genes and either five or six out of 13 AMR genes. The most frequent AMR genes were *ssmE*, *smdB*, *smdA*, *sdeY*, *sdeA* (27 each), *smfY*, *sdeB* (26 each), *aac(6′)* (25), *blaSRT* (22), and *tet(41)* (21).

Acinetobacter baumannii complex

Among the isolates of the *A. baumannii* complex, four were *A. baumannii* isolates and eight were *A. pittii*. Among the *A. baumannii* isolates, the MLST types represented were ST1, ST40, and ST213, with one isolate unassigned and with an average nucleotide identity to each other ranging from 97.54 to 97.69 and from 96.52 to 98.03 among the *A. pittii* isolates. No virulence genes were identified; however, between one and five stress tolerance genes were identified in all isolates and either five or six AMR genes in *A. pittii* and between 3 and 12 in *A. baumannii*. In both species, the most frequently encoded stress tolerance genes were *nreB* (12), *clpK* (6), *yfdX2* (5), *trxLHR* (5), and *hdeD-GI* (5), while the most frequent AMR genes were *amvA* (12) and *ant(3″)-IIa* (10). The available data were insufficient to perform statistical analyses pertaining to hypotheses involving 30-day and 90-day outcomes and survival rates.

Pseudomonas aeruginosa

The average nucleotide identity of *P. aeruginosa* isolates was 98.85 and ranged from 98.66 to 98.94. For 11 isolates, no MLST type could be determined, while three belonged to ST234, ST253, and ST823 each, two to ST308, ST316, and ST446 each, and all the remaining isolates to different MLST types. Isolates belonged mainly to serogroups O11 (13), O6 (9), O1 (5), and O10 (4). No virulence genes were detected, and stress response genes were only detected in less than half of the isolates in which they ranged from 2 to 14 in number. The most frequently encoded were *merE* (14), *merT* (12), *merR* (12), *merP* (12), *merD* (11), and *merA* (11). No isolate had fewer than 8 AMR genes with some carrying up to 18. By far, the most frequent were *mexE* (38), *mexA* (38), *fosA* (38), *aph(3′)-llb* (38), *mexX* (37), *catB7* (37), and *crpP* (29). Thirty-five isolates carried *blaOXA* genes and four *blaVIM* (three *blaVIM-2* and one *blaVIM-11*). Five isolates were classified as 4MRGN and three as 3MRGN. One *blaVIM-2* encoding isolate did not previously classify as 4MRGN as it had below-resistant MICs to piperacillin–tazobactam and cefepime and the *blaVIM-2* gene was neither investigated nor detected in routine diagnostics. It was resistant to imipenem, meropenem, ciprofloxacin, and ceftazidime. No significant differences were found in the 30-day and 90-day survival linked to the presence or absence of certain genes. Isolates exhibited a distinct clustering pattern, roughly partitioning into three distinct branches. Notably, one of these branches encompassed all but one isolate from patients with negative 30-day survival (see Figure A5 in the Supplementary Material) ($p = 0.038$).

Stenotrophomonas maltophilia

Stenotrophomonas maltophilia isolates only had an average nucleotide identity of 93.15 to each other, ranging from 92.09 to 94.17. One MLST type could not be determined, while all remaining were of different ones: ST4, ST23, ST224, and ST233. Only one did not have any stress response genes, the others had from three to nine, and all isolates carried either seven or eight antimicrobial resistance genes. Those shared among all isolates were *aph(6)*, *emrA*, *emrB*, *emrC*, and *smeF*.

Discussion

The present study aimed to conduct a comprehensive analysis of blood culture isolates' genomes, with a retrospective correlation between the detected virulence genes, resistance genes, and stress tolerance genes with the phenotypical susceptibilities and patient outcomes. The findings of this investigation yielded extensive and informative data on the distribution of genes implicated in bloodstream infections across various species. However, the interpretation of these results necessitates careful consideration due to several noteworthy factors. The intriguing observed associations between survival outcomes and the presence of specific resistance genes warrant caution, particularly given the limited knowledge regarding many of the identified virulence factors. The c^2 test assumptions were persistently violated due to the relatively small sample sizes in the groups of patients with negative 30-day and 90-day survival available for each species, making it imperative to validate and strengthen these associations by replicating the study with larger sample sizes for different species. One key consideration is the possibility that host factors may play a more significant role than pathogen factors in determining the outcomes of bloodstream infections and the inherent difficulty in fully accounting for such factors in studies of this nature (Hekker et al., 2000; Casadevall and Pirofski, 2001; Tseng et al., 2002; Beck et al., 2004; Tseng et al., 2005; Newman et al., 2017).

For example, research on *splE* in *S. aureus* still aims to uncover its precise functions and mechanisms. It seems that *splE* plays a significant role in *S. aureus* pathogenesis by promoting immune evasion through the degradation of immune components and facilitating tissue invasion by breaking down extracellular matrix proteins (Stach et al., 2018). Confirming its role in survival would further emphasize the importance of understanding *splE* for developing effective strategies against staphylococcal infections. The *IncFII(pSE11)* and *p0111* plasmids that were linked to lower survival in *E. coli* are known for disseminating AMR genes (Tasleem Jan and Tiwari, 2017; Wang et al., 2022), and their likely correlation with factors such as prolonged hospitalizations cannot be excluded to confound results, emphasizing that interpretation of the results warrants considered caution. The lower survival in *aph(3′)-la* encoding *P. mirabilis* isolate is probably the most questionable finding, because aside from resistance to aminoglycosides, it is not reported to contribute to virulence (Shaw et al., 1993), and aminoglycosides are infrequently used in our setting.

The study highlights important differences in genetic variation across different species and might be representative to some degree for these pathogens involved in BSI, while it is certainly important to exercise caution in generalizing these findings or applying them to other settings. The identity and coverage values for *E. faecium* ST117 and *E. coli* ST131 were both 100% for their exact alleles. Notably, ECO 131 has been highlighted in several studies as a rapidly expanding multidrug-resistant pathogen with high receptivity and the potential to

develop resistance to last-resort antibiotics such as carbapenems and colistin (Pitout and Finn, 2020; Li et al., 2021; Taati Moghadam et al., 2021; Brumwell et al., 2023). Serotypes H4 and O25 were found to match all isolates in our investigation, consistent with previous studies (Brumwell et al., 2023), but none of the isolates exhibited the antimicrobial MDR profile of 3MRGN or 4MRGN. *Enterococcus faecium* ST117 was previously identified as a rising factor in vancomycin-resistant enterococci (VRE) (Weber et al., 2020), and five out of six isolates in our study were found to carry *vanB* genes. A comparison with this recent comprehensive study involving 120 isolates revealed that all the genes mentioned in the study (*msrC*, *efmA*, *erm(B)*, *dfrG*, *aac(6′)-Ii*, *gyrA*, *parC*, and *pbp5*) matched those present in our isolates, except for *efmA*, which was absent in the NCBI database used. The notable clustering of ST50 *E. quasihormaechei* isolates calls for focused investigations, given the escalating local and global issue of sewage-related clonal colonization in hospital sanitary facilities (Babouee Flury et al., 2016; Kehl et al., 2022; Stokes et al., 2022). Similarly, the recurring identification of ST147 *K. pneumoniae* and ST235 and ST823 *P. aeruginosa* isolates, even in wards geographically distant from those assumed to have a contamination source in our setting (Kehl et al., 2022; Neidhöfer et al., 2023a), urges us to implement transmission dynamics monitoring networks (Bohl et al., 2022; Ko et al., 2022; Neidhöfer et al., 2023b).

Conclusion

Conducting replication studies with larger sample sizes for each species is imperative to cautiously interpret associations between survival outcomes and the presence of specific genes, emphasizing the need for further research in this area. A deeper understanding of virulence and host factors will not only aid in the interpretation of results but also pave the way for the development of more targeted therapeutic and preventive measures, thus enhancing patient outcomes. In addition, implementing more efficient transmission dynamics monitoring holds significant promise as a valuable tool in preventing bloodstream infections caused by certain pathogens, highlighting the importance of establishing robust surveillance systems.

Data availability statement

All data and original contributions presented in the study are included in the article/Supplementary Material, except for genomes that cannot be readily made available because of binding collaboration agreements. These can, however, be made available upon request directed to the corresponding author.

Ethics statement

The studies involving humans were approved by the Ethics Committee of the University Hospital Bonn. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent for participation was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and institutional requirements.

Author contributions

CN: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. MN: Formal Analysis, Investigation, Methodology, Software, Visualization, Writing – original draft, Writing – review & editing, Conceptualization, Data curation. RJ: Data curation, Formal Analysis, Methodology, Project administration, Writing – review & editing. BA: Data curation, Writing – review & editing. SU: Data curation, Methodology, Software, Writing – original draft, Writing – review & editing. UN: Data curation, Methodology, Software, Writing – original draft, Writing – review & editing. SG: Data curation, Methodology, Software, Writing – original draft, Writing – review & editing. MP: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

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Conflict of interest

SG is coinventor of one pending international patent on diagnostic algorithms and chief scientific officer and cofounder of Noscendo GmbH in Duisburg, Germany. SU and UN are employed by Noscendo GmbH.

The remaining 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2023.1274573/full#supplementary-material

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3.5 Condic M*, **Neidhöfer C***, Ralser DJ, Wetzig N, Thiele R, Sieber M, Otten LA, Warwas LK, Hoerauf A, Mustea A, Parčina M. Analysis of the cervical microbiome in women from the German national cervical cancer screening program. J Cancer Res Clin Oncol. 2023 Aug;149(9):6489-6500.

Zielsetzung der Arbeit – Gebärmutterhalskrebs (CC) wird durch eine persistierende Hochrisiko-Infektion mit dem humanen Papillomavirus (hrHPV) verursacht. Das zerviko-vaginale Mikrobiom kann die Entwicklung von (Prä-) Krebsläsionen beeinflussen. Ziel der Studie war es, (i) das neue CC-Screening-Programm in Deutschland hinsichtlich der Erkennung von hochgradigen CC-Vorläuferläsionen zu evaluieren und (ii) die Rolle des zerviko-vaginalen Mikrobioms und seinen potenziellen Einfluss auf die zervikale Dysplasie zu erforschen.

Methoden und Ergebnisse – Das Mikrobiom von 310 Patientinnen, die zur Kolposkopie überwiesen wurden, wurde mittels Amplikonsequenzierung bestimmt und mit klinisch-pathologischen Parametern korreliert. Die meisten Patientinnen wurden zur Kolposkopie überwiesen, weil sie in zwei aufeinanderfolgenden Jahren einen positiven hrHPV-Befund in Kombination mit einem normalen PAP-Abstrich hatten. In 2,1 % dieser Fälle wurde eine CIN-III-Läsion festgestellt. Es bestand ein signifikanter positiver Zusammenhang zwischen dem PAP-Stadium und der Besiedlung mit *Lactobacillus vaginalis* sowie zwischen dem Schweregrad der CC-Vorläuferläsionen und *Ureaplasma parvum*.

Schlussfolgerungen – In unserer Kohorte führte das neue Gebärmutterhalskrebs-Screeningprogramm zu einer geringen Rate an zusätzlich entdeckten CIN III. Es ist fraglich, ob diese Fälle nur durch zusätzliche HPV-Tests vor dem Auftreten zytologischer Anomalien früher erkannt wurden, oder ob das neue Screening-Programm die Entdeckungsrate von CIN III langfristig wirklich erhöht. Die Besiedlung mit *U. parvum* war mit histologischen dysplastischen Läsionen verbunden. Ob eine gezielte Therapie dieses Erregers oder eine Optimierung des Mikrobioms Dysplasien verhindert, bleibt spekulativ.

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RESEARCH

Analysis of the cervical microbiome in women from the German national cervical cancer screening program

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Abstract

Purpose Cervical cancer (CC) is caused by a persistent high-risk human papillomavirus (hrHPV) infection. The cervico-vaginal microbiome may influence the development of (pre)cancer lesions. Aim of the study was (i) to evaluate the new CC screening program in Germany for the detection of high-grade CC precursor lesions, and (ii) to elucidate the role of the cervico-vaginal microbiome and its potential impact on cervical dysplasia.

Methods The microbiome of 310 patients referred to colposcopy was determined by amplicon sequencing and correlated with clinicopathological parameters.

Results Most patients were referred for colposcopy due to a positive hrHPV result in two consecutive years combined with a normal PAP smear. In 2.1% of these cases, a CIN III lesion was detected. There was a significant positive association between the PAP stage and *Lactobacillus vaginalis* colonization and between the severity of CC precursor lesions and *Ureaplasma parvum*.

Conclusion In our cohort, the new cervical cancer screening program resulted in a low rate of additional CIN III detected. It is questionable whether these cases were only identified earlier with additional HPV testing before the appearance of cytological abnormalities, or the new screening program will truly increase the detection rate of CIN III in the long run. Colonization with *U. parvum* was associated with histological dysplastic lesions. Whether targeted therapy of this pathogen or optimization of the microbiome prevents dysplasia remains speculative.

Keywords Cervicovaginal microbiome · Cervical cancer screening · HPV diagnostic · Colposcopy

Introduction

In recent years, the human microbiome has increasingly become the focus of scientific interest. The colonization of our body with microbiota is at least as diverse and complex as our somatic cell physiology (Sender et al. 2016). It is estimated that about 500–1000 different microorganisms simultaneously colonize our body (Turnbaugh et al. 2007). Alterations in the human microbiome, as well as interactions with the immune, endocrine, and nervous systems, have been linked to a variety of health changes and diseases, including cancer and their precursor lesions (Kostic et al. 2013; Helmink et al. 2019). The precise manner in which the microbiome influences the maintenance of health or the development of disease, however, is still far from being answered.

CC is predominantly caused by infection with human papillomavirus (HPV), in ≥ 99% with the high-risk (hr) HPV types 16 and 18 (Walboomers et al. 1999). About 90% of women are exposed to HPV infections during the course of their lives, and in only 10% of the cases the infection persists with a high risk of developing precancerous cervical intraepithelial lesions and CC (Shulzhenko et al. 2014). HPV persistence is co-induced by impaired immune reactions, and adverse accompanying effects exerted by the

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cervico-vaginal microbiome (Garrett 2015). There is growing scientific evidence for a relationship between a cervico-vaginal microbiome dominated by species other than lactobacilli, and a higher risk of HPV infection, HPV persistence and the development of CC and its precursor lesions (Mitra et al. 2015; Laniewski et al. 2020; Lin et al. 2020; Norenhag et al. 2020).

The development and improvement of molecular methods, in particular represented by bacterial 16S ribosomal RNA gene sequencing, has led to a deeper understanding of the cervico-vaginal microbiome (van de Wijgert et al. 2014). According to the presence of distinct bacterial species that are identified by 16S RNA sequencing, the cervico-vaginal microbiome is sometimes classified into five groups, designated as community state types (CST). In detail, CST I–III and CST V are characterized by an abundance of *Lactobacillus crispatus*, *L. gasseri*, *L. iners*, and *L. jensenii*, respectively, whereas, in contrast, CST IV shows a combination of diverse facultative anaerobes with low abundances of lactobacilli (Ravel et al. 2011). In reproductive-aged women, shifts from the *Lactobacillus*-dominated microenvironment are commonly observed during menses and sexual activity caused by a reduction of lactobacilli (Gajer et al. 2012). With increasing age and the decrease of estrogen and glycogen levels, *Lactobacillus* species are replaced by diverse anaerobes (Gliniewicz et al. 2019). This transformation of the cervico-vaginal site flora is associated with the genitourinary syndrome of menopause (Hummelen et al. 2011). Dysbiosis of the lower female reproductive tract increases the risk for infections with STD (Martin et al. 1999). Further, the absence of Lactobacilli is associated with the increase risk of HIV and HSV transmission (Cherpes et al. 2003). Recent studies confirm that changes of the human microbiome can impair the symbiotic relationship between microorganisms and host, leading to the development of different cancer types and suggesting a role for microbiota in genesis of various malignancies (Bhatt et al. 2017; Lin et al. 2020; Norenhag et al. 2020).

For early detection of CC, an annual cytological examination program (PAP smear) has been introduced in Germany in 1971. Since then, incidence rates of CC dropped remarkably by 75% in the first decades but, however, incidence rates have stagnated in recent years. The PAP smear has a low sensitivity (60–80%), a false negative rate of 30% and false-positive rates ranging from 15 to 50% (Yim and Park 2007). Hence, in some European countries, a switch to primary HPV-DNA testing was established recently. HPV testing is a highly sensitive approach and the absence of hrHPV infection indicates a low risk for CC precursor lesions and CC development (Dillner et al. 2008). As part of the German National Cancer Plan, the Federal Joint Committee (G-BA) implemented an updated organized cervical cancer screening program starting in January 2020. Annual cytology screening remained unchanged for women between 20 and 34 years. For women of 35 years and older, a co-testing, comprising a Pap smear and an HPV test was introduced. In case of positive findings, women are referred for colposcopy (Bujan Rivera and Klug 2018). The aim of the present study was to evaluate the new screening program for the detection of high-grade precursor lesions and to investigate whether microbiome analyses could have a potential role in this screening.

Methods

Study design and population

The study cohort included women who were referred for colposcopy to the certified Colposcopy Centre at the Department of Gynecology and Gynecological Oncology of the University Hospital Bonn from November 2021 until February 2022. Colposcopy was indicated according to the guidelines of the new national cancer screening program (abnormal PAP smear finding and/or a positive result for hrHPV).

Routine colposcopy was performed including the application of acetic acid. In cases of TZ type 1 or TZ type 2 (Quaas et al. 2013), a targeted biopsy was performed from the most conspicuous lesion. In case of a TZ type 3 with no visible lesion on the ecto-cervix, an endo-cervical curettage was performed.

Clinical data regarding nicotine abuse, menopause status, HPV vaccination, the application of local suppositories, the intake of hormonal contraceptives or hormone replacement therapy, the presence of an intrauterine device (IUD-copper or hormonal) and the last sexual intercourse were obtained from patient questionnaires and the clinical database.

Histopathological analysis and HPV diagnostics

The taken biopsies were histopathologically classified into benign, low squamous intraepithelial lesions (LSIL/CIN I), and high squamous intraepithelial lesions (HSIL) according to the 2014 WHO classification. HSIL lesions were further sub-classified into CIN II and CIN III lesions according to Richart (Richart 1973).

In women above 35 years, HPV status was available as a part of the new cancer screening program. Due to the use of different HPV molecular detection assays and, therefore, inconsistent data for specific HPV types, analyses with respect to HPV were limited to low risk (lr) and hrHPV. In the presence of hrHPV, it was differentiated whether hrHPV types 16 and/or 18 were present.

HPV diagnostics were repeated from all samples with the Anyplex II HPV28 Detection (Seoul, South Korea) that detects 19 hrHPV: 16, 18, 26, 31, 33, 35, 39, 45,51, 52, 53,

56, 58, 59, 66, 68, 69, 73, 82; and nine low carcinogenic risk HPV types: 6, 11, 40, 42, 43, 44, 54, 61, 70. It was performed strictly following the manufacturer's instructions. DNA was extracted on a Seegene NIMBUS (Seoul, South Korea) and analyzed on a CFX96 real-time PCR instrument (Bio-Rad Laboratories, Inc., Hercules, California, USA). Data recording and analysis were automated using the Seegene Viewer software.

Women, in which no biopsy was taken for histological analysis and women with pathologies of the vulva were excluded from the study.

Sample collection and preparation for sequencing

During the colposcopic examination, before the application of acetic acid, a flocked swab (eNAT® system, Copan Italia, Brescia, Italy) was taken by three experienced gynecologists from the cervical canal. The swabs were stored at 4 °C and subsequently processed within 2–9 days.

Highly purified DNA was extracted from all samples using the column-based ZymoBIOMICS DNA Miniprep Kit (Zymo Research Europe GmbH, Freiburg, Germany). The isolation was performed strictly according to the manufacturer's instructions. The crucial mechanical lysis step of the samples was performed by Precellys® Evolution homogenizer from Bertin Technologies SAS (Bretonneux, France). At the end of the extraction process, the DNA was eluted to 100 uL volume and qualitatively and quantitatively evaluated using the NanoDrop OneC, Thermo Fisher Scientific Inc. (Waltham, MA, USA).

16S rRNA gene sequencing libraries were constructed from each sample using the Quick-16S NGS Library Prep Kit (Zymo Research Europe GmbH, Freiburg, Germany) with its included V1–V2 primer pairs. Each run included 94 samples, the positive control included in the kit, and a negative control. For quantitative PCR, quality control, and normalization purposes, the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA) was utilized.

After pooling, the DNA was quantified with the QuantiFluor® dsDNA System on the Quantus™ Fluorometer (both: Promega GmbH, Walldorf, Germany) and diluted strictly according to the Illumina-protocol for MiSeq sample preparation. For the final library, a loading concentration of 10 pm was chosen and a 10% Illumina v3 PhiX spike-in control was added before running it on the Illumina MiSeq platform with a 500cycle v2 Illumina MiSeq Reagent Kit (all three: Illumina, San Diego, CA, USA).

Bioinformatic analysis

The bioinformatic analysis included three main parts, starting with the preprocessing of raw paired end reads. Following the preprocessing, the sequences were assigned to taxonomies. Finally, a statistical and graphical evaluation was performed on the resulting taxa.

QIIME2 (Bolyen et al. 2019) was used for both preprocessing and classification of the data. With the plugin tool DADA2 (Callahan et al. 2016), forward and reverse reads were trimmed from the 3' end at position 249, while shorter reads as well as low-quality reads got discarded. DADA2 was also used to perform error correction, merging of forward and reverse reads if there was an overlap of at least 12 base pairs, and chimera removal.

The processed sequences were clustered into OTUs (operational taxonomic units) of 100% sequence identity and assigned to taxa, using a classifier trained on full-length sequences of SILVA (Quast et al. 2013). The trained classifier was provided by QIIME2 using scikit-learn 0.24.1 and the plugin tool q2-feature-classifier (Bokulich et al. 2018; Robeson et al. 2021).

Statistical analysis

Statistical analysis was performed using Stata version 14 for the clinical data and Datatab version 1.12.1 for taxa frequency comparisons and correlation with clinical parameters. P values less than 0.05 were considered statistically significant.

Ethics statement

The study was approved by the Ethics Committee of the Medical Faculty of the University of Bonn (vote: 128/21). All methods were carried out in accordance with relevant guidelines and regulations. Informed consent was obtained from all subjects.

Results

Participant characteristics and clinical results

The study cohort included 310 women. All relevant clinicopathological parameters are summarized in Table 1. The mean age of the study cohort was 44.6 years (± standard deviation (SD) 12.4 years). 72.9% of the women were premenopausal, and 27.1% were postmenopausal. 30.3% of the patients were active smokers. Within the whole cohort, 12.3% of the patients had been vaccinated against HPV. Among the subgroup of women ≤ 30 years, 62.2% had been vaccinated against HPV. In the subgroup of premenopausal women, 29.2% used hormonal contraceptives, and 13.3% had an IUD. Of these, 26.7% had a copper, 63.3% a Mirena ® IUD, 6.7% a Jaydess ®, and 3.3% a Kyleena ® IUD. In the subgroup of postmenopausal women, 8.3% received

Table 1 Clinicopathological characteristics of the entire cohort

Clinicopathological parameter	
Age (years)	
Mean (± SD)	44.6 ± 12.4
Min–max	20–82
Menopausal status	
Pre	226 (72.9%)
Post	84 (27.1%)
Smoker	
No	216 (69.7%)
Yes	94 (30.3%)
HPV vaccination	
No	272 (87.7%)
Yes	38 (12.3%)
HPV vaccination < 30 years	
No	14 (37.8%)
Yes	23 (62.2%)
Hormonal contraceptives (premenopausal)	
No	160 (70.8%)
Yes	66 (29.2%)
Intrauterine device (IUD) (premenopausal)	
No	196 (86.7%)
Yes	30 (13.3%)
IUD	
Cooper	8 (26.7%)
Hormonal	22 (73.3%)
Hormonal replacement therapy (postmenopausal)	
No	77 (91.7%)
Yes	7 (8.3%)
Vaginal suppositories (postmenopausal)	
No	66 (78.6%)
Yes	18 (21.4%)
HPV high-risk status	
Negative	31 (10.0%)
Positive	262 (84.5%)
Unknown	17 (5.5%)
HPV 16/18	
Negative	183 (69.8%)
Positive	79 (30.2%)
Clinicopathological parameter	
Pap smear cytology	
I/IIa	162 (52.3%)
IIp	40 (12.9%)
IIg	6 (1.9%)
IIIp	11 (3.5%)
IIIg	4 (1.3%)
IIID1	46 (14.8%)
IIID2	22 (7.1%)
IVa-p	18 (5.8%)
IVa-g	1 (0.3%)
Histological diagnosis	
No CIN	201 (64.8%)
LSIL CIN I	51 (16.5%)
HSIL CIN II	36 (116%)
HSIL CIN III	22 (7.1%)
Histological diagnosis, Pap I/IIa, HP' high-risk pos	
No CIN	112 (77.8%)
LSIL CIN I	20 (139%)
HSIL CIN II	9 (6.3%)
HSIL CIN III	3 (2.1%)
Surgical therapy	
No	263 (84.8%)
Yes	47 (15.2%)
Type of surgical therapy	
LEEP conization	38 (80.9%)
Hysteroscopy/curettage	3 (6.4%)
Hysterectomy	2 (4.3%)
Laser vaporization	4 (8.5%)
Therapy of CIN III	
LEEP conization	19 (86.4%)
Hysterectomy	1 (4.5%)
No surgical therapy	2 (9.1%)
Therapy of CIN II	
LEEP conization	19 (52.8%)
Hysterectomy	1 (2.8%)
No surgical therapy	16 (44.4%)

hormone replacement therapy. The proportion of postmenopausal women who used vaginal suppositories (estriol) was 21.4%. Our analysis included five pregnant women.

84.5% of the whole study cohort were positive for a hrHPV type. The most prevalent subtypes were HPV types 16 und 18 in 30.2% of the cases. In 5.5% of the study cohort, HPV status was not available and 10.0% were negative for hrHPV types.

Most interestingly, 52.3% of the study cohort had a regular Pap smear (I or IIa, according to the Munich III classification). They were referred to a colposcopy due to a positive status for hrHPV in two consecutive years. This approach corresponds to the new guidelines. Among the subgroup with a positive hrHPV status and normal cytology, 8.4% of the women had an HSIL. A CIN III was detected in only 2.1% (3/144).

In the entire study cohort, histological examination revealed in 16.5% of the cases a CIN I, in 11.6% a CIN II, and in 7.1% a CIN III. 15.2% of all patients received surgical therapy due to precursor lesions of the cervix, with 80.9% receiving a LEEP conization, 6.4% a hysteroscopy with curettage, 4.3% a hysterectomy, and 8.5% laser vaporization. Among the 22 patients diagnosed with a CIN III lesion, 19 received conization and one a hysterectomy. Two patients

with CIN III did not receive surgical treatment: one woman was pregnant at the time of diagnosis, and one was 20 years old. In this case, a close surveillance every three months was scheduled, which is in line with guidelines. Among the CIN II subgroup, 52.8% received a LEEP conization and one woman a hysterectomy. In 44.4% of the cases, a follow-up was scheduled in 6 months according to the guidelines. In cases with LSIL/CIN I, no surgical therapy was performed, and a follow-up colposcopy in 6 months was scheduled.

HPV Diagnostic

HPV diagnostic was repeated in all patients. In 252 cases, HPV status determined within the CC screening program was in concordance with our analysis. In 18 cases, the comparison was not possible as these patients did not receive prior HPV testing or our analysis was invalid. In 40 cases, HPV HR diagnostic showed a discrepancy in the results: In 20 cases, that were initially tested negative for HPV HR within the CC screening program, a positive hrHPV status was determined in our analysis. Among these cases, 11 were positive for HPV 16. The medical history showed that 9 patients had cervical dysplasia before. In the actual biopsy, none of the patients had an HSIL. In 20 cases that were initially hrHPV-positive, no hrHPV infection was detected in our analysis. Five of these patients were initially positive for HPV 16/18. As observed for the counterpart subgroup, none of the patients had an HSIL.

In 17 cases, HPV diagnostic was not performed previously, as patients were younger than 35 years. These patients were referred for colposcopy due to an abnormal PAP smear. We found a positive HPV HR status in 16 patients; histology showed in 5 cases an LSIL and in 7 cases an HSIL.

Cervical microbiome profiles

The 310 sequenced cervical samples generated a total of 31,881,480 reads with a mean read count of 102,843 per sample. Of these samples, 293 passed the minimum quality filter (> 1500 reads and > 1000 merged reads).

Cervical microbiota profiles were classified into 5 groups based upon the dominant (or at least > 30% relative abundance) taxa observed within each sample at the genus level (see Fig. 1). Accordingly, 194 microbiomes were predominated by *Lactobacillus* (66.21%), 52 by *Gardnerella* (17.75%), 9 by *Bifidobacterium*, 6 by *Streptococcus*, 2 by *Pseudomonas*, 1 by *Prevotella* and in 29 cases (9.9%) none of these genera predominated.

Nine bacterial species had an average prevalence of > 0,5%, namely *Lactobacillus crispatus* (mean prevalence 33,21%), *Lactobacillus iners* (23,12%), *Gardnerella vaginalis* (5,98%), *Lactobacillus jensenii* (2,84%), *Bifidobacterium breve* (1,47%), *Streptococcus agalactiae* (1,13%), *Bifidobacterium longum* (1,01%), *Streptococcus anginosus* (0.72%), and *Prevotella bivia* (0.58%) (Fig. 1B).

The relative abundance of *L. crispatus* was negatively correlated with *L. iners* ($r = -0.41$, $p < 0.001$), *G. vaginalis* ($r = -0.36$, $p < 0.001$), *L. jensenii* ($r = -0.12$, $p = 0.043$), and *P. bivia* ($r = -0.13$, $p = 0.028$). The relative abundance of *L. iners* was negatively correlated with *L. crispatus and G. vaginalis* ($r = -0.21$, $p < 0.001$). No other significant correlations between these species were observed.

Correlations between clinical and demographic variables and the microbiome

The patient's age was correlated negatively with *L. crispatus* ($r = -0.3$, $p < 0.001$) and positively with *G. vaginalis* ($r = 0.15$, $p = 0.011$) and *B. longum* ($r = 0.14$, $p < 0.016$). However, performing multiple linear regression analysis to examine the influence of the menopausal state, revealed that only the *B. longum* was associated with age ($p < = 0.027$). As depicted in Fig. 2, being postmenopausal correlated significantly negatively with *L. crispatus* (rpb =− 0.32, $n = 293$, $p = < 0.001$) and positively with the genus *Pseudomonas* (rpb = 0.2, $p = 0.001$), *Prevotella* (rpb = 0.15, $p = 0.011$), *Cutibacterium* (rpb = 0.14, $p = 0.015$), *Atobium* (rpb = 0.13, $p = 0.027$), *Staphylococcus* (rpb = 0.14, $p = 0.014$), *Dialister* (rpb = 0.16, $p = 0.008$), *Acinetobacter* (rpb = 0.14, $p = 0.017$), *Oscillospirales* (rpb = 0.19, $p = 0.001$), and *Fusobacterium* (rpb = 0.15, $p = 0.008$) ($n = 293$ for all). The cervical microbiomes of postmenopausal patients displayed higher richness (t(124.46) = − 2.71, $p = 0.008$, 95% confidence interval [− 40.29, − 6.23]) and higher fisher-alpha diversity (t(116.25) = − 3.13, $p = 0.002$, 95% confidence interval [− 8.11, − 1.82]).

Premenopausal women with an IUD displayed lower richness in their cervical microbiome (t(45.87) = 2.27, $p = 0.028$, 95% confidence interval [2.19, 36.46]) than those without. However, one-factor analysis of variance showed that there was no significant difference between not having an IUD, having a hormonal IUD, or a copper IUD and the variable richness $F = 1.84$, $p = 0.161$ (Fig. A1 in the Appendix displays differences among IUDs that were not statistically significant). Among premenopausal women, intake of oral contraceptives was linked to a higher prevalence of *L. crispatus* (t(209) = − 3.42, $p = 0.001$, 95% confidence interval [− 35.48, − 9.43]) and a lower prevalence of *L. iners* t(182.96) = 4.45, $p = < 0.001$, 95% confidence interval [10.58, 27.58]. Among postmenopausal women taking hormone replacement therapy, no such differences were observed (Fig. A2 in the Appendix displays these differences that were not statistically significant). Smoking was positively correlated with the genus *Veillonella* (rpb = 0.16, $n = 293$, $p = 0.008$).

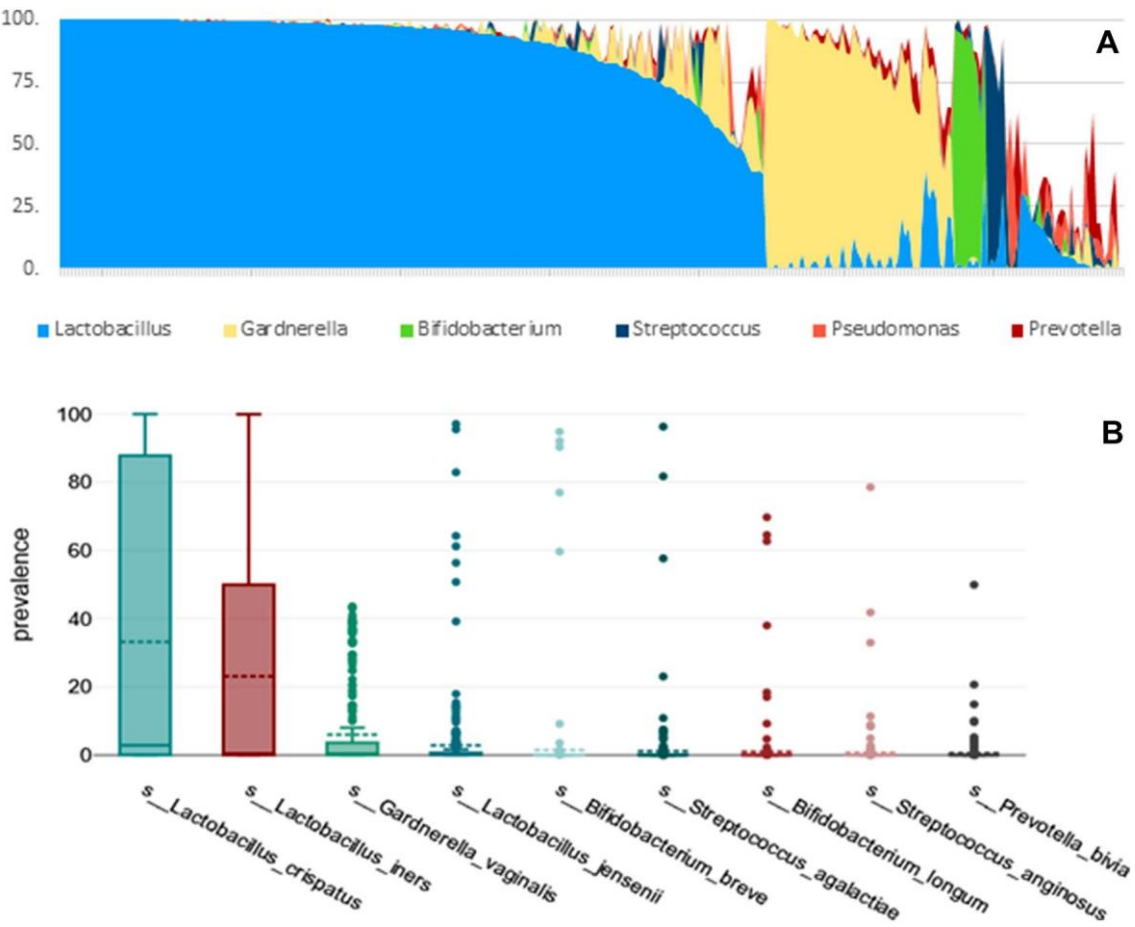

Fig. 1 Genus-level cervical microbiota profiles **A**. Prevalence of species with an average prevalence > 0.5% **B**

Correlations between cytology/histology and the microbiome

The result of the Pearson correlation showed that there was a significant low positive association between Pap stage and the order *Lactobacillales* (r(291) = 0.15, $p = 0.008$), the genus *Lactobacillus* (r(291) = 0.15, $p = 0.008$), the genus *Bacillus* (r(291) = 0.21, $p = < 0.001$) and with *Lactobacillus vaginalis* (r(291) = 0.22, $p = < 0.001$). When excluding postmenopausal patients from the analysis, only the positive associations with the genus *Bacillus* (r(209) = 0.24, $p = < 0.001$) and with *Lactobacillus vaginalis* (r(209) = 0.24, $p = < 0.001$) remained significant.

Further, there was a significant low positive association between histological stage and the genus *Lactobacillus* (r(291) = 0.12, $p = 0.038$), the genus *Bacillus* (r(291) = 0.17, $p = 0.004$),and *L. vaginalis* (r(291) = 0.16, $p = 0.006$). In addition, there was a low, positive correlation between histological stage and *Sneathia sanguinegens* (r(291) = 0.14, $p = 0.02$), the order *Mycoplasmatales* (r(291) = 0.19, $p = 0.001$), the genus *Ureaplasma* (r(291) = 0.18, $p = 0.002$), and *Ureaplasma parvum* (r(291) = 0.17, $p = 0.004$). Multiple linear regression analysis to examine the influence of age, menopausal state, smoking, and IUD in addition to Pap stage and histology, revealed that Pap stage remained associated with the genus *Bacillus* ($p = 0.033$), and *L. vaginalis* ($p = 0.021$). Histological stage remained associated with the order *Mycoplasmatales* ($p = 0.015$), the genus *Ureaplasma* ($p = 0.016$), and *U. parvum* ($p = 0.15$) (see Fig. 3).

Discussion

This study was conducted to evaluate the newly implemented German national CC screening program combined with an analysis of the cervical microbiome in patients referred to colposcopy according to the new guidelines.

There is broad scientific evidence from randomized controlled trials and meta-analyses that screening for HPV is more sensitive in the detection of cervical intraepithelial neoplasia grade III (CIN III) and CC than conventional cervical cytology (Naucler et al. 2007; Anttila et al. 2010;

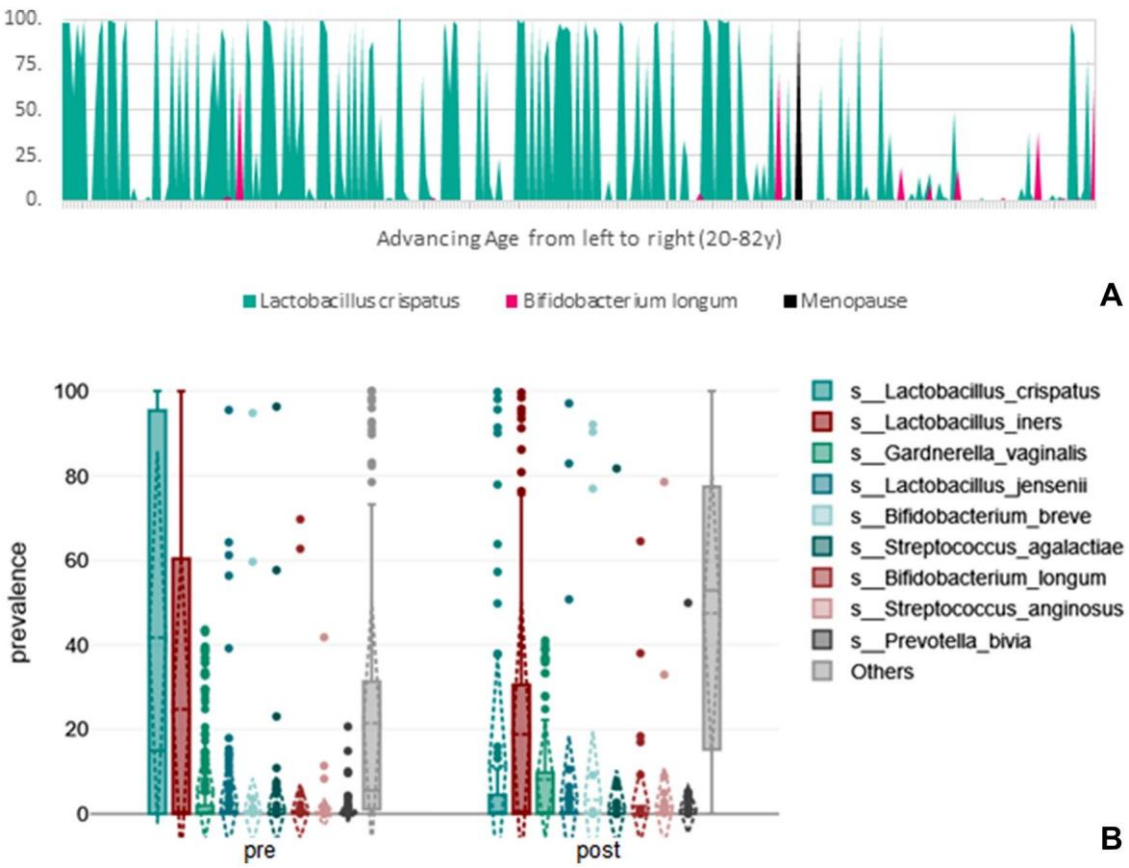

Fig. 2 Visualization of the influence of menopause and age on *L. crispatus* and *B. longum*, respectively (A), and the displacement of *L. crispatus* by other species **B**

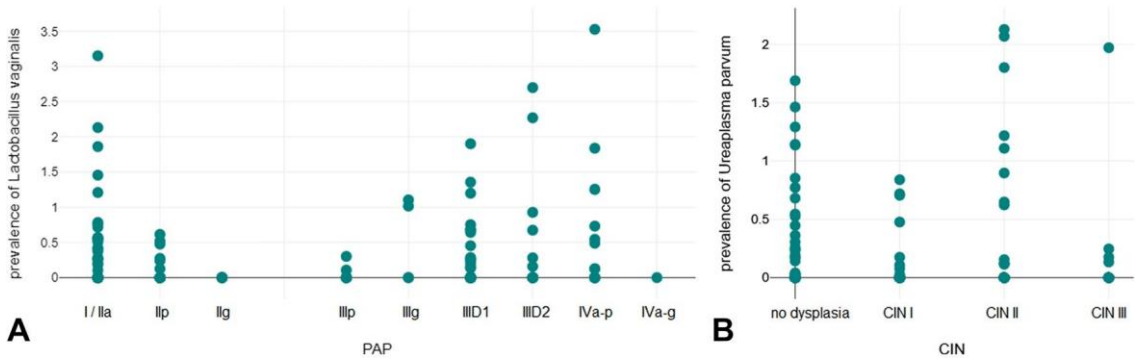

Fig. 3 Scatter diagram on the prevalence of *L. vaginalis* by cytological stage (**A**) and *U. parvum* by histological stage (**B**)

Ronco et al. 2010; Rijkaart et al. 2012). Integration of HPV testing into CC screening programs led to a decrease in CC incidence (Kjaer et al. 2010). This is, in particular, attributable to higher detection rates of cervical adenocarcinoma and its precursor lesions, as this subgroup is often underdiagnosed by cytological methods (Castle et al. 2010; Katki et al. 2011). Trials showed that women that are negative for hrHPV display a very low risk for the development of CIN III CC precursor lesions or CC (Dillner et al. 2008; Mesher et al. 2010). Based on these data, the new national CC screening program was set up in Germany with the implementation of a Pap smear/HPV co-testing for women aged 35 years and older.

Our study showed that 52.3% of the patients were referred for colposcopy due to an hrHPV-positive result in two consecutive years and normal cytology in both years. Among this subgroup, histological examination revealed only 3 cases of CIN III (2.1%) and 9 cases of CIN II (6.3%). Data from other studies reported a CIN III incidence range of 3–7% in women with normal cytology and a positive high-risk HPV test (Petry et al. 2003; Thrall et al. 2010). Another study from Germany that evaluated co-testing in women older than 30 years showed CIN III lesions in 9.2% of hrHPV-positive/normal-cytologic cases (Luyten et al. 2014). The implementation of colposcopy for Pap-normal/hrHPV-positive women in two consecutive years had the goal of diagnosing approximately 10% CIN III detected lesions. One finding is that the new screening program leads to an increased need for colposcopies and histological examinations performed. In our study, the supplementary HPV testing identified 3 cases of CIN III that would not have received histological assessment in the old screening program. Whether the number of CIN III remains the same in the long term, and additional HPV testing only detects them earlier before cytological abnormalities are detectable, must be clarified in further studies.

In 17 cases, the HPV status was not available as women were younger than 35 years and referred for colposcopy due to an abnormal Pap smear. Histological examination revealed in 5 cases a LSIL and 7 cases an HSIL lesion. HPV analyses showed a positive result for hrHPV in 16/17 cases. As all precursor lesions among these patients were detected by Pap smear, there was no additional benefit of HPV testing. However, this must be interpreted with caution as women < 35 with unremarkable PAP smears but positive hrHPV status were not included in the study and are not referred for a colposcopy within the current screening algorithm.

In our study, we repeated the HPV diagnostic with a test based on the Anyplex II HPV28 Detection for all enrolled patients. In 40 cases, there were deviating results from the initial testing for hrHPV. In 20 cases, that initially tested positive, the new negative result can be explained by spontaneous regression, as the time difference between the two analyses was 3–5 months. In 20 initial hrHPV negative cases, we found a positive result for hrHPV, with even 11 cases being positive for HPV 16. Divergence of these results can be explained by different sensitivity of HPV tests, a new infection with hrHPV, or a reactivation of a hrHPV infection in the meantime. A crucial step in an HPV-based cervical cancer screening program is the selection of an appropriate HPV test (Arbyn et al. 2015). As HPV infections are very common, with a high tendency for spontaneous regression, the positive predictive value for all HPV tests is relatively low. On the global market, 82% of the HPV tests lack any published analytical and/or clinical evaluation (Poljak et al. 2016). In the case of this study, Anyplex II HPV28 Detection was chosen as the broadest CE/IVD PCR assay, and extensively validated in the Vigilant Framework settings (Bonde et al. 2018).

Most of the available HPV tests are DNA-based and can only discriminate between the presence and absence of HPV-specific DNA. Hence, these tests are not able to discriminate between an active or inactive infection (Benevolo et al. 2011). Tests that use E6/E7 mRNA detection demonstrated higher clinical specificities than DNA-based tests, as E6/E7 mRNA is only found in actively infected cells (Ratnam et al. 2010; Arbyn et al. 2013). Currently, there are a variety of approved HPV tests available in Germany for screening, both DNA- and mRNA-based, with most using DNA test kits. Caution is needed when interpreting HPV results, as there are many different assays and positive HPV-DNA does not necessarily mean that an active infection is present. Future studies evaluating the new cancer screening program will need to clarify whether supplemental HPV testing improves the detection rate of CIN III in the long term and not just increases the number of examinations performed (colposcopies and histologic assessments). In future, more emphasis should be given to the selection of HPV assays, as the conclusions from mRNA and DNA assays differ significantly.

The production of lactic acid leading to a pH below 4,5 and antimicrobial substances such as bacteriocins, the competition for nutrients to counteract the overgrowth by other microorganisms, and the modulation of the local immune response are the main mechanisms of the protective role of lactobacilli (Aroutcheva et al. 2001). High estrogen levels and especially the glycogen content of the vaginal epithelium (Mirmonsef et al. 2016) lead to an environment dominated primarily by *L. crispatus*, *L. gasseri*, and *L. jensenii* (Gajer et al. 2012). The production of lactic acid is one central mechanism by which microorganisms protect themselves from viruses and competitors (Mitra et al. 2016). Further, *L. iners* dominance over *L. crispatus* has been associated with a higher risk for intraepithelial squamous lesions and cancer (Norenhag et al. 2020). A study analyzing the microbiota of HPV-positive and negative women demonstrated that *L. gasseri* is associated with a higher HPV elimination (Brotman et al. 2014). Accordingly, CST IV is associated with cervical abnormalities, low-grade squamous intraepithelial lesions (LSIL), high-grade SIL (HSIL), and cervical cancer. Comparing LSIL and HSIL samples, there was a microbiome shift to a greater abundance of *Sneathia sanguinegens*, *Anaerococcus tetradius*, and *Peptostreptococcus anaerobius* and a lower abundance of *L. jensenii* with HSIL (Mitra et al. 2015). These data suggest a major role of the vaginal and cervical microbiome in the development of precancerous lesions of the cervix. Nevertheless, the most important previously published papers on the role of the cervical microbiome in the development of cervical carcinoma were

limited to 169 (Mitra et al. 2015), 137 (Zhang et al. 2018), 126 (Seo et al. 2016), 120 (Oh et al. 2015), 94 (Wu et al. 2021), 92 (Tango et al. 2020), 47 (Kwon et al. 2019) and 32 (Audirac-Chalifour et al. 2016) patients, respectively. While despite all limitations in comparing results of microbiome studies, the relative patterns in various conditions would be expected to be reasonably consistent (Berman et al. 2020). This does not necessarily hold true if different primer pairs targeting 16 s-rDNA are used. On the one hand, universal V3/V4 Primer pairs do, for example, allow for better vaginal community state types assignment than universal V1/V2-based primers, detect more taxa, and generally present a higher abundance of *Gardnerella vaginalis* (Graspeuntner et al. 2018). In silico, on the other hand, V1/V3 primers seemed to perform at least as good as V3/V4 (Hugerth et al. 2020), and optimized V1/V2 primers, such as those used in our study, cover *Bifidobacteria* and disease-associated taxa, such as *G. vaginalis* and *Chlamydia trachomatis* (Frank et al. 2008; Zhang et al. 2019), while better differentiating among *Lactobacilli* (Zhang et al. 2019). In our study, we not only see more *G. vaginalis* than would be expected with universal V1/V2 primers, but also similar proportions of *Lactobacillus*-, *Gardnerella*-, and mixed-flora-dominated microbiomes to those observed in the largest shotgun metagenomics study performed to date of the cervical microbiome (Jie et al. 2021).

Primers previously used included, most importantly, universal V1/V2 (Mitra et al. 2015), V1/V3 (Oh et al. 2015; Seo et al. 2016), V3/V4 (Audirac-Chalifour et al. 2016; Zhang et al. 2018), and such targeting the V4 region (Wu et al. 2021). Nevertheless, even among studies using the same primer pairs, results substantially differed. While some argued that anaerobes, greater alpha diversity, and consequently lower levels of *Lactobacilli* seemed associated with a bad prognosis (Mitra et al. 2015; Audirac-Chalifour et al. 2016; Wu et al. 2021), others did not find differences linked to *Lactobacilli* or associations with anaerobes found in earlier studies (Oh et al. 2015; Seo et al. 2016; Zhang et al. 2018). Both studies targeting the V1/V3 region found excessively large proportions of *Fannyhessea (Atopobium) vaginae* and, respectively, little *G. vaginalis.* All studies agreed, however, that more studies involving larger sample sizes are needed, given the possible bias occurring with smaller sample sizes.

We are skeptical of differences linked to rare taxa in previous studies, also due to partially small study populations. Despite the V3/V4 primers being described as detecting more taxa (Graspeuntner et al. 2018), meta-transcriptome analyses have shown that only a couple of dominant genera contribute to most of the bacterial transcripts (Arroyo Muhr et al. 2021).

Rather than with neoplasia, we see the most remarkable differences in terms of alpha diversity and *L. crispatus* associated with menopause. Seeing that the study that most closely reflected our findings was a meta-genomic analysis of 516 women to evaluate the effect of lifestyle on the cervical microbiome (Jie et al. 2021), we find confirmation on the one hand and point out on the other hand that microbiome-based studies need to be conducted on large sample sizes. Moreover, just as in mentioned study, we find a significantly larger prevalence of *L. crispatus* among premenopausal women on oral contraceptives and no linkage between *G. vaginalis* and a disturbed microenvironment. In the same study, *L. vaginalis* was positively correlated with irregular menstruation, while in ours, with increasing PAP-score.

Regarding the role of *U. parvum* in the progression of neoplasia, we found three reports in the literature on the possible association between *U. parvum*, HPV, and intraepithelial neoplasia of the cervix (Biernat-Sudolska et al. 2011; Szostek et al. 2014; Drago et al. 2016), indicating the need for further investigation, as the detection of *U. parvum* is currently not indicative for therapy (Patel and Nyirjesy 2010; Kokkayil and Dhawan 2015).

In our study, only a few additional CIN III cases were identified. Results from other centers must be awaited to determine whether the addition of HPV testing will improve cervical cancer screening in Germany. In contrast to many other studies, dyplastic changes were only associated with *U. parvum*. We believe that there is currently insufficient data to support modulation of the vaginal microbiome, which is currently heavily marketed to counter dysplastic changes.

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Author contributions MC, CN and MP conceived and designed the study. MC, CN, MP, NW, RT and MS performed data analysis and interpretation. MC, LW and LO collected data. CN and MP performed lab experiments. AM, AH and DJR provided scientific insight and/or contributed to the interpretation of parts of the data. All authors read, edited, and reviewed the manuscript.

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Data availability The datasets generated during the current study are available from the corresponding author on reasonable request.

Code availability Software packages are published, available to the public, and sources are cited in the Methods section.

Declarations

Conflict of interests The authors declare no financial or commercial conflict of interest.

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3.6 **Neidhöfer C**, Nkwetta DL, Fuen BR, Yenban NF, Mbiatong N, Nchanji GT, Korir P, Wetzig N, Sieber M, Thiele R, Parcina M, Klarmann-Schulz U, Hoerauf A, Wanji S, Ritter M. Tropical leg lymphedema caused by podoconiosis is associated with increased colonisation by anaerobic bacteria. Sci Rep. 2023 Aug 23;13(1):13785.

Zielsetzung der Arbeit – Die nicht-filariale und nicht übertragbare Krankheit Podokoniose betrifft etwa 4 Millionen Menschen und ist durch ein schweres Lymphödem an den Beinen gekennzeichnet, das mit schmerzhaften, intermittierenden akuten Entzündungsschüben einhergeht, die als akute Dermatolymphangioadenitis (ADLA)-Anfälle bezeichnet werden. Es wurden Risikofaktoren mit der Krankheit in Verbindung gebracht, aber die Mechanismen der Pathophysiologie sind nach wie vor unklar. Lymphödeme können zu Hautläsionen führen, die als Eintrittspforten für Bakterien dienen können, die ADLA-Schübe verursachen können, die zu einem Fortschreiten des Lymphödems führen. Das Mikrobiom der Haut der betroffenen Beine von Podokoniose-Patienten bleibt jedoch ungeklärt. Daher haben wir das Hautmikrobiom von Podokoniose-Beinen mittels Next Generation Sequencing analysiert.

Methoden und Ergebnisse – In einer Querschnittsstudie, die im Bamenda Clinical Trial Centre in der nordwestlichen Region Kameruns durchgeführt wurde, die für ihre hohe Prävalenz von Podokoniose bekannt ist, wurden Personen mit und ohne Lymphödem aus denselben Gemeinden rekrutiert und soziodemografische Daten sowie die Krankengeschichte erhoben sowie Hautabstrichproben extrahiert, sequenziert und analysiert, um die mikrobielle Zusammensetzung zu bestimmen. Wir konnten eine positive Korrelation zwischen dem Schweregrad des Lymphödems und nicht-kommensalen anaeroben Bakterien, insbesondere *Anaerococcus provencensis*, sowie eine negative Korrelation mit dem Vorhandensein von *Corynebacterium*, einem Bestandteil der normalen Hautflora, feststellen. Krankheitssymptome waren im Allgemeinen mit einer höheren mikrobiellen Vielfalt und einem größeren Reichtum verbunden, die von der normalen Zusammensetzung der Haut abwichen.

Schlussfolgerungen – Diese Ergebnisse zeigen eine Assoziation verschiedener Bakterientaxa mit Lymphödemstadien, was die wichtige Rolle von Bakterien bei der

Pathogenese der Podokoniose unterstreicht und die Auswahl besserer Behandlungsschemata zur Bewältigung von ADLA-Anfällen und des Krankheitsverlaufs ermöglichen könnte. Ob und inwieweit diese Bakterien für die Fieberschübe oder das Fortschreiten der Krankheit verantwortlich sind, muss dringend geklärt werden, da maßgeschneiderte Behandlungsoptionen wahrscheinlich nicht schwer umzusetzen wären.

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Tropical leg lymphedema caused by podoconiosis is associated with increased colonisation by anaerobic bacteria

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The non-filarial and non-communicable disease podoconiosis affects around 4 million people and is characterized by severe leg lymphedema accompanied with painful intermittent acute inflammatory episodes, called acute dermatolymphangioadenitis (ADLA) attacks. Risk factors have been associated with the disease but the mechanisms of pathophysiology remain uncertain. Lymphedema can lead to skin lesions, which can serve as entry points for bacteria that may cause ADLA attacks leading to progression of the lymphedema. However, the microbiome of the skin of affected legs from podoconiosis individuals remains unclear. Thus, we analysed the skin microbiome of podoconiosis legs using next generation sequencing. We revealed a positive correlation between increasing lymphedema severity and non-commensal anaerobic bacteria, especially *Anaerococcus provencensis*, as well as a negative correlation with the presence of *Corynebacterium*, a constituent of normal skin flora. Disease symptoms were generally linked to higher microbial diversity and richness, which deviated from the normal composition of the skin. These findings show an association of distinct bacterial taxa with lymphedema stages, highlighting the important role of bacteria for the pathogenesis of podoconiosis and might enable a selection of better treatment regimens to manage ADLA attacks and disease progression.

Podoconiosis causes bilateral lymphedema of the lower limbs and it is estimated that worldwide around 4 million individuals are affected, especially in Africa but also in parts of Latin America and South East Asia[1–3]. Until now, 32 countries reported cases of podoconiosis with the highest prevalence rates in Ethiopia, Cameroon and Uganda[4–8]. Control and elimination are integrated into lymphatic filariasis morbidity management and disability prevention, but the WHO has not officially recognized podoconiosis as a neglected tropical disease. Risk factors like exposure to volcanic red clay soils, which are formed by weathering of rocks predominately in areas characterised by high rainfall and altitude as well as low temperature, heritability and genetic factors, and profession as a farmer were associated with podoconiosis[9–12]. Nevertheless, environmental and socioeconomic factors cannot be used as strict causes of the disease since podoconiosis has been reported also in areas with low altitude, less rainfall, high temperature and in individuals who were not farmers. Diagnosis of podoconiosis and especially discrimination from other infectious (e.g., co-endemic lymphatic filariasis) and non-infectious lymphedema forms depends on a combination of different diagnostic tools including assessment of medical history and chronic diseases accompanied with a detailed physical examination[5,13]. Diagnosis of podoconiosis requires trained health workers in rural areas of low- and middle-income countries and thus, research on podoconiosis-specific biomarkers needs to be performed to facilitate the diagnosis of the disease.

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However, contact with volcanic clay in combination with the major risk factors seem to be the cause of the disease but until now specific component(s)/molecule(s) within the soil that drive podoconiosis remain unknown. The bilateral lymphedema caused by podoconiosis can be divided into five stages according to the de novo clinical staging system postulated by Tekola and colleagues[14]. Moreover, podoconiosis is characterised by nodule formation accompanied with extensive sclerosis, loss of elastic fibres, verrucous acanthosis and changes of eccrine structures[14–16]. Consequently, individuals with podoconiosis suffer from skin lesions that serve as entry points for pathogens like bacteria causing inflammatory episodes called acute dermatolymphangioadenitis (ADLA) attacks driving inflammation, progression of the lymphedema and are characterised by malaise, fever, chills and even skin peeling (scaling off of the skin)[17–19]. However, bacterial composition of the legs of podoconiosis individuals has not been analysed and/or compared to the skin microbiome of filarial-driven lymphedema legs which have been shown to harbour distinct patterns of bacterial species[20,21]. Therefore, this study analysed the bacterial composition on the skin of podoconiosis-driven lymphedema legs in the North West Region (NRW) of Cameroon.

Results

Characterisation of the study population. As shown in Table 1, 82 participants were enrolled in the study including 8 lymphedema-free individuals who lived in the same communities and 9 individuals with one healthy leg (n = 25 stage 0). The majority of individuals were women (n = 66, 80.49%) and worked as farmers (n = 47, 57.32%). The median age of the participants was 43.2 years and most of the participants (n = 27, 32.93%) belong to the oldest age group of 51–73 years.

Microbiome profiles. In total, 164 sequenced skin samples generated a total of 17,791,210 reads with a mean read count of 108,483 per sample. Of these samples, 163 passed the minimum quality filter (> 30,000 reads and > 20,000 merged reads). The 91 most prominent taxa (average prevalence > 0.3%) were considered for statistical analysis (5 phyla, 7 classes, 15 orders, 21 families, 27 genera, 16 species). The most common bacterial class was the Actinobacteria (61.95%). These mainly consisted of *Corynebacteriales* (42.83%) and to a lesser degree of *Staphylococcales* (13.79%) and *Micrococcales* (13.61%). The relative abundance of the six most important bacterial classes and families per sample is depicted in Fig. 1. Based on the predominant bacterial family, the majority of samples could be classified into three primary groups of roughly equal size: those primarily consisting of *Corynebacteriaceae*, those dominated by *Staphylococcaceae*, and those dominated by *Peptostreptococcales-Tissierellales*. A few samples were mildly dominated by *Micrococcaceae* or *Aerococcaceae* and some other samples were without any significant dominant taxa.

Lymphedema stage and correlation to distinct taxa. To correlate disease progression with higher or lower abundance of distinct taxa we used both Pearson and Spearman correlation coefficients. This approach accounted for the unique nature of disease stages as both metric and ordinal variables, detected non-linear associations, and ensured reliable results. As depicted in Fig. 2, the results of the Pearson correlations showed

	Character	Number	Frequency (%)
Gender	Female	66	80.49
	Male	16	19.51
Age	18–30	15	18.29
	31–40	21	25.61
	41–50	19	23.17
	51–73	27	32.93
Occupation	Business	14	17.07
	Farmer	47	57.32
	Student	4	4.88
	Teacher	4	4.88
	Others	13	15.85
Left leg stage	0	12	14.63
	1	7	8.54
	2	31	37.80
	3	32	39.02
	4	0	0
Right leg stage	0	13	15.85
	1	12	14.63
	2	30	36.59
	3	25	30.49
	4	2	2.44

Table 1. Characteristics of study population (n = 82).

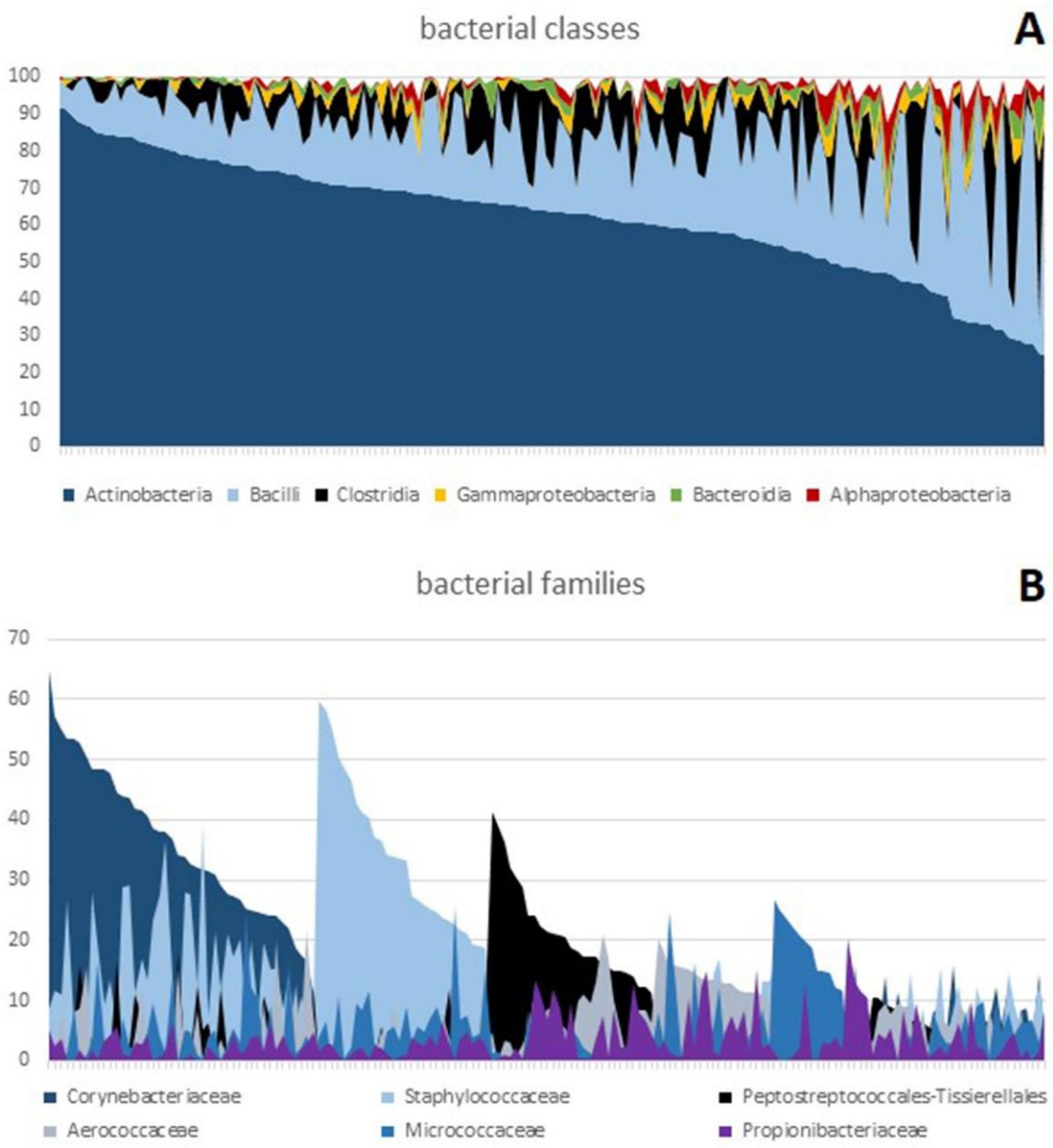

Figure 1. Abundance of bacteria classes and families on the legs of enrolled participants. (**A**) Relative abundance of the six most important bacterial classes. (**B**) Relative abundance of the six most important bacterial families ordered according to the dominant taxa. Each line on the x-axis represents one of the 163 samples.

that there was a significant positive association between disease stage and the class *Clostridia* (r(161) = 0.17, p = 0.031), the order *Peptostreptococcales-Tissierellales* (r(161) = 0.18, p = 0.021), the family *Peptostreptococcales-Tissierellales* (r(161) = 0.18, p = 0.02), the genus *Anaerococcus* (r(161) = 0.22, p = 0.006), genera other than the 27 evaluated ones (r(161) = 0.16, p = 0.046), and the species *Anaerococcus provencensis* (r(161) = 0.16, p = 0.045). There was a significant negative correlation between disease stage and the family *Corynebacteriaceae* (r(161) = −0.17, p = 0.034), the genera *Corynebacterium* (r(161) = −0.17, p = 0.035) and *Propioniferax* (r(161) = -0.26, p = 0.001), as well as the species *Propioniferax innocua* (r(161) = −0.26, p = 0.001). The results of the Spearman correlations showed that there was a significant positive association between disease stage and the family *Dysgonomonadaceae* (r(161) = 0.16, p = 0.039) and *Anaerococcus provencensis* (r(161) = 0.21, p = 0.008), while there was a significant negative association between disease stage and the class *Bacilli* (r(161) = −0.18, p = 0.02), the order *Staphylococcales* (r(161) = −0.16, p = 0.038), the families *Corynebacteriaceae* (r(161) = −0.18, p = 0.024), *Staphylococcaceae* (r(161) = −0.16, p = 0.037), and *Dermabacteraceae* (r(161) = −0.17, p = 0.028), the genera *Corynebacterium* (r(161) = −0.16, p = 0.037) and *Staphylococcus* (r(161) = −0.19, p = 0.017), as well as the species *Corynebacterium jeikeium* (r(161) = −0.18, p = 0.02). Hence, both correlation coefficients revealed that the family of *Corynebacteriaceae*, the genus *Corynebacterium* and the species *Ananerococcus provencensis* were correlated with disease progression. Moreover, there was a significant positive association between increasing

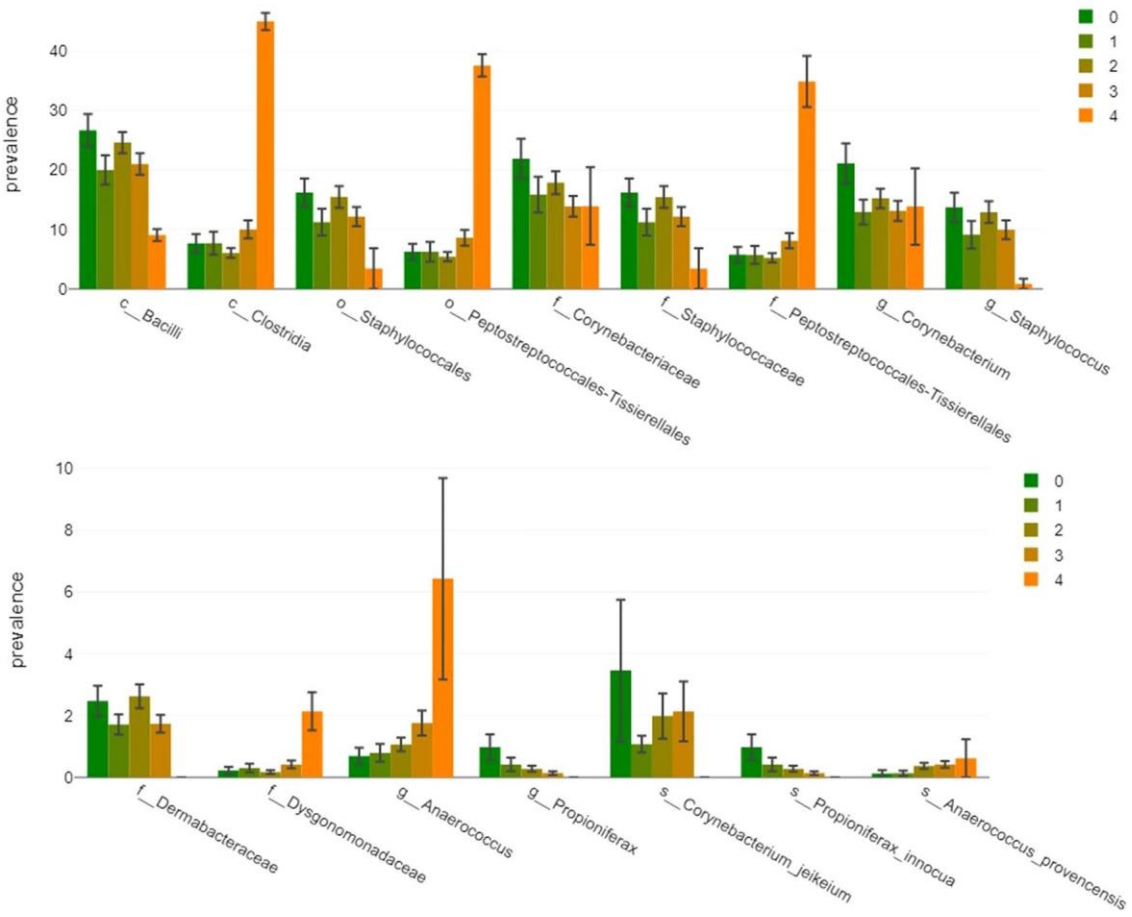

Figure 2. Prevalence of bacteria according to lymphedema stage. Graphs show prevalence of the 16 taxa that were found to be significantly correlated with disease progression by Person correlation, Spearman correlation, or both stratified according to the lymphedema stages (1–4).

lymphedema stage and the most important anaerobic genera *Peptoniphilus* and *Anaerococcus* taken together in both the Pearson ($r(161) = 0.2$, $p = 0.009$) and Spearman correlation ($r(161) = 0.17$, $p = 0.034$) (Fig. 3). No other taxa correlations reached the significance threshold and no significant correlations were observed in terms of richness, Shannon- or Simpson-diversity index or evenness.

Clinical picture and correlation to distinct taxa. A two-tailed t-test for independent samples showed that individuals that suffered from ADLA attacks in the year previous to sample collection had a significantly richer microbiome (taxa: $t(118.84) = -2.94$, $p = 0.004$; OTU: $t(124.78) = -2.68$, $p = 0.008$) and a higher Shannon- (taxa: $t(104.37) = -2.38$, $p = 0.019$; OTU: $t(114.63) = 2.19$, $p = 0.03$) and Fisher-alpha-diversity (taxa: $t(119.47) = -3.01$, $p = 0.003$; OTU: $t(126.23) = -2.74$, $p = 0.007$). These participants were less prominently colonized by the order *Corynebacteriales* ($t(99.62) = 2.41$, $p = 0.018$) and more prominently colonized by the phylum *Deinococcota* ($t(138.54) = -2.6$, $p = 0.01$), the classes *Alphaproteobacteria* ($t(129.38) = -2.4$, $p = 0.018$) and *Deinococci* ($t(138.54) = -2.6$, $p = 0.01$), the order *Micrococcales* ($t(127.72) = -2.9$, $p = 0.004$), *Rhizobiales* ($t(141) = -2.49$, $p = 0.014$), *Deinococcales* ($t(138.55) = -2.6$, $p = 0.01$), and *Sphingomonadales* ($t(130.88) = -2.7$, $p = 0.008$), the families *Intrasporangiaceae* ($t(134.9) = -5.23$, $p < 0.001$), *Nocardiaceae* ($t(117.01) = -4.67$, $p < 0.001$), *Dermacoccaceae* ($t(139.6) = -2.96$, $p = 0.004$), *Deinococcaceae* ($t(138.9) = -2.57$, $p = 0.011$), and *Sphingomonadaceae* ($t(130.88) = -2.7$, $p = 0.008$), the genera *Micrococcus* ($t(137.25) = -3.58$, $p < 0.001$), *Kocuria* ($t(138.53) = -3.01$, $p = 0.003$), *Janibacter* ($t(138.51) = -3.81$, $p < 0.001$), *Nocardioides* ($t(114.48) = -4.75$, $p < 0.001$), *Deinococcus* ($t(138.9) = -2.57$, $p = 0.011$), *Tessaracoccus* ($t(140.95) = -2.55$, $p = 0.012$), *Ornithinimicrobium* ($t(129.25) = -5.56$, $p < 0.001$), and *Microbacterium* ($t(125.72) = -3.33$, $p = 0.001$), the species *Staphylococcus hominis* ($t(112.53) = -2.96$, $p = 0.004$), and phyla ($t(124.49) = -2.42$, $p = 0.017$), classes ($t(118.55) = -2.52$, $p = 0.013$), and orders ($t(112.03) = -2.75$, $p = 0.007$) other than those considered for statistical analysis.

Pearson correlation analysis revealed the number of the ADLA attacks in the year previous to sampling to be negatively correlated with *Lactobacillales* ($r(135) = 0.26$, $p = 0.002$) and *Microbacteriaceae* ($r(141) = -0.17$, $p = 0.038$) and positively with *Aerococcaceae* ($r(135) = 0.24$, $p = 0.005$), *Micrococcaceae* ($r(135) = 0.17$, $p = 0.042$), and *Dermabacter hominis* ($r(135) = 0.2$, $p = 0.018$). The duration of the last ADLA attacks positively correlated with *Intrasporingiaceae* ($r(135) = 0.22$, $p = 0.011$), *Dermacoccaceae* ($r(135) = 0.2$, $p = 0.022$), *Brachybacterium* ($r(135) = 0.24$, $p = 0.004$), *Kocuria* ($r(135) = 0.18$, $p = 0.036$), *Janibacter* ($r(135) = 0.24$, $p = 0.004$),

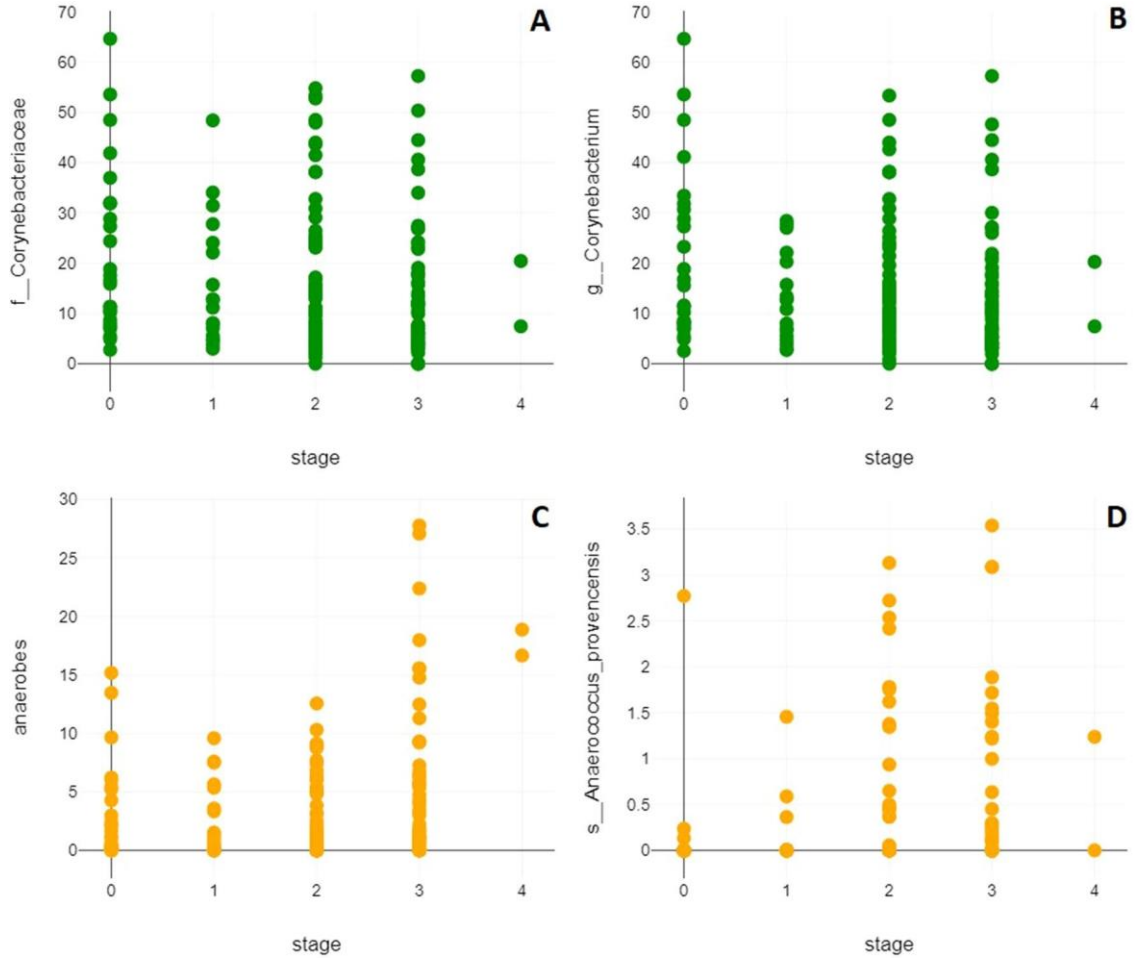

Figure 3. Severity of lymphedema is correlated to distinct bacteria species. (**A**) Increased lymphedema stage is negatively correlated with the family *Corynebacteriaceae* (Pearson: r(161) = −0.17, p = 0.034; Spearman: r(161) = −0.18, p = 0.024) and (**B**) the genus *Corynebacterium* (Pearson: r(161) = −0.17, p = 0.035; Spearman: r(161) = −0.16, p = 0.037), whereas (**C**) the presence of anaerobic genera *Anaerococcus* and *Peptoniphilus* (Pearson: r(161) = 0.2, p = 0.009; Spearman: r(161) = 0.17, p = 0.034) and specifically (**D**) *Anaerococcus provencensis* is positively correlated with increased lymphedema stage (Pearson: r(161) = 0.16, p = 0.045; Spearman: r(161) = 0.21, p = 0.008).

Ornithinimicrobium (r(135) = 0.24, p = 0.004) and species (r(135) = 0.25, p = 0.003) other than those considered for statistical analysis and negatively correlated with *Corynebacteriaceae* (r(135) = −0.24, p = 0.005), *Corynebacterium* (r(135) = −0.19, p = 0.023), and *Dermabacter* (r(135) = −0.23, p = 0.007).

Skin peeling was associated with an increased richness (taxa: t(87.81) = 3.57, p = 0.001; OTU: t(83.11) = -3.22, p = 0.002) and higher Shannon- (OTU: t(141) = 2.54, p = 0.012) and Fisher-alpha-diversity (taxa: t(87.75) = 3.53, p = 0.001; OTU: t(82.82) = 3.21, p = 0.002). It was also associated more prominently with *Bacilli* (t(141) = −2.83, p = 0.005), *Staphylococcales* (t(141) = −3.64, p < 0.001), *Enterobacterales* (t(141) = −2.06, p = 0.041), *Rhizobiales* (t(66.29) = −2.46, p = 0.016), *Sphingomonadales* (t(94.72) = −2.24, p = 0.028), *Staphylococcaceae* (t(141) = −3.64, p < 0.001), *Sphingomonadaceae* (t(94.72) = −2.24, p = 0.028), *Staphylococcus* (t(97.62) = −2.57, p = 0.012), *Macrococcus* (t(69.85) = −2.3, p = 0.025), *Staphylococcus pettenkoferi* (t(58.26) = −2.22, p = 0.03), and *Brachybacterium conglomeratum* (t(89.84) = −2.82, p = 0.006), and less prominently with *Clostridia* (t(140.99) = 2.64, p = 0.009), order and family *Peptostreptococcales-Tissierellales* (t(140.74) = 2.51, p = 0.013), *Bacteroidales* (t(108.97) = 2.66, p = 0.009), *Dysgonomonadaceae* (t(111.35) = 3.45, p = 0.001), *Peptoniphilus* (t(140.6) = 2.1, p = 0.038), *Gallicola* (t(139.43) = 2.79, p = 0.006), *Tessaracoccus* (t(120.12) = 3.11, p = 0.002), and *Ignavigranum ruoffiae* (t(139.24) = 3.04, p = 0.003).

Finally, fever was associated with a higher richness (taxa: t(42.9) = 4.79, p < 0.001; OTU: *t*(44.15) = 4.88, *p* = < 0.001), a higher Shannon- (taxa: t(34.45) = 4.51, p < 0.001; OTU: *t*(47.93) = 6.37, *p* < 0.001), Simpson- (taxa: t(17.42) = 2.95, p = 0.009; OTU: *t*(48) = 4.91, *p* < 0.001), and Fisher-alpha-diversity (taxa: t(41.97) = 5.04, p < 0.001; OTU: t(41.14) = 5.45, p < 0.001), and a higher evenness (OTU: t(48) = 2.08, p = 0.043). Furthermore, fever was associated more prominently with *Pseudomonadota* (t(46.85) = −4.13, p < 0.001), *Bacteroidota* (t(47.88) = −2.79, p = 0.007), *Gammaproteobacteria* (t(47.96) = −3.89, p < 0.001), *Bacteroidia* (t(47.88) = −2.79, p = 0.007), *Alphaproteobacteria* (t(46.76) = −3.39, p = 0.001), *Rhodobacterales* (t(47.72) = −2.59, p = 0.013), *Pseudomonadales*

(t(47.45) = −3.21, p = 0.002), *Rhizobiales* (t(44.48) = −2.69, p = 0.01), *Sphingomonadales* (t(46.7) = −3.55, p = 0.001), *Flavobacteriales* (t(45.99) = −2.87, p = 0.006), *Intrasporingiaceae* (t(43.24) = −3.51, p = 0.001), *Rhodobacteracea* (t(47.72) = −2.59, p = 0.013), *Pseudomonadaceae* (t(47.34) = −2.74, p = 0.009), *Sphingomonadaceae* (t(46.7) = −3.55, p = 0.001), *Micrococcus* (t(45.88) = −2.45, p = 0.018), *Janibacter* (t(47.93) = −2.76, p = 0.008), *Pseudomonas* (t(47.23) = –2.7, p = 0.01), *Ornithinimicrobium* (t(36.78) = −2.13, p = 0.04), *Microbacterium* (t(46.9) = −2.49, p = 0.016), *Corynebacterium resistens* (t(35) = −2.3, p = 0.027) and classes (t(42.32) = −2.41, p = 0.021) other than those considered for statistical analysis.

A summary of all correlations between leg skin microbiome and clinical symptoms of podoconiosis individuals is shown in Table 2.

Discussion

The microbiome of the skin varies greatly depending on the anatomical location. The toe web space has been described as being represented mainly by *Corynebacteriaceae*, and to a slightly lesser degree, by *Micrococcaceae* and *Staphylococcaceae*[22,23]. These three bacterial families represent three out of the five most prominently detected ones in our study. *Peptostreptococcales-Tissierellales* are not frequently found in human skin microbiome analyses, but represent the third most common family in our study. One cannot rule out the possibility that Cameroonians may simply be underrepresented in skin microbiome studies to date. The only study we found that specifically looked at the skin microbiome of Cameroonians included 27 healthy Cameroonians and compared this to that of healthy Japanese[24]. The taxa that were correlated with progressive disease stages in our study were either absent or present at very low levels in the skin microbiomes of these healthy Cameroonians. The high prevalence of *Peptostreptococcales-Tissierellales* may thus be more closely related to the disease of the patient group studied than to their geography. Anaerobic bacteria other than *Cutibacterium acnes* and some anaerobic *Staphylococci*, are rarely detected in this context and mostly associated with disease or chronic wounds[22,23,25,26]. *Staphylococci* have been already isolated from wounds of patients with lower limb lymphedema from Ethiopia[27]. Future studies need to be performed to analyse if the microbiome differs between wounds and skin of the affected legs. Nevertheless, available literature is far from exhaustive. The fact that our study mainly included individuals affected by podoconiosis at different disease stages could explain this finding. Our study revealed a significant correlation between disease progression and the rising prevalence of anaerobic gram-positive cocci, such as *Anaerococcus provencensis*. This bacterium was first isolated from a cerebral abscess sample of a patient from Marseille[28], while scarcely anything else is known about this pathogen. As with all 16s amplicon studies, even significant taxa should be interpreted with caution despite the suitably large sample size. Nevertheless, we do see a trend in regards to disease symptoms which were all associated with a higher microbial richness and diversity. In particular long

Characteristics	Positively associated	Negatively associated
Presence of ADLA attack 1 year before sampling	Richness and diversity of leg skin microbiome Phyla: *Deinococcota* Classes: *Alphaproteobacteria, Deinococci* Orders: *Micrococcales, Rhizobiales, Deinococcales, Sphingomonadales* Families: *Intrasporangiaceae, Nocardiaceae, Dermacoccaceae, Deinococcaceae, Sphingomonadaceae* Genera: *Micrococcus, Kocuria, Janibacter, Nocardioides, Deinococcus, Tessaracoccus, Ornithinimicrobium, Microbacterium* Species: *Staphylococcus hominis*	Orders: *Corynebacteriales*
Number of last ADLA attacks	Families: *Intrasporangiaceae, Micrococcaceae* Species: *Dermabacter hominis*	Orders: *Lactobacillales* Families: *Microbacteriaceae*
Duration of last ADLA attack	Families: *Intrasporangiaceae, Dermacoccaceae* Genera: *Brachybacterium, Kocuria, Janibacter, Ornithinimicrobium*	Families: *Corynebacteriaceae* Genera: *Corynebacterium, Dermabacter*
Skin peeling	Richness and diversity of leg skin microbiome Classes: *Bacilli* Orders: *Staphylococcales, Enterobacterales, Rhizobiales, Sphingomonadales* Families: *Staphylococcaceae, Sphingomonadaceae* Genera: *Staphylococcus, Macrococcus* Species: *Staphylococcus pettenkoferi, Brachybacterium conglomeratum*	Classes: *Clostridia* Orders: *Peptostreptococcales-Tissierellales, Bacteroidales* Families: *Peptostreptococcales-Tissierellales, Dysgonomonadaceae* Genera: *Peptoniphilus, Gallicola, Tessaracoccus* Species: *Ignavigranum ruoffiae*
Fever	Richness, diversity and evenness of leg skin microbiome Phyla: *Pseudomonadota, Bacteroidota,* Classes: *Gammaproteobacteria, Bacteroidia, Alphaproteobacteria,* Orders: *Rhodobacterales, Pseudomonadales, Rhizobiales, Sphingomonadales, Flavobacteriales* Families: *Intrasporangiaceae, Rhodobacteraceae, Pseudomonaceae, Sphingomonadaceae* Genera: *Micrococcus, Janibacter, Pseudomonas, Ornithinomicrobium, Microbacterium* Species: *Corynebacterium resistens*	

Table 2. Correlations of clinical symptoms and leg skin microbiome of podoconiosis individuals.

and frequent ADLA attacks and fever were associated with smaller proportions of microbes usually expected on intact skin and higher prevalence of bacteria that possibly do not belong on the skin.

Previous studies found *Staphylococcus*, *Enterococcus*, *Micrococcus* and *Bacillus* spp. in tissue and lymph fluid as well as lymph nodes from lymphatic filariasis patients and suggested that these bacteria may be responsible for progression of lymphedema and ADLA attacks[20,21]. However, future investigations need to be performed to narrow down distinct bacterial species that are important for the progression of the disease by collecting lymph fluid or blood of individuals with different lymphedema stages before and during ADLA attacks. The investigation of distinct bacterial patterns might also reveal potential biomarkers for the discrimination of podoconiosis and lymphatic filariasis from other infectious and non-infectious forms of lymphedema. Nevertheless, this study has some limitations such as the small number of individuals with high lymphedema stages (especially leg stage 4) and thus, future studies should obtain samples from stage 4 and 5 to proof the positive correlation of anaerobic bacteria and severity of lymphedema. In addition, the majority of enrolled participants were women (80%). Indeed, previous publications already showed that women are more affected than men[4,29–32], since they often have less income than men and thus cannot afford proper shoes and moreover, spent longer periods of time in the fields and therefore are more exposed to irritant soil[31,33]. Further studies should focus on sampling additional skin sites and most importantly skin folds for detailed discrimination of skin bacteria and should include more male participants to decipher if gender influences the skin microbiome of podoconiosis legs.

In summary, we conclude that the microbiome of the skin from the ankle to the interdigital spaces of podoconiosis individuals with steady deterioration is increasingly characterized by the presence of anaerobic bacteria, which are not described to be part of the healthy skin flora. Whether and to what extent these bacteria are responsible for the fever episodes or the progression of the disease is urgently to be clarified, as tailored treatment options would most likely not be challenging to implement.

Methods

Ethics approval and consent to participate. The ethical clearance was sought and obtained from the Faculty of Health Sciences Institutional Review Board of University of Buea (No: 2021/1458-05/UB/SG/IRB/FHS) and the Ethics Ctwoommittee at the University Hospital Bonn, Germany (Lfd Nr. 359/17). The administrative clearance was obtained from North West Regional Delegation of Public Health (No: 2021/203/ATT/NWR/RDPH/BRIGAD). All experiments and methods were performed in accordance with the relevant guidelines and regulations.

Study area and design. This cross-sectional study was conducted at the Bamenda Clinical Trial Centre (BCTC) in NRW of Cameroon, which is characterised by a mean altitude of 1403 m above sea level, mean annual rainfall of 2500 mm and presence of fertile clay soils used to grow rice, maize, beans and other cash crops by farmers[34]. This region has been shown to have the highest prevalence of podoconiosis among 10 regions of Cameroon[7,35].

Study population. Podoconiosis individuals and contacts without lymphedema living in the same communities visiting the BCTC were recruited in this study. Eligible individuals had to be at least 18 years old, to have lived in the NWR for at least 2 years, to not be pregnant and to have no evidence of severe or systematic co-morbidities. Leg stages of the individuals affected by podoconiosis were classified according to the de novo clinical staging system[14]. In addition, we enrolled 8 lymphedema-free individuals from the same communities and obtained 9 individuals with one healthy leg. Thus, in total we obtained 25 control samples (stage 0). Information about the study was provided and eligible individuals willing to participate in the study signed or provided a thumb print on informed consent forms. Then, a questionnaire-based survey was applied to collect sociodemographic data like gender, age, occupation, duration in the community, and educational level from each participant and medical history especially information about ADLA attacks (number and duration of attacks within the last year before sample collection and occurrence of skin peeling and fever during ADLA attacks), which was then confirmed by medical records, if available.

Collection of skin swabs. Samples were collected from both legs of each participant by swabbing the skin areas between toes, skin folds and around the anklebone under strict aseptic techniques to avoid contamination using pre-labelled sterile flocked swabs (Copan, Brescia, Italy). In total, we obtained two swabs per participant (one swab per leg). The swabs were immediately transferred into 1 ml eNAT medium tubes and stored at −20 °C. For consecutive analysis samples were shipped to the Institute for Medical Microbiology, Immunology and Parasitology (IMMIP) at the University Hospital Bonn (UKB), Bonn, Germany.

Skin swab DNA extraction and sequencing. Skin swab samples were homogenized using the bead beating precellys evolution homogenizer (Bertin Technologies SAS Bretonneux, France). Highly purified DNA was then extracted using the column-based PureLink Microbiome DNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. At the end of the extraction process the DNA was eluted to 100 µL volume and qualitatively and quantitatively evaluated using the NanoDrop OneC (Thermo Fisher Scientific). Then, 16S rRNA gene sequencing libraries were constructed from each sample using the Quick-16S NGS Library Prep Kit (Zymo Research Europe GmbH, Freiburg, Germany) with its included optimized V1–V2 primer pairs. Each run included 94 samples, the positive control included in the kit, and a negative control. For quantitative PCR, quality control, and normalization purposes the Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Inc., Hercules, California, USA) was used. After pooling, the DNA was quantified with the QuantiFluor dsDNA System on the Quantus Fluorometer (Promega GmbH,

Walldorf, Germany) and diluted strictly according to the Illumina protocol for MiSeq sample preparation. For the final library a loading concentration of 10pm was chosen and a 10% Illumina v3 PhiX spike-in control added before running it on the Illumina MiSeq platform with a 500cycle v2 Illumina MiSeq Reagent Kit. All reagents and equipment for sequencing of samples were obtained from Illumina, San Diego, CA, USA.

Bioinformatic analysis. The bioinformatic analysis included three main parts, starting with the preprocessing of raw paired end reads. Following the preprocessing, the sequences were assigned to taxonomies. Finally, a statistical and graphical evaluation was performed on the resulting taxa. QIIME2[36] was used for both preprocessing and classification of the data. With the plugin tool DADA2[37] forward and reverse reads were trimmed from the 3' end at position 249, while shorter reads, as well as low-quality reads, got discarded. DADA2 was also used to perform error-correction, merging of forward and reverse reads if there was an overlap of at least 12 base pairs and chimera removal.

The processed sequences were clustered into OTUs (operational taxonomic units) of 100% sequence identity and assigned to taxa, using a classifier trained on full-length sequences of SILVA[38]. The trained classifier was provided by QIIME2 using scikit-learn 0.24.1 and the plugin tool q2-feature-classifier[39,40]. Based on the quantified OTUs and taxa, different diversity indices were calculated using Python and the skbio.diversity library. The Richness, Shannon-, Simpson- and Fisher-index as a measurement for alpha diversity and the Bray–Curtis- and Jaccard-index as a measurement for beta diversity. The Shannon index quantifies the level of entropy in predicting the species identity of a randomly selected individual from a community, whereas the Simpson index assesses the likelihood that two randomly selected individuals belong to the same species. Fisher's alpha is a measure of species richness that adjusts for variations in sample size. The relative abundance of the taxa and the measurements of alpha diversity were tested for statistically significant differences between groups of samples. This analysis was performed in Python using the Mann–Whitney-U test from the statsmodels library[41]. Additionally, taxa frequency comparison and correlation analysis were performed using Datatab version 1.12.1. with the Pearson correlation coefficient used for continuous and normally distributed variables and Spearman correlation coefficient used for ordinal or non-normally distributed variables.

Data availability

The datasets generated during and/or analysed during the current study are not publicly available due to ethical restrictions since they contain information that could compromise the privacy of research participants but are available from the corresponding author on reasonable request.

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Author contributions

M.R., P.K., S.W., A.H. and U.K.S. designed and/or conceived the study. D.L.N., B.R.F., N.F.Y., N.M., G.T.N. and S.W. conducted the fieldwork and collected samples. C.N., N.W., M.S., R.T., M.P. performed the NGS and analysed the data. C.N., N.W., M.S., R.T. and M.R. drafted the manuscript, which was then edited and critically assessed by all authors.

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Competing interests

The authors declare no competing interests.

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4. Diskussion

Die Beziehung zwischen Geschlecht, ethnischer Zugehörigkeit und Anfälligkeit für Infektionskrankheiten, einschließlich der Besiedlung durch multiresistente Erreger, ist komplex und wird von einer Vielzahl verhaltensbedingter und biologischer Faktoren beeinflusst, wobei neue Erkenntnisse auf eine entscheidende Rolle des Mikrobioms hindeuten (Kim et al. 2017; Libertucci & Young 2018; Seekatz et al. 2022; Shah et al. 2021). Das Mikrobiom spielt eine entscheidende Rolle bei der Besiedlungsresistenz, wobei eine ausgewogene mikrobielle Gemeinschaft die Besiedlung mit Krankheitserregern verhindern kann. Störungen, wie sie beispielsweise durch Antibiotika verursacht werden, können zu einer erhöhten Anfälligkeit für Infektionen führen, auch für solche durch multiresistente Erreger (Baron et al. 2018; Kim et al. 2017). Die in unserer und anderen Studien beobachtete erhöhte Prävalenz von multiresistenten Bakterien bei männlichen Patienten (Gomila et al. 2018; Sahm et al. 2001; Wang et al. 2019) oder die Prävalenz unterschiedlciher multiresistenter Erreger in Abhängigkeit von der Ethnizität wirft wichtige Fragen bezüglich der zugrundeliegenden Ursachen auf. Es ist bemerkenswert, dass sowohl Verhaltensfaktoren als auch biologische Differenzen zwischen den Geschlechtern zu diesen Unterschieden beitragen könnten. Es wurde diskutiert ob Ernährung und geringere Handhygiene dazu beitragen könnten, was bereits in mehreren Studien als signifikanter Faktor für die Übertragung von Krankheitserregern identifiziert wurde (Eckmanns et al. 2006; Tschudin-Sutter et al. 2010). Zusätzlich könnten immunologische und hormonelle Unterschiede die Anfälligkeit für bestimmte Infektionen beeinflussen (Klein 2000; McClelland & Smith 2011). In der Praxis bedeutet dies, dass präventive Strategien und Aufklärungsprogramme möglicherweise geschlechtsspezifisch angepasst werden müssen, um effektiver zu sein. Beispielsweise könnte die Implementierung zielgerichteter Hygiene-Schulungsprogramme für männliche Patienten und medizinisches Personal in Krankenhäusern eine Senkung der MRSA-Raten bewirken. Darüber hinaus könnte die Forschung in diesem Bereich von einer tieferen Untersuchung der endokrinologischen Profile und ihrer Korrelation mit Infektionsanfälligkeiten profitieren, um personalisierte Therapieansätze zu entwickeln, die auf das individuelle Risikoprofil der Patienten abgestimmt sind. Die Erkenntnis, dass das Geschlecht einen solchen Einfluss auf die Infektionsraten hat, sollte auch in die

Gestaltung klinischer Studien einfließen. Genauso wichtig diagnostische und präventive Strategien auf die spezifischen Bedürfnisse verschiedener ethnischer Gruppen zuzuschneiden. Darüber hinaus könnte eine vertiefte Untersuchung der sozioökonomischen Faktoren, die mit verschiedenen ethnischen Gruppen verbunden sind, dazu beitragen, die Dynamik der Resistenzverbreitung besser zu verstehen. Es ist bekannt, dass sozioökonomische Bedingungen wie Zugang zu Gesundheitsversorgung, Wohnverhältnisse und allgemeine Hygienebedingungen einen erheblichen Einfluss auf die Gesundheitsoutcomes haben können (Adler et al 1994), und selbst über Jahrzehnte hinweg (Conroy et al. 2010). Das Zusammenspiel dieser Faktoren - Zusammensetzung des Mikrobioms, Antibiotikaeinsatz, genetische Veranlagung und Geschlecht - zeigt, dass personalisierte Ansätze zur Prävention und Behandlung von Infektionen, insbesondere im Zusammenhang mit der zunehmenden Antibiotikaresistenz, von entscheidender Bedeutung sind. Das Verständnis dieser Dynamik ermöglicht eine bessere Risikostratifizierung und maßgeschneiderte Therapien, die diese verschiedenen Faktoren berücksichtigen.

Die aktuelle Resistenzlage in Krankenhäusern verdeutlicht die dringende Notwendigkeit, unser Verständnis für die Verbreitung und Dynamik von multiresistenten Pathogenen zu vertiefen. Unsere und auch andere Studien zeigen, dass spezielle Bereiche innerhalb der Krankenhäuser, insbesondere jene mit intensivem Antibiotikaeinsatz, als kritische Hotspots für die Entstehung und Erhaltung von antimikrobiellen Resistenzen fungieren (Breathnach et al. 2012; Fridkin et al. 1999; Guo et al. 2019; Shaw et al. 2017;). Dieses Wissen ist von entscheidender Bedeutung, um effektivere Reinigungs- und Desinfektionsstrategien zu entwickeln sowie Antibiotikaverwaltungspolitiken entsprechend anzupassen, um die Verbreitung resistenter Erreger einzudämmen. Die Implementierung von neuartigen, gezielten Hygienemaßnahmen, die auf molekulargenetischen und metagenomischen Analysen von Erregern basieren, könnte ein wesentlicher Schritt zur Kontrolle der Resistenzentwicklung sein. Solche Maßnahmen würden es besser als bisher ermöglichen, spezifische Erreger und ihre Resistenzmuster direkt zu identifizieren und daraufhin zielgerichtete Desinfektionsprotokolle zu entwickeln, die auf die jeweiligen Erreger abgestimmt sind. Beispielsweise könnten Oberflächen in Bereichen, in denen häufig resistente Bakterien nachgewiesen werden, mit speziellen antimikrobiellen Beschichtungen versehen werden, um die Keimlast zu reduzieren.

Weiterhin könnte die Anpassung der Antibiotikaverwaltung auf Basis zusätzlicher Daten über die vorherrschenden Resistenzmuster innerhalb des Krankenhauses erfolgen. Die Nutzung von metagenomischen Daten ermöglicht es zudem, die mikrobielle Diversität innerhalb der Krankenhausumgebung zu überwachen und zu verstehen, wie sich diese durch verschiedene Interventionen verändert. Solche Informationen sind essenziell, um zu erkennen, ob Maßnahmen effektiv sind oder ob sie möglicherweise unbeabsichtigte Konsequenzen haben, wie beispielsweise die Selektion von besonders resistenten Mikroorganismen.

Die Integration von Whole Genome Sequencing (WGS) und anderen fortgeschrittenen genetischen Analysemethoden in die Infrastruktur von Krankenhäusern eröffnet nicht nur Möglichkeiten zur Bekämpfung von antimikrobiellen Resistenzen, sondern erweitert auch das Spektrum der diagnostischen Möglichkeiten. Die gleichen Geräte, die für das WGS verwendet werden, können ebenfalls für Mikrobiom- und Metagenomanalysen eingesetzt werden. Diese Technologien ermöglichen es, die gesamte mikrobielle Gemeinschaft in einer Probe zu charakterisieren, was über die bloße Identifizierung einzelner Pathogene hinausgeht. Mikrobiom- und Metagenomanalysen bieten tiefere Einblicke in das komplexe Zusammenspiel verschiedener Mikroorganismen innerhalb des Krankenhausumfelds. Durch die Analyse des gesamten Mikrobioms kann man gezielter untersuchen, wie sich Mikroorganismen gegenseitig beeinflussen und welche Umweltfaktoren das Wachstum resistenter Stämme begünstigen könnten. Diese umfassenden Daten erlauben es Risikoprofile besser zu definieren, die nicht nur auf der Präsenz einzelner resistenter Erreger basieren, sondern auch auf der Zusammensetzung der gesamten mikrobiellen Gemeinschaft. Solche Risikoprofile sind entscheidend für die Entwicklung präziserer und personalisierterer Behandlungsstrategien. Indem sie die spezifischen mikrobiellen Gemeinschaften erkennen, die mit höheren Infektions- oder Resistenzrisiken verbunden sind, können Gesundheitsdienstleister gezielte Präventions- und Interventionsstrategien entwickeln, die auf die jeweilige mikrobielle Umgebung des Patienten abgestimmt sind. Durch die Nutzung der gleichen Technologieplattformen für unterschiedliche Zwecke maximieren Krankenhäuser ihre Ressourceneffizienz und unterstreichen die Bedeutung dieser Technologie als zentrales Werkzeug in der modernen medizinischen Forschung und Praxis.

Die Mikrobiomforschung hat in den letzten Jahren zunehmend an Bedeutung gewonnen, insbesondere im Hinblick auf das Verständnis der komplexen Wechselwirkungen zwischen menschlichem Körper und den kolonisierenden Mikroorganismen. Diese Forschung bietet potenziell revolutionäre Ansätze für die Prävention, Diagnose und Behandlung verschiedener Krankheiten. Trotz des enormen Potenzials steckt die Anwendung der Mikrobiomforschung in klinischen Settings jedoch noch in den Kinderschuhen. Es gibt bedeutende Herausforderungen und Limitationen, die adressiert werden müssen, um die Ergebnisse aus der Mikrobiomforschung effektiv in die klinische Praxis zu integrieren. Ein wesentliches Hindernis ist das Fehlen von umfangreichen, longitudinalen und reproduzierbaren Daten, die notwendig sind, um die oft subtilen Veränderungen im Mikrobiom zu verstehen, die mit spezifischen Krankheitsbildern assoziiert sind (Martin et al. 2018; Zmora et al. 2019 . Zudem bedarf es des Engagements seitens ärztlicher und mikrobiologischer Kollegen sich der interdisziplinären Thematik anzunehmen (Liwinski et al. 2021; Massart et al. 2015). Hierbei spielen Studien wie unsere eine entscheidende Rolle. Um jedoch die Mikrobiomforschung effektiv in klinische Anwendungen zu übersetzen, bedarf es weiterer umfangreicher Studien, die nicht nur die mikrobiellen Gemeinschaften charakterisieren, sondern auch die funktionellen Auswirkungen dieser Gemeinschaften auf den Wirt verstehen. Dazu gehören verbesserte methodologische Ansätze, standardisierte Protokolle zur Probenahme und Analyse sowie fortgeschrittene bioinformatische Tools, die es ermöglichen, große Datenmengen effizient zu analysieren und zu interpretieren.

5. Zusammenfassung

Die präsentierten Ergebnisse unterstreichen die Bedeutung des Mikrobioms nicht nur als Indikator für gesundheitliche Zustände, sondern auch als aktiven Teilnehmer in der Krankheitsentwicklung. Durch die detaillierte Analyse der genetischen Merkmale von Mikroorganismen konnten spezifische Risikofaktoren identifiziert werden, die durch Wohnort, Ethnizität und spezifische Krankenhausumgebungen beeinflusst werden. Diese Erkenntnisse sind entscheidend für die Entwicklung gezielter präventiver Maßnahmen und Therapieansätze, die auf die spezifischen Bedürfnisse der Patienten und die Bedingungen ihrer unmittelbaren Umgebung zugeschnitten sind. Neue Molekulargenetische Technologien ermöglichen eine noch nie dagewesene Präzision in der Infektionsforschung und bieten die Grundlage für die Entwicklung personalisierter medizinischer Lösungen, die auf die mikrobielle Zusammensetzung und genetische Disposition des einzelnen Patienten abgestimmt sind. Durch die Nutzung der gleichen Technologieplattformen für unterschiedliche Zwecke bieten sich die molekulargenetischen Ansätze als zentrales Werkzeug in der modernen medizinischen Forschung und Praxis an und erlauben es Krankenhäuser ihre Ressourceneffizienz zu maximieren. Die generierten Daten erlauben es Risikoprofile besser zu definieren, die nicht nur auf der Präsenz einzelner resistenter Erreger basieren, sondern auch auf der Zusammensetzung der gesamten mikrobiellen Gemeinschaft. Zusammenfassend bestätigen diese Studien die kritische Bedeutung des Mikrobioms und genetischer Analysen in der modernen Medizin und legen nahe, dass eine weiterführende Erforschung und Anwendung dieser Erkenntnisse essenziell ist, um die Herausforderungen im Umgang mit Infektionskrankheiten und Antibiotikaresistenzen in Zukunft erfolgreich zu bewältigen.

6. Darstellung der Überlappung durch geteilte Autorenschaften

Die vorliegende Habilitationsschrift hat sechs publizierte Originalarbeiten zur Grundlage. Vier Arbeiten habe ich als alleiniger Erstautor veröffentlicht.

Eine Arbeit habe ich als geteilter Erstautor zusammen mit Herrn Marcel Neuenhoff zusammen veröffentlicht. Herr Neuenhoff war ein menschlich und fachlich herrausragender bioinfromatischer Kollege und mein Masterstudent. Herr Neuenhoff untersuchte die Bakteriengenome auf Virulenzfaktoren, Resistenzgene und Klonalität mithilfe verschiedener Softwaretools. Ich plante und leitete die Studie, von der Vorbereitung über die Sequenzierung der Genome und das Sammeln der klinischen und mikrobiologischen in-vitro Daten bis hin zur statistischen Analyse und dem Verfassen des Artikels. Entsprechend seines Engagements wurde die Autorenschaft geteilt.

Eine Überlappung mit anderen Habilitationsschriften ist nicht gegeben.

Eine weitere Arbeit habe ich als Erstautor zusammen mit Dr. Mateja Condic, eine geschätze Kollegin aus de Gynäkologie, geteilt. Dr. Condic leitete die Sammlung der Proben und der klinischen Daten. Ich führte die Mikrobiomanalysen für die Proben durch, übernahm die bioinformatische Analyse und die statistische Auswertung der Mikrobiomanalysen bezüglich der Korrelationen mit klinischen Parametern. So übernahm Dr. Condic die Beantwortung der ersten Fargestellung der Arbeit, das neue Screening-Programm für Zervixkrebs in Deutschland hinsichtlich der Erkennung von hochgradigen Vorstufen von Zervixkrebs zu bewerten, und ich die der zweiten, die Rolle des zervikovaginalen Mikrobioms sowie dessen potenziellen Einfluss auf die zervikale Dysplasie zu untersuchen.

Eine Überlappung mit anderen Habilitationsschriften ist auch hier nicht gegeben.

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8. Erklärung

Hiermit bestätige ich, dass ich die Richtlinien zur guten wissenschaftlichen Praxis der Universität Bonn laut Habilitationsordnung zur Kenntnis genommen habe und ich versichere, dass ich sie beim Verfassen der Habilitationsschrift beachtet habe. Insbesondere versichere ich, dass ich alle in der Habilitationsschrift benutzten Quellen und Hilfsmittel angegeben habe.

Bonn 23.04.2024

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